SUMMARY

An analytical method, employing gas chromatography with electron capture detection (GC/ECD), for the determination and confirmation of OK-5203, OC 53276 and OC 56574 in soil was developed and validated at Covance Laboratories Limited.

The method involved extraction by ultrasonic processing with aqueous acetonitrile, removal of the acetonitrile before partitioning into organic solvent followed by silica solid phase extraction (SPE) clean-up. Reconstitution in toluene enabled determination of OK-5203, OC 53276 and OC 56574 by GC/ECD.

A further analytical method, employing Liquid Chromatography with Tandem Mass Spectrometry (LC/MS-MS), for the determination and confirmation of OC 56635 in soil was developed and validated at Covance Laboratories Limited.

This method involved extraction by ultrasonic processing with aqueous acetonitrile: removal of the acetonitrile and reconstitution in water: acetonitrile: acetic acid enabled determination of OC 56635 by LC/MS-MS.

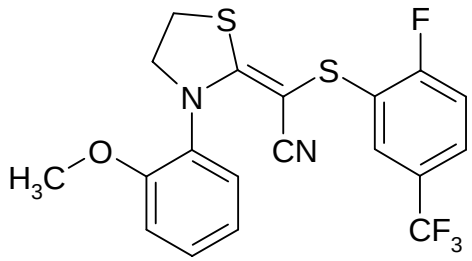
Determination of OK-5203, OC 53276 and OC 56574
Control samples of one soil type were fortified with OK-5203, OC 53276 and OC 56574 at 0.01 and 0.1 mg/kg in quintuplicate and analysed. Recovery of OK-5203, OC 53276 and OC 56574 was determined and the validity of the analytical method was assessed. For the remaining three soil types, control samples were fortified with OK-5203, OC 53276 and OC 56574 at 0.01 mg/kg, in triplicate and analysed. At this fortification level, the overall accuracy and precision was also assessed across all four soil types.

Response of the GC/ECD instrument to OK-5203, OC 53276 and OC 56574 was linear over the concentration range 0.0008 to 0.25 µg/mL. The coefficient of determination (r^2) for each calibration line was > 0.99.

No apparent residues of OK-5203, OC 53276 or OC 56574 were observed in the control extracts above 30% of the limit of quantification (LOQ).. The method is therefore considered specific for OK-5203, OC 53276 and OC 56574.

INTRODUCTION

OK-5203 is a development fungicide and has the following chemical structure:



Chemical Name (IUPAC): (Z)-2-[3-(2-methoxyphenyl)-2-thiazolidinylidene]-2-(α, α, α, 6-tetrafluoro-*m*-tolylthio)acetonitrile

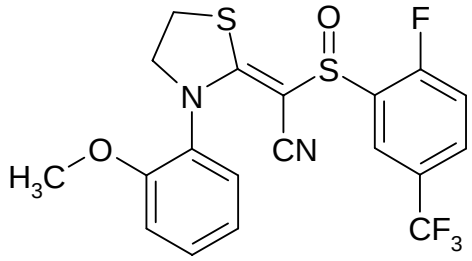
CAS Registry Number: [304900-25-2]

Chemical Formula: $C_{19}H_{14}F_4N_2OS_2$

Molecular Weight: 426.45

The following three metabolites have also been identified for analysis.

OC 53276 is a soil metabolite of OK-5203 and has the following chemical structure:

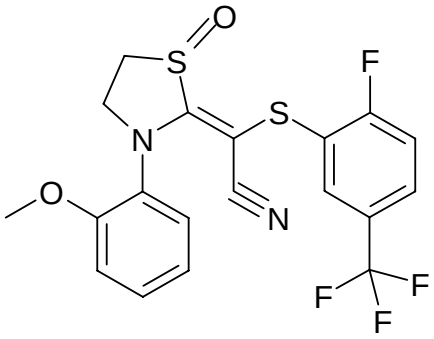


Chemical Name (IUPAC): (z)-2-[fluoro-5-(trifluoromethyl)phenylsulfinyl]-2-[3-(2-methoxyphenyl)thiazolidinylidene]acetonitrile

Chemical Formula: $C_{19}H_{14}F_4N_2O_2S_2$

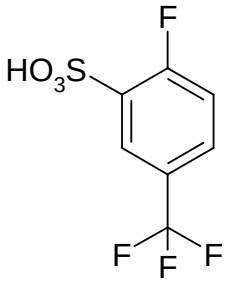
Molecular Weight: 442.45

OC 56574 is a soil metabolite of OK-5203 and has the following chemical structure:



Chemical Name (IUPAC):	(z)-2-[2-fluoro-5-(trifluoromethyl) phenylthio]-2-[3-(2-methoxyphenyl)-2-thiazolidinylidene]acetonitrile 1'-oxide
Chemical Formula:	$C_{19}H_{14}F_4N_2O_2S_2$
Molecular Weight:	442.45

OC 56635 is a soil metabolite of OK-5203 and has the following chemical structure:



$(2H_2O)$

Chemical Name (dihydrate):	2-fluoro-5-(trifluoromethyl)benzenesulfonic acid (dihydrate)
Chemical Formula (dihydrate):	$C_7H_4F_4O_3S$ $(2H_2O)$
Molecular Weight (dihydrate):	244.2 (36.0)
Chemical Name (anhydrous):	2-fluoro-5-(trifluoromethyl)benzenesulfonic acid
Chemical Formula (anhydrous):	$C_7H_4F_4O_3S$
Molecular Weight (anhydrous):	244.2

EXPERIMENTAL PROCEDURES

Protocol Adherence

The study was performed in accordance with the protocol and nine amendments, with no major deviations. A minor deviation, which did not affect the quality or integrity of the study, is presented in Appendix 6.

