1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM007.10A is suitable for the determination of mesotrione and its metabolites AMBA and MNBA (Figures 1 - 3) in soil. The limit of quantification (LOQ) of the method has been established at 0.002 mg/kg (or 2 ppb).

This method satisfies US EPA guidelines OCSPP 850.6100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 8.1.

1.2 Method Summary

Soil samples (10 g) are extracted by shaking with 0.05M NH_4OH at room temperature, centrifuging and decanting the supernatant liquid into a separate polypropylene bottle. This procedure is repeated with 0.05M NH_4OH:acetone (50:50, v/v) and finally acetone. The extracts are combined and centrifuged to settle suspended solids. An aliquot of extract is taken and the organic solvent removed by evaporation (N-Evap). The samples are diluted with formic acid. After centrifugation, an aliquot of the extract is transferred to an LC sample vial. Final determination is by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

The limit of quantification of the method is 0.002 mg/kg (0.002 ppb).

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

2.3.1 Stock Solutions

Prepare individual 100 µg/mL stock solutions for mesotrione, AMBA and MNBA by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient mesotrione, AMBA and MNBA analytical standard into separate "Class A" amber volumetric flasks (100 mL size). Dilute to the mark with methanol to give individual 100 µg/mL stock solutions of mesotrione, AMBA and MNBA.

Alternatively, the appropriate volume of methanol to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity.

$$V = \frac{W \times P}{C} \times 1000$$

P	=	Standard purity in decimal form (P(%)/100)
V	=	Volume of methanol required (mL)
W	=	Weight, in mg, of the solid analytical standard
C	=	Desired concentration of the final solution, (µg/mL)
1000	=	Unit conversion factor

In this case, the standard material is weighed directly into an appropriate storage vessel.

2.3.2 Fortification Solutions

Sample fortification solutions containing mesotrione, AMBA and MNBA should be prepared by serial dilution in water/acetonitrile/acetic acid (95/5/0.1, v/v/v) down to 10 µg/mL. Subsequent dilutions to 0.1 µg/mL should be performed in methanol.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

A significant matrix effect (suppression) of the instrument response for AMBA has been observed in the soil types tested using the procedures described in Section 3 during method validation. Therefore, matrix-matched calibration standards should normally be used for quantification of all analytes.

To prepare for example an LOQ equivalent matrix-matched standard, take an additional 10 g aliquot of untreated soil extract and take it through the analytical procedure to step 3.3 i). Take 20 µL of a 0.01 µg/mL mixed mesotrione, AMBA and MNBA standard in methanol and adjust the final volume to 1 mL with untreated soil extract, ultrasonicate

briefly to mix thoroughly. Transfer an aliquot into a suitable autosampler vial for final determination by LC-MS/MS.

A calibration curve may also be generated to quantify mesotrione, AMBA and MNBA residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volume of mesotrione, AMBA and MNBA standards in methanol.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored between 2 and 8°C when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of 87 days for mesotrione, AMBA and MNBA in methanol is recommended (Reference 2) unless additional data are generated to support a longer expiration date.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as 'Hazards in the Chemical Laboratory', edited by S G Luxon, The Royal Society of Chemistry, Cambridge (Reference 3).

Solvent and Reagent hazards

	Acetonitrile	Methanol	Acetic acid	Ammonium Hydroxide	Acetone	Formic acid
Harmful Vapour	✓	✓	✓	✓	✓	✓
Highly Flammable	✓	✓	✗	✗	✓	✗
Harmful by Skin Absorption	✓	✓	✓	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓	✓	✓	✓
Causes severe burns	✗	✗	✓	✓	✗	✓
OES Short Term (mg/m^3)	105	310	37	27	3560	19
OES Long Term (mg/m^3)	70	260	25	17	1780	9

Suitable personal protective equipment should be worn when handling chemicals and reagents and a local risk assessment carried out. In all cases avoid breathing vapour. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form in Appendix 4.

3.1 Sample Preparation

All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis.

3.2 Sample Fortification

To each pre-weighed soil sample (10 g), add the appropriate amount of mixed standard solution containing mesotrione, AMBA and MNBA in methanol. Mix the sample thoroughly by shaking. At least one untreated control and two fortified control samples should be analysed with each sample set.

3.3 Experimental Procedure

A summary of the method is included in flow chart form in Appendix 4.

a) Weigh representative amounts of soil (10 ± 0.1 g) into separate 250 mL polypropylene bottles. At least one untreated control and two control samples fortified with known amounts of mesotrione, AMBA and MNBA in methanol should be analysed with each sample set, using the same procedure, to verify method performance. No more than 1.0 mL of fortification solution should be added. Allow fortified control samples to equilibrate for at least 5 minutes before proceeding with the extraction.

   Add 20 mL 0.05M NH_4OH, cap and shake on a reciprocal shaker at a speed that visibly agitates the samples for a minimum of 30 minutes. Bottles should be placed in an upright or vertical orientation.

b) Centrifuge samples at 10000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes. Decant the supernatant liquid into a separate 250 mL polypropylene bottle.