Analytical Standards (Test Substance)

Analytical grade standards (OK-5203, OC 53276, OC 56574 and OC 56635) were supplied to Covance by the Sponsor. Certificates of Analysis including purity, expiry date and batch number were supplied.

All precautions required in the handling, storage and disposal of the test substances were outlined by the supplier.

The date of receipt and expiry date, plus batch number and purity details are recorded in the table below along with the allocated Covance identification and lot number. The Sponsor changed the code names in November 2006 and to avoid confusion, old and new codes are listed below. The analytical standards were stored refrigerated (2 to 8°C). The Certificates of Analysis are presented in Appendix 1.

Old Code	New Code	Covance CHD number (lot number)	Date received	Supplier Batch Number	Chemical purity	Expiry date
OK-5203	NA	350/05-2554 (3)	11 May 2006	04AF1-1	99.13%	28 June 2007
AB-6	OC 53276	1424/05-2554 (1)	5 December 2006	HN0510016	99.7%	26 Oct 2009
AB-13	OC 56574	1163/06-2554 (1)	5 December 2006	YaE0610001	99.0%	26 Oct 2009
B-3	OC 56635	1172/06-2554 (1)	30 October 2006	06102301	95.6%	26 Oct 2009

NA = Not applicable

OC 56635 was supplied as the dihydrate. All concentrations are expressed as anhydrous OC 56635, not as the dihydrate. A correction factor of 0.87 (244.2/280.2) was applied to the calibration standard concentrations and fortification levels prior to determination of the anhydrous OC 56635 recovery in the validation samples.

A summary of the corrected calibration standard concentrations and fortification levels is presented in the following table:

Identification	OC 56635 concentration as the dihydrate	OC 56635 anhydrous concentration
Fortification level (mg/kg)	0.001	0.00087
Fortification level (mg/kg)	0.0025	0.00218
Fortification level (mg/kg)	0.005	0.00436
Fortification level (mg/kg)	0.10	0.08715
Calibration standard (µg/mL)	0.006	0.005229
Calibration standard (µg/mL)	0.005	0.004358
Calibration standard (µg/mL)	0.002	0.001743
Calibration standard (µg/mL)	0.001	0.000872
Calibration standard (µg/mL)	0.00075	0.000654
Calibration standard (µg/mL)	0.0005	0.000436
Calibration standard (µg/mL)	0.0002	0.000174
Calibration standard (µg/mL)	0.0001	0.000087
Calibration standard (µg/mL)	0.000075	0.000065
Calibration standard (µg/mL)	0.00006	0.000052

Receipt and Storage of Study Samples
Control soil samples were obtained for use in this study from European field trials being conducted by Covance for the same Sponsor under study number 2554/016[1]. These trials are located in Germany, Northern France, Southern France and Spain.

The samples were given unique Covance numbers on receipt (06/2554/92 or 06/2554/250 for soil A from Germany, 06/2554/93 or 06/2554/240 for soil B from Northern France, 06/2554/94 or 06/2554/256 for soil C from Southern France and 06/2554/95 or 06/2554/246 for soil D from Spain) in accordance with departmental Standard Operating Procedures (SOP). Soil samples 06/2554/92-94 were received ambient and stored at room temperature (10 to 30°C). Soil samples 06/2554/250, 240, 256 and 246 were received frozen and stored under frozen conditions (< -10°C, nominally – 20°C).

Using different storage conditions for the study samples did not affect the quality or integrity of the study.

Preparation of Study Samples
Control samples of all four soil types were homogenised by hand mixing followed by sieving (2 mm mesh size) according to departmental SOP.

Water Content
Fortification levels and analytical results in this study are expressed as mg/kg dry weight of soil for OK-5203, OC 53276, OC 56574 and OC 56635. The percent water content for the eight soils analysed were determined and are presented in the following table:

[1] Covance study number 2554/016: OK-5203: The Dissipation and Accumulation of Residues in Soil in Northern and Southern Europe. Protocol issued 30 May 2006. Study currently on-going.

Soil reference	Sample identification	Country of origin	% moisture (w/w)
Soil A	06/2554/92	Germany	17.48
Soil A	06/2554/250	Germany	12.56
Soil B	06/2554/93	Northern France	24.08
Soil B	06/2554/240	Northern France	17.37
Soil C	06/2554/94	Southern France	10.80
Soil C	06/2554/256	Southern France	5.28
Soil D	06/2554/95	Spain	12.20
Soil D	06/2554/246	Spain	7.95

In order to correct the OK-5203, OC 53276 and OC 56574 results for dry weight, a correction factor was applied when calculating the detected residue levels in mg/kg. The correction factor was calculated as follows:

$$\%\ \text{moisture (w/w)} = \frac{\text{weight of wet sample} - \text{weight of dry sample}}{\text{weight of wet sample}} \text{ x } 100$$

$$\text{Correction factor} = \frac{100}{100 - \%\ \text{moisture (w/w)}}$$

Using the above calculations, the following correction factors were applied for the analysis of residues of OK-5203, OC 53276 and OC 56574:

Soil reference	Sample identification	Country of origin	Correction factor
Soil A	06/2554/92	Germany	1.21
Soil B	06/2554/93	Northern France	1.32
Soil C	06/2554/94	Southern France	1.12
Soil D	06/2554/95	Spain	1.14

Residue levels in mg/kg were calculated using the following calculation:

$$\text{Residue (mg/kg)} = \frac{\text{extract concentration (µg/mL) x final volume (mL) x soil moisture factor}}{\text{sample weight (g)}}$$

For the analysis of OC 56635, the moisture content of each soil was taken into account when fortifying the samples. The dry weight of each soil was determined using the following calculation:

$$\text{Dry weight of soil (g)} = \text{Wet weight of soil (g)} \text{ x } \frac{100 - \%\ \text{moisture (w/w)}}{100}$$

Analytical Procedures (CLE 2554/015-01V)

Analytical procedure CLE 2554/015-01V was developed by Covance and was based on existing analytical procedures, supplied by the Sponsor. The analytical method employed gas chromatography with electron capture determination (GC/ECD).

Summary of the Analytical Procedure for Soil

The method involved extraction by ultrasonic processor initially with acetonitrile and subsequently with acetonitrile:water (3:1, v/v), followed by centrifugation. The acetonitrile:water mix was evaporated to less than 5 mL and the extract partitioned with hexane:ethyl acetate (8:2, v/v). Clean up was then achieved by silica (Si) solid phase extraction (SPE). OK-5203, OC 53276 and OC 56574 were quantified by GC using electron capture detection (GC/ECD). The limit of quantification for this procedure was 0.01 mg/kg.

Analytical procedure CLE 2554/015-01V is presented in Appendix 2.

Analytical Procedures (CLE 2554/015-01V (M))

Analytical procedure CLE 2554/015-01V (M) was developed by Covance and was based on existing analytical procedures, supplied by the Sponsor. The analytical method employed Liquid Chromatography with Tandem Mass Spectrometry (LC/MS-MS).

Summary of the Analytical Procedure for Soil

The method involved extraction by ultrasonic processor initially with acetonitrile and subsequently with acetonitrile:water (3:1, v/v), followed by centrifugation. The acetonitrile:water mix was evaporated to *ca* 1 mL. OC 56635 was quantified by LC/MS-MS. The limit of quantification for this procedure was 0.00436 mg/kg.

The analytical procedure CLE 2554/015-01V (M) is presented in Appendix 3.

Validation Procedure

The analytical method was validated by fortifying aliquots of untreated control soil (all four soil types) with known amounts of OK-5203, OC 53276, OC 56574 and OC 56635. The fortified samples were then analysed using the defined analytical procedure and recovery of OK-5203, OC 53276, OC 56574 and OC 56635 from each sample was determined.

Validation included consideration of the criteria in the following sections.

Linearity
In order to establish linearity of response of the analytical chromatographic system to OK-5203, OC 53276 and OC 56574, at least six standard solutions of increasing concentration were prepared over the range 0.0008 to 0.25 µg/mL for validation of all four soil types.

In order to establish linearity of response of the analytical chromatographic system to OC 56635, at least six standard solutions of increasing concentration were prepared over the range 0.000052 to 0.005229 µg/mL for validation of all four soil types.

For each compound, the lowest concentration was equivalent to less than 50% of a sample at the limit of quantification (LOQ) and the highest concentration was equivalent to greater than 120% of the highest level to be analysed.

Single determinations at each concentration were made by injecting each solution into the chromatograph in random order, and concentration/response curves were prepared.

Specificity
The ability of the method to distinguish between OK-5203, OC 53276, OC 56574, OC 56635 and other substances present in each control sample was investigated. Components present in a control sample that interfered with the analysis were not present at levels greater than 30% of the limit of quantification.

Precision
Repeatability of each method was demonstrated by analysing each validation level in quintuplicate for soil A. For soils B, C and D determination was in triplicate at the LOQ validation level only. Relative standard deviation (RSD) was determined at each validation level and overall and was considered acceptable if ≤ 20%.

Recovery
Recovery from control soil A fortified at 0.01 mg/kg (LOQ) and 0.1 mg/kg (10 × LOQ) for OK-5203, OC 53276 and OC 56574, was determined in quintuplicate. Recovery was also determined from control soil A fortified at 0.00436 mg/kg (LOQ) and 0.08715 mg/kg (10 × LOQ) for OC 56635 in quintuplicate. These fortification levels related to the 'dry' weight of the soil as received, with adjustment made for soil moisture. Recovery from control soils B, C and D fortified at 0.01 mg/kg (LOQ) for OK-5203, OC 53276 and OC 56574 and 0.00436 mg/kg (LOQ) for OC 56635, was determined in triplicate.

In addition, control samples of all soil types were extracted and analysed in duplicate. Where there was an apparent response to OK-5203, OC 53276, OC 56574 or OC 56635 from a control soil, the equivalent concentration was subtracted from each of the relevant validation levels, before calculation of the recovery values.