   Note: With some soils, particularly those with high clay content, the solution may still be visibly cloudy even after centrifugation. This is normal and will not affect results.

c) Repeat extraction using 20 mL 0.05M NH_4OH:acetone (50:50, v/v). Add extraction solvent to the solid soil remaining in the centrifuge bottle from the first extraction at 3.3 (b). Cap and shake by hand or vortex to mix. If shaking cannot break up the compacted soil, use a suitable implement (*i.e.* a spatula) to facilitate this process. Shake on a mechanical shaker at a speed that visibly agitates the sample for a minimum of 30 minutes. Once again, bottles should be placed in an upright or vertical orientation.

Analytical Method (Continued)

d) Centrifuge samples at 10000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes. Decant the supernatant liquid into the polypropylene bottle containing the first extract.

e) Extract a third time using 20 mL acetone. Add extraction solvent to the solid soil remaining in the centrifuge tube from the second extraction at 3.3 (d). Cap and shake by hand or vortex to mix. If shaking cannot break up compacted soil, use a suitable implement (*i.e.* a spatula) to facilitate this process. Shake on a mechanical shaker at a speed that visibly agitates the samples for a minimum of 30 minutes. Once again tubes must be placed in an upright or vertical orientation.

f) Centrifuge samples at 10000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes. Decant the supernatant liquid into the plastic bottle containing the first two extracts and adjust the volume to 60 mL with 0.05M NH_4OH:acetone (50:50, v/v).

Cap and shake briefly to mix.

g) Centrifuge samples at 3500 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes.

h) Remove a 6 mL aliquot (equivalent to 1 g soil) and transfer to a 15 mL disposable, graduated centrifuge tube. Place on an N-Evap unit with a bath temperature of *ca.* 40 ºC and reduce to aqueous. Adjust nitrogen evaporation gas flow and position so that an indentation of 2-4 mm is observed in the top of the sample extract. Reduce to a volume of *ca.* 2.5 mL.

i) Add 2% formic acid to adjust each sample to a final volume of 10 mL. Cap and shake well to mix. Sonicate for 3-5 minutes. Centrifuge at 3500 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes.

j) Transfer an aliquot of sample to an amber glass autosampler vial for analysis by LC with triple quadrupole mass spectrometric detection (LC-MS/MS).

3.4 Experimental Precautions

a) Bottled HPLC grade ultra-pure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

3.5 Time Required for Analysis

The methodology is normally performed with a batch of 12 or more samples. One person can complete the analysis of 12 samples in 1 day (8 hour working period).

3.6 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

The method has been developed for use on an AB Sciex API 5000. The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 Instrument Description

Pump	:	Agilent 1100 series G1312A
Degasser	:	Agilent 1100 series G1379A
Column Oven	:	Agilent 1100 series G1316A
Detector	:	API 5000, AB Sciex with Analyst™ software version 1.4.2
Autosampler	:	Agilent 1100 series G1329A
Nitrogen generator	:	Peak Scientific N300DR

4.2 Chromatography Conditions

Column	:	Phenomenex PLRP-S 100 Å (50 x 4.6 mm, 5µm)
Column Oven Temperature	:	35 ^{o}C
Injection volume	:	100 µL
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Mobile phase	:	Solvent 1 = 0.1% Acetic Acid in Water (HPLC grade) Solvent 2 = 0.1% Acetic Acid in Acetonitrile (HPLC grade)

Mobile Phase Composition

Time (mins)	% solvent 1	% solvent 2	Flow rate (mL/min)
0.0	98	2	0.8
0.1	98	2	0.8
4.1	50	50	0.8
6.0	25	75	0.8
7.0	5	95	0.8
8.0	5	95	0.8
8.2	95	5	0.5
10.0	95	5	0.5

Notes: Under these conditions the retention time of mesotrione is approximately 5.8 minutes, the retention time of AMBA is approximately 3.9 minutes and the retention time of MNBA is approximately 2.6 minutes. If required, a 2 minute equilibration step matching the starting mobile phase composition and flow rate can be added to the timetable.

Valco Valve Diverter Programme

Time	Position
0.0	Waste
1.0	Mass spectrometer
7.0	Waste

4.3 Mass Spectrometer Conditions for Mesotrione, AMBA and MNBA

Interface	:	Turbo Ion Spray
Polarity	:	Negative
Curtain gas (CUR)	:	Nitrogen set at 25 (arbitrary units)
Temperature (TEM)	:	700 °C
Ionspray voltage	:	-4000 V
Collision gas setting (CAD)	:	Nitrogen set at 10 (arbitrary units)
Gas 1 (GS1)	:	Air set at 50 (arbitrary units)
Gas 2 (GS2)	:	Air set at 30 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**Mesotrione primary transition**	**Mesotrione confirmatory transition**	**AMBA primary transition**	**AMBA confirmatory transition**
Q1 *m/z*	:	338.2	338.2	213.8	213.8
Q3 *m/z*	:	291.0	212.1	170.1	64.0
Dwell time	:	150 ms	150 ms	150 ms	150 ms
Resolution Q1	:	High	High	High	High
Resolution Q3	:	High	High	High	High
Declustering potential (DP)	:	-65 V	-65 V	-65 V	-65 V
Entrance potential (EP)	:	-10 V	-10 V	-10 V	-10 V
Collision energy (CE)	:	-14 V	-42 V	-20 V	-58 V
Collision cell exit potential (CXP)	:	-9 V	-13 V	-23 V	-9 V