Mean recoveries of OK-5203, OC 53276, OC 56574 and OC 56635 at each level and overall, were considered acceptable if they fell within the range 70 to 110%.

Limit of Quantification (LOQ)

The limit of quantification in each soil type was defined as the lowest fortification level where an acceptable mean recovery for OK-5203, OC 53276, OC 56574 and OC 56635 was obtained (70 to 110%), and a relative standard deviation of ≤ 20% was achieved. For analytical method CLE 2554/015-01V, the limit of quantification for OK-5203, OC 53276 and OC 56574 was initially proposed as 0.01 mg/kg in all four soils. For analytical method CLE 2554/015-01V (M), the limit of quantification for OC 56635 was initially proposed as 0.004361 mg/kg in all four soils.

Further analysis was conducted at fortification levels equivalent to 0.001, 0.0025 and 0.005 mg/kg for OC 53276 and OC 56574 and at fortification levels equivalent to 0.00087 and 0.00217 mg/kg for OC 56635 in an attempt to lower the LOQ for each compound below the initially proposed levels.

Limit of Detection (LOD)

The limit of detection was defined as the lowest concentration of the test compound measurable in soil extracts, calculated (by estimation) from the lowest concentration of the calibration standards. The LOD was 30% of the LOQ or lower, and its signal to noise ratio (S/N) should be greater than three.

Method Confirmation

Determination using GC/ECD was employed in this study for the analysis of OK-5203, OC 53276 and OC 56574 since testing confirmed that this gave better sensitivity and precision than GC/MSD determination. Therefore as the selected method did not involve determination using GC/MS (3 ions), re-analysis of final extracts, using a suitably selective and sensitive alternative chromatographic technique, was required. The same criteria for precision and recovery (see above) were used to assess this confirmatory method.

Determination using LC/MS-MS was employed in this study for the analysis of OC 56635. This technique is considered to be highly specific and therefore no additional confirmation analysis was required.

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been made of those procedural steps involving preparation of solutions, reagents and analysis of matrix samples. Appropriate safety codes have been included in the text (e.g. category [1a/b, 4b/4d]) and are defined in the section titled General Handling Control Categories (on page 54 of this report).

The hazards and risks of the substances hazardous to health used in this method have been considered. Provided the method is accurately followed and the control measures specified in the method are correctly used, there should be no foreseeable hazards to health.

INTRODUCTION

This method describes the procedure for determining residues of OK-5203, OC 53276 and OC 56574 in soil. Residues in soil are extracted by ultrasonic processor and acetonitrile followed by acetonitrile: water (3:1, v/v), followed by centrifugation. The acetonitrile: water mix is evaporated to less than 5 mL and the extract partitioned with hexane: ethyl acetate (8:2, v/v). Clean up is then achieved by silica (Si) solid phase extraction (SPE). OK-5203, OC 53276 and OC 56574 are quantified by GC using ECD detection (GC/ECD). The limit of quantification for this procedure is 0.01 mg/kg.

Summary of the Procedure
The method of analysis comprises the following stages:

1. Extraction from soil using ultrasonic processor and acetonitrile followed by acetonitrile: water (3:1, v/v).
2. Centrifugation.
3. Evaporation of acetonitrile.
4. Clean up by hexane: ethyl acetate partition.
5. Evaporation.
6. Further clean up by silica SPE.
7. Evaporation and reconstitution in toluene.
8. Quantification by GC/ECD.

All procedures will be carried out in compliance with departmental SOP.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- GC/ECD system: Agilent 6890 µECD
- GC Column: J&W HP5 30 m x 0.32 mm i.d, 0.25 µm film
- GC confirmation column: VF17MS 15 m x 0.25 mm i.d, 0.25 µm film
- Centrifuge: MES, Mistral 3000E
- Ultrasonic processor: Sonics -Vibra Cell VC130
- VISIPREP 24 box: Supelco
- 15 mL glass tubes
- Sample concentrator: Zymark Turbovap LV sample concentrator
- Glass jars
- 50 mL polypropylene tubes: Costar
- 40 mL glass tubes
- Various laboratory glassware
- Various pipettes
- Short form pipettes

Equivalent equipment may be used.

Materials

• Acetone	Rathburn, Glass distilled grade
• Acetonitrile	Rathburn, HPLC grade
• Water	In-house, Ultrapure Deionised
• Toluene	Rathburn, Glass Distilled grade
• Hexane	Rathburn, HPLC grade
• Ethyl acetate	Rathburn, Glass Distilled grade
• Sodium chloride	BDH, AnalaR
• Bond Elut SI (3 mL/500 mg) SPE cartridge	Varian

Equivalent or better grade reagents/solvents may be used.

Reagents and Solutions [Category 1a/b, 4d]

Acetonitrile: water (3:1, v/v)
Mix 1500 mL acetonitrile with 500 mL water.

Hexane: ethyl acetate (8:2, v/v)
Mix 800 mL hexane with 200 mL of ethyl acetate.

5% sodium chloride
Dissolve 50 g NaCl in 1 L of water.

Hexane: ethyl acetate (3:1, v/v)
Mix 300 mL hexane with 100 mL of ethyl acetate.

Hexane: ethyl acetate (1:2, v/v)
Mix 100 mL hexane with 200 mL of ethyl acetate.

Preparation of Standard Solutions [Category 1a/b, 4b/4d]
Duplicate primary stock standard solutions of OK-5203, OC 53276 and OC 56574 are prepared in acetone. Fortification solutions are prepared in acetone from dilutions of the stock solutions. Standard solutions must be stored glass, under refrigerated conditions, when not in use. Standard solutions should be allowed to warm to room temperature prior to use.

Preparation of Stock Solutions [Category 1a/b, 4d]
In duplicate, accurately weigh *ca* 20 mg (corrected for purity) of OK-5203, OC 53276 and OC 56574 into separate 20 mL volumetric flasks and dilute each to the mark using acetone to give primary stock standards of concentration 1000 µg/mL. (Note: Smaller amounts may be weighed to conserve test substance, final volume should be adjusted such that the final concentration is still 1000 µg/mL.)

Note: Duplicate solutions of each test substance are prepared to check both the accuracy of weighing and solubility of the test substances. Correlation is achieved by GC/ECD quantification of appropriately diluted solutions. Only one primary stock standard is used for the preparation of both fortification and calibration solutions.

Calibration Standards [Category 1a/b, 4b]
Prepare appropriate mixed calibration standards in toluene to cover the range 0 to 0.25 µg/mL, with a lowest calibration level of 0.0008 µg/mL. Calibration standards may be stored refrigerated for up to three months.

Fortification Solutions [Category 1a/b, 4b]
Prepare suitable mixed fortification solutions in acetone by serial dilution from the primary stock solutions (1000 µg/mL in acetone).

PROCEDURES

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Extraction From Soil [Category 1a/b, 4a]
1. Weigh 10 g of soil sample into a 50 mL polypropylene tube. Untreated control samples may be fortified at this point for the determination of procedural recovery (with up to 500 µL of a suitable fortification solution in acetone).
2. Add 25 mL of acetonitrile to the tube. Extract using an ultrasonic processor set to approximately 50% power pulsed for approximately one minute.
3. Centrifuge sample for 2 minutes at 2500 rpm (1400 g). Decant the supernatant into a glass jar.
4. Add 25 mL of acetonitrile: water (3:1, v/v) to the soil pellet and repeat the ultrasonic processor extraction for a further minute.
5. Centrifuge the second extraction (2500 rpm for 2 minutes) and combine the supernatants.
6. Repeat steps 4 and 5 with a further 25 mL of acetonitrile: water (3:1, v/v).
7. Make to 80 mL final volume with acetonitrile.

8. Take a 2 g equivalent aliquot (16 mL) and evaporate to less than 5 mL using a Turbovap set to 40°C.

Partition Clean-up [Category 1a/b, 4a]

9. Transfer the sample to a 50 mL polypropylene tube and add 25 mL 5% NaCl solution and 10 mL hexane: ethyl acetate (8:2, v/v)
10. Shake for approximately 1 minute.
11. Centrifuge for 2 minutes at 2500 rpm to remove any emulsions.
12. Transfer the top hexane: ethyl acetate layer to a clean 40 mL tube.
13. Repeat the partition with a further 2 x 10 mL of hexane: ethyl acetate (8:2, v/v) combining the top layers.
14. Evaporate the extract just to dryness using a Turbovap at 35°C.
15. Reconstitute samples in 2 mL hexane.

SPE Clean-up [Category 1a/b, 4a]

16. Condition 500 mg silica Bond Elute cartridges with 3 mL hexane. A small amount of anhydrous sodium sulphate may be added to the cartridges to remove any moisture present.
17. Load samples from step 15 onto cartridges. Discard the eluant to waste. Do not allow the cartridges to go dry.
18. Rinse the flask with hexane (2 mL) and add the rinse to the cartridges. Elute as before and discard the eluant to waste.
19. Rinse the cartridges with hexane: ethyl acetate (3:1, v/v) (3 mL). Elute as before and discard the eluant to waste.
20. Add hexane: ethyl acetate (1:2, v/v) (3 mL) to the cartridges and elute as before. Collect the eluant in a glass tube. Briefly dry the cartridge for 1 to 2 seconds then elute with a further 3 mL hexane: ethyl acetate (1:2 v/v), collecting in the same glass tubes.
21. Evaporate the extract just to dryness using a Turbovap at 35°C.
22. Reconstitute samples with 10 mL of toluene sonicate and vortex mix to dissolve.
23. Further dilute samples as necessary with toluene to be within the calibration range

All extracts derived during or from this procedure may be stored refrigerated or frozen if storage is required.