MRM Conditions		**MNBA primary transition**	**MNBA confirmatory transition**
Q1 *m/z*	:	244.1	244.1
Q3 *m/z*	:	200.0	170.1
Dwell time	:	150 ms	150 ms
Resolution Q1	:	High	High
Resolution Q3	:	High	High
Declustering potential (DP)	:	-65 V	-65 V
Entrance potential (EP)	:	-10 V	-10 V
Collision energy (CE)	:	-12 V	-20 V
Collision cell exit potential (CXP)	:	-11 V	-11 V

Typical chromatograms are shown in the Figures Section.

4.4 Confirmatory Procedures for Mesotrione, AMBA and MNBA

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

5.0 CALCULATION OF RESULTS

5.1 Multi-Point Calibration Procedure

Mesotrione, AMBA and MNBA residues may be calculated in mg/kg for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 30% LOQ to 20 x LOQ). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to mesotrione, AMBA and MNBA. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient of the line of best fit ("X-variable 1" in MS Excel) and c is the intercept value. An example of this equation generated using the experimental values of m and c should be included in the raw data, as should the "R-Squared" value for the regression.

Re-arrangement for *x* gives

$$x = \frac{y - c}{m}$$

e) Calculate the mesotrione, AMBA and MNBA residues in the sample, expressed as mg/kg, as follows

$$\text{Residue } (m\text{g/kg}) = \frac{\text{Analyte found } (\mu\text{g/mL})}{\text{Sample conc.} (\text{g/mL})}$$

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in g/mL, accounting for any concentration in the SPE step where used.

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \ (m\text{g/kg})$$

5.2 Single -Point Calibration Procedure

Mesotrione, AMBA and MNBA residues may be calculated in mg/kg for each sample using a mean standard response from each of the injections bracketing the sample as follows.

a) Make repeated injections of a standard Mesotrione, AMBA and MNBA at an appropriate concentration into the LC-MS/MS operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for mesotrione, AMBA and MNBA.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to mesotrione, AMBA and MNBA.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) Calculate the mesotrione, AMBA and MNBA residues in the sample, expressed as mg/kg using a mean standard response from each of the injections bracketing the sample as follows.

$$\text{Residue (mg/kg)} = \frac{\text{PK area (SA)}}{\text{PK area (STD)}} \times \frac{\text{Standard Conc.}}{\text{Sample Conc.}}$$

PK area (SA) = Peak response for sample
PK area (STD) = Average peak response for bracketing standards
Standard Conc. = Concentration of standard (μg/mL)
Sample Conc. = Sample concentration (g/mL)

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \text{ (mg/kg)}$$

Although single point calibration may be used to quantify residues it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 4).

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analysed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analysed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts of mesotrione, AMBA and MNBA in methanol) should also be analysed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 120% and with a relative standard deviation of $\leq$20%.

Where the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

7.1 Matrix

LC-MS/MS is a highly specific detection technique. Interference arising from the matrices tested has not been observed.

7.2 Reagent and Solvent Interference

Using high purity solvents and reagents no interference has been found.

7.3 Labware Interference

This method uses mainly disposable labware. All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

APPENDIX 4 METHOD FLOW CHART

Weigh 10 ± 0.1g of soil into 250 mL polypropylene bottles.

↓

Add 20 mL 0.05M NH_4OH to the sample and shake on a mechanical shaker for a minimum of 30 minutes

↓

Centrifuge Samples at 10000 rpm for 5 minutes and decant supernatant to separate 250 mL polypropylene bottle.

↓

Repeat extraction by adding 20 mL 0.05M NH_4OH: acetone (50:50, v/v) to solid soil remaining and shake on a mechanical shaker for a minimum of 30 minutes

↓

Centrifuge Samples at 10000 rpm for 5 minutes and decant supernatant to bottle containing first extract

↓

Repeat extraction third time by adding 20 mL acetone to solid soil remaining and shake on a mechanical shaker for a minimum of 30 minutes

↓

Centrifuge Samples at 10000 rpm for 5 minutes and decant supernatant to bottle containing first two extracts

↓

Adjust the volume to 60 mL with 0.05M NH_4OH: acetone (50:50, v/v)

↓

Transfer 6 mL aliquot to 15 mL centrifuge tube and place on an N-Evap unit to reduce to a volume of *ca*. 2.5 mL.

↓

Add 2% formic acid in UPW to adjust final volume to 10 mL.

↓

Cap, shake, sonicate for 3-5 mins and centrifuge for 5 mins at 3500 rpm.

↓

Transfer an aliquot into an amber glass autosampler vial

↓

Analysis by LC-MS/MS