Conditions for GC Analysis with MS Detection

Instrumentation:	Agilent 6890 series GC/µECD with Agilent 7683 series autosampler
Column#:	J&W HP5 30 m x 0.32 mm i.d, 0.25 µm film
Confirmation column:	Varian VF17MS 15 m x 0.25 mm i.d, 0.25 µm film
Injection volume:	1 µL, Split 5:1
Injector temperature:	90°C, hold for 0.2 mins. Ramp at 700°C/minute to 310°C, hold 5 mins. Ramp 50°C/minute to 290°C,
Carrier gas:	He at 20 PSI
Make up gas:	N_2 approx.60 mL/min
Oven temperature:	90°C, hold for 1 min. Ramp at 50°C/minute to 290°C, hold 10 mins.
Detector temperature:	300°C
Retention time:	OK-5203 approx. 7.7 minutes OC 53276 approx. 10.2 minutes. OC 56574 approx. 8.7 minutes. (Confirmation conditions OK-5203 approx. 8.0 minutes OC 53276 approx.13.4 minutes OC 56574 approx. 10.1 minutes)

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

CALCULATION OF RESULTS

The presence of OK-5203, OC 53276 and OC 56574 in a sample is confirmed if the resulting peaks arising from the test sample have the same chromatographic retention times as the appropriate standard. All peak measurements and calculations are performed using a data system (Atlas 2000 R2).
Residues of OK-5203, OC 53276 and OC 56574 are determined by the interpolation of the peak area of OK-5203, OC 53276 and OC 56574, from the standard regression equation, as follows:

The calibration line is determined by plotting the responses from the calibration solutions (R) against the of amount test substance injected (A) to generate a straight line graph.

$R = B0 + B1 \times A$
where B1 is the gradient and B0 is the intercept.

Concentrations of test substance (A) in sample extracts are calculated from their response using the equation:

Concentration of extract A (µg/mL) = (Peak area – intercept)/slope

The residue of OK-5203, OC 53276 and OC 56574 in each test sample is calculated as follows:

$$\text{Residue (mg/kg)} = \frac{\text{extract concentration (µg/mL) x final volume (mL) x soil moisture factor}}{\text{sample weight (g)}}$$

Where the final volume includes dilution steps, if applicable and:

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{soil moisture factor} = \frac{100}{100 - \%\,\text{moisture}}$$

$$\text{Recovery (\%)} = \frac{A - C}{S} \times 100$$

Where:-

A = concentration found in fortified sample (mg/kg)
C = concentration (or interference) found in control sample (mg/kg)
S = concentration added to fortified sample (mg/kg)

Linearity of Response
The linearity of response of the GC/ECD system should be determined with at least six different concentration standard solutions across the range 0 to 0.25 µg/mL, with the lowest calibration level being 0.0008 µg/mL.

METHOD CRITERIA

Analysis by GC/ECD will be considered successful only if the following criteria are met.

- A procedural recovery of 70 to 110% will be obtained for each batch of analysis
- Control sample contains a concentration ≤ 30% of the limit of quantification
- At least 6 calibration standards will be used in the determination of each calibration line. A 1/x weighting should be used.
- A coefficient of determination (r^2) for each calibration line will be ≥ 0.98
- All test samples will be within the appropriate calibration standards range

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main	Division	CONTROL Name and Specification
1		GLOVES
	a	Disposable latex
	b	Disposable nitrile
	c	Rubber gloves
	d	Specific type for the job (see assessment giving details)
2		PROTECTIVE CLOTHING
	a	Laboratory coat or equivalent
	b	Disposable overalls
	c	Oversleeves
	d	Overshoes
	e	Plastic apron
3		EYE/FACE PROTECTION
	a	Safety glasses to BS 2092/2 C or better
	b	Face shied to BS 2092/2 C or better
	c	Safety goggles to BS 2092/2 C or better
4		ENGINEERING CONTROLS
	a	Open bench in ventilated area
	b	Fume cupboard to BS 7258
	c	Laminar flow cabinet to BS 5295 Class 1
	d	Re-circulating fume chamber
	e	Radioisotope lab
	f	Biohazard lab
	g	Glove box
5		RESPIRATORY PROTECTIVE EQUIPMENT
	a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
	b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
	c	Respirator with specified canister (HSE approved)
6		SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7		ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8		REFER TO MATERIAL SAFETY DATA SHEET
9		KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10		POISON – ensure antidote is available and is within its expiry date (must specify details)

Appendix 3
Methodology of Analytical Procedure CLE 2554/015-01V (M)

Procedure Title: OC 56635: An Analytical Method for the Determination of Residues in Soil

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been made of those procedural steps involving preparation of solutions, reagents and analysis of matrix samples. Appropriate safety codes have been included in the text (e.g. category [1a/b, 4b/4d]) and are defined in the section titled General Handling Control Categories (on page 9 of this analytical procedure).

The hazards and risks of the substances hazardous to health used in this method have been considered. Provided the method is accurately followed and the control measures specified in the method are correctly used, there should be no foreseeable hazards to health.

INTRODUCTION

This method describes the procedure for determining residues of OC 56635 in soil. Residues in soil are extracted by ultrasonic processor and acetonitrile followed by acetonitrile:water (3:1, v/v), followed by centrifugation. The acetonitrile:water mix is evaporated to approximately 1 mL and the extract reconstituted in water:acetonitrile:acetic acid (9:1:0.1, v/v/v). OC 56635 is quantified by LC/MS-MS. The limit of quantification for this procedure is 0.005 mg/kg.

Summary of the Procedure
The method of analysis comprises the following stages:

1. Extraction from soil using ultrasonic processor and acetonitrile followed by acetonitrile:water (3:1, v/v).
2. Centrifugation.
3. Evaporation of acetonitrile.
4. Reconstitution in water:acetonitrile:acetic acid (9:1:0.1, v/v/v)
5. Quantification by LC/MS-MS.

All procedures will be carried out in compliance with departmental SOP.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- LC/MS-MS system:

Name	Specification (Model, Brand etc)	Supplier/Manufacturer
Pump	LC-10ADVP	Shimadzu
	Acquity – UPB	Waters
	Series 200	Perkin Elmer
Autosampler	Acquity – UPS	Waters
Mass Spectrometer	API 4000	Applied Biosystems
Column oven	Acquity	Waters
Switching Valve	EMMA 10 port-2 position	VICI
System Controller	SCL-10AVP	Shimadzu

- Centrifuge: MES, Mistral 3000E
- Ultrasonic processor: Sonics -Vibra Cell VC130
- Sample concentrator: Zymark Turbovap LV sample concentrator
- Reciprocating shaker: Edmund Buhler
- Glass jars
- 50 mL polypropylene tubes: Costar
- 40 mL glass tubes
- Various laboratory glassware
- Various pipettes
- Short form pipettes

Equivalent equipment may be used

Materials

- Acetone — Rathburn, Glass distilled grade
- Acetonitrile — Rathburn, HPLC grade
- Water — In-house, Ultrapure Deionised
- Acetic Acid — BDH AnalaR
- 0.2 µm PTFE membrane filter — Arcodisk

Equivalent or better grade reagents/solvents may be used.

Reagents and Solutions [Category 1a/b, 4d]
Acetonitrile: water (3:1, v/v)
Mix 1500 mL acetonitrile with 500 mL water.

Water: acetonitrile: acetic acid (9:1:0.01, v/v)
Mix 900 mL water with 100 mL of acetonitrile and 1mL acetic acid.

0.1% acetic acid in acetonitrile
Add 1mL acetic acid to 1L acetonitrile and mix well.

0.1% acetic acid in water
Add 1mL acetic acid to 1L water and mix well.

Preparation of Standard Solutions [Category 1a/b, 4b/4d]
Duplicate primary stock standard solutions of OC 56635 are prepared in acetone. Fortification solutions are prepared in acetone from dilutions of the stock solutions. Standard solutions must be stored in glass, under refrigerated conditions, when not in use. Standard solutions should be allowed to warm to room temperature prior to use.

Preparation of Stock Solutions [Category 1a/b, 4d]
In duplicate, accurately weigh ca 10 mg (corrected for purity) of OC 56635 into separate 10 mL volumetric flasks and dilute each to the mark using acetone to give primary stock standards of concentration 1000 µg/mL. (Note: Smaller amounts may be weighed to conserve test substance, final volume should be adjusted such that the final concentration is still 1000 µg/mL.)

Note : Duplicate solutions of each test substance are prepared to check both the accuracy of weighing and solubility of the test substances. Correlation is achieved by LC/MS-MS quantification of appropriately diluted solutions. Only one primary stock standard is used for the preparation of both fortification and calibration solutions.

Calibration Standards [Category 1a/b, 4b]
Prepare appropriate mixed calibration standards in water:acetonitrile:acetic acid (9:1:0.1 v/v/v) to cover the range 0 to 0.006 µg/mL, with a lowest calibration level of 0.00006 µg/mL. Calibration standards may be stored refrigerated for up to three months.

Fortification Solutions [Category 1a/b, 4b]
Prepare suitable fortification solutions in acetone by serial dilution from the primary stock solution (1000 µg/mL in acetone).

PROCEDURES

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Extraction From Soil [Category 1a/b, 4a]

1. Weigh 10 g of soil sample into a 50 mL polypropylene tube. Untreated control samples may be fortified at this point for the determination of procedural recovery (with up to 500 µL of a suitable fortification solution in acetone).
2. Add 25 mL of acetonitrile to the tube. Extract using an ultrasonic processor set to approximately 50% power pulsed for approximately one minute.
3. Centrifuge sample for 2 minutes at 2500 rpm (1400 g). Decant the supernatant into a glass jar.
4. Add 25 mL of acetonitrile: water (3:1, v/v) to the soil pellet and shake on a reciprocating shaker for approximately 10 minutes. Repeat the ultrasonic processor extraction for a further minute.
5. Centrifuge the second extraction (2500 rpm for 2 minutes) and combine the supernatants.
6. Repeat steps 4 and 5 with a further 25 mL of acetonitrile: water (3:1, v/v).
7. Make to 80 mL final volume with acetonitrile.
8. Take a 0.5 g equivalent aliquot (4 mL) and evaporate to approximately 1 mL using a Turbovap set to 40°C.
9. Make samples to volume (10 mL) with water:acetonitrile:acetic acid (9:1:0.1, v/v/v).
10. Further dilute samples as necessary with water:acetonitrile:acetic acid (9:1:0.1, v/v/v) to be within the calibration range

All extracts derived during or from this procedure may be stored refrigerated or frozen if storage is required.

Conditions for UPLC Analysis with MS MS Detection

	LC Conditions
Analytical column#	Waters, C18, 1.7µm, 50 x 2.1mm Part n/o 186002350
In line filter (Acquity)	Supplier: Waters Part n/o 700002775
Column oven temperature#	Nominal 40°C
Autosampler temperature	Nominal 4°C
Mobile phase A#	0.1% acetic acid in water
Mobile phase B#	0.1% acetic acid in acetonitrile
Flow rate#	0.6 mL/min
Split	N/A
Gradient settings:	See table below

Time	A (%)	B (%)
0.0	90	10
0.2	90	10
0.8	65	35
1.1	65	35
1.2	10	90
1.7	10	90
1.8	90	10
2.5	90	10

Acquity run time	2.5 minutes
Switching Valve times	0 – 0.5 mins – To waste 0.5 – 1.5 mins – To MS
Slave pump solvent	MeCN :Water (50:50 v/v)
Slave pump flow rate	0.6 mL/min
Wash solvent 1#	Water: acetonitrile: acetic acid (90:10.0.1, v/v/v)
Weak Wash (Acquity)	
Wash solvent 2#	Mobile phase B
Strong Wash (Acquity)	
Injection mode (Acquity)	Partial loop with needle over-fill
Needle placement	1.0 mm from bottom
Injection volume (Recommended)	10 µL (this may vary depending on instrument performance)

no alternative to be used

Waters Acquity

Weak Wash Volume (µL)	2000 (Range 200 to 5000)
Strong Wash Volume (µL)	2000 (Range 0 to 5000)

Mass Spectrometer Parameters API 4000

Mode of operation#	Turbo IonSpray (negative ion) (MS/MS)
Collision gas setting (CAD)	6 (± 5)
Curtain gas setting (CUR)	20 psi (± 5)
Ion source gas 1 (GS1)	60 psi
Ion source gas 2 (GS2)	70 psi
IonSpray Voltage (IS)	-4500 V (± 2000 V)
Temperature (TEM)	450°C (400-500°C)
Interface Heater Status	On
Analysis time	1.5 minutes (± 1 minute)

Compound name	Ions monitored (± 0.5 Da)	Dwell time (ms)	Declustering Potential (DP) Volts	Collision Energy (CE) Volts	Collision Cell Exit Potential (CXP) Volts
OC 56635	242.7→ 178.9	100	-50	-34	-13

Voltages and dwell times may require optimisation therefore the above values are to be used for reference only.

Entrance Potential (EP)	10 V (± 5 V)
Pause time#	5 ms
Collision gas#	Nitrogen

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

CALCULATION OF RESULTS

The presence of OC 56635 in a sample is confirmed if the resulting peaks arising from the test sample have the same chromatographic retention times as the appropriate standard. All peak measurements and calculations are performed using the current version of Analyst data system.

Residues of OC 56635 are determined by the interpolation of the peak area of OC 56635, from the standard regression equation, as follows:

The calibration line is determined by plotting the responses from the calibration solutions (R) against the of amount test substance injected (A) to generate a straight line graph.

$R = B0 + B1 \times A$
where B1 is the gradient and B0 is the intercept.

Concentrations of test substance (A) in sample extracts are calculated from their response using the equation:

Concentration of extract A (µg/mL) Peak area – intercept)/slope

The residue of OC 56635 in each test sample is calculated as follows:

$$\text{Residue (mg/kg)} = \frac{\text{extract concentration (µg/mL) x final volume (mL) x soil moisture factor}}{\text{sample weight (g)}}$$

$$\text{soil moisture factor} = \frac{100}{100 - \%\,\text{moisture}}$$

Where the final volume includes dilution steps, if applicable and:

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery} \quad (\%) = \frac{A - C}{S} \times 100$$

Where:-

A = concentration found in fortified sample (mg/kg)
C = concentration (or interference) found in control sample (mg/kg)
S = concentration added to fortified sample (mg/kg)

Linearity of Response
The linearity of response of the LC/MS-MS system should be determined with at least six different concentration standard solutions across the range 0 to 0.005 µg/mL, with the lowest calibration level being 0.00006 µg/mL.

METHOD CRITERIA

Analysis by LC/MS-MS will be considered successful only if the following criteria are met.

- A procedural recovery of 70 to 110% will be obtained for each batch of analysis
- Control sample contains a concentration ≤ 30% of the limit of quantification
- At least 6 calibration standards will be used in the determination of each calibration line. A 1/x weighting should be used.
- A coefficient of determination (r^2) for each calibration line will be ≥ 0.98
- All test samples will be within the appropriate calibration standards range

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main	Division	CONTROL Name and Specification
1		GLOVES
	a	Disposable latex
	b	Disposable nitrile
	c	Rubber gloves
	d	Specific type for the job (see assessment giving details)
2		PROTECTIVE CLOTHING
	a	Laboratory coat or equivalent
	b	Disposable overalls
	c	Oversleeves
	d	Overshoes
	e	Plastic apron
3		EYE/FACE PROTECTION
	a	Safety glasses to BS 2092/2 C or better
	b	Face shied to BS 2092/2 C or better
	c	Safety goggles to BS 2092/2 C or better
4		ENGINEERING CONTROLS
	a	Open bench in ventilated area
	b	Fume cupboard to BS 7258
	c	Laminar flow cabinet to BS 5295 Class 1
	d	Re-circulating fume chamber
	e	Radioisotope lab
	f	Biohazard lab
	g	Glove box
5		RESPIRATORY PROTECTIVE EQUIPMENT
	a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
	b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
	c	Respirator with specified canister (HSE approved)
6		SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7		ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8		REFER TO MATERIAL SAFETY DATA SHEET
9		KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10		POISON – ensure antidote is available and is within its expiry date (must specify details)