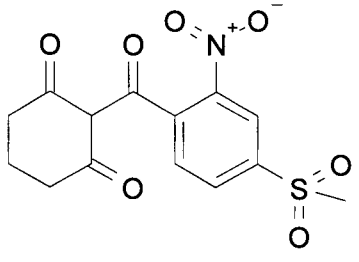
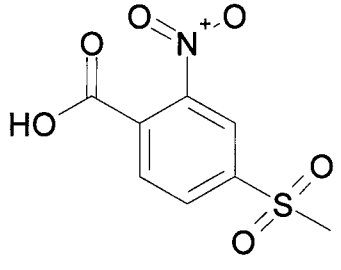
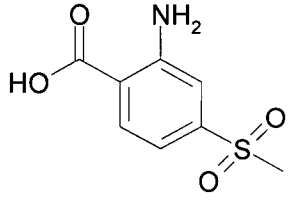
1.0 Introduction and Summary

1.1 Scope and Chemical Structures

Analytical method 1200-03 is suitable for the determination of residues of mesotrione and its metabolites AMBA and MNBA in soil samples. The method LOQ has been established at 2.0 ppb (ng/g). Chemical structures and CAS information for the method analytes are shown below.

Name/Synonym:	Mesotrione
CAS Name:	1,3-Cyclohexanedione, 2-[4-(methylsulfonyl)-2-nitrobenzoyl]-
CAS Number:	104206-82-8
Structure:	

Name/Synonym:	AMBA
CAS Name:	Benzoic acid, 2-amino-4-(methylsulfonyl)-
CAS Number:	393085-45-5
Structure:	

Name/Synonym:	MNBA
CAS Name:	Benzoic acid, 4-(methylsulfonyl)-2-nitro-
CAS Number:	110964-79-9
Structure:	

1.2 Method Summary

Soil samples (10 g) are extracted three times by shaking with solvent at room temperature. 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 acidified and diluted with formic acid to precipitate soil acids. After centrifugation, an aliquot of the extract is transferred to an LC sample vial. Final determination is by high performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS). External standards in 10:90 (v/v) methanol:ultra-pure water are used for calibration.

2.0 Materials and Apparatus

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

2.1 Reagents and Analytical Standards

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. See Appendix 2 for a list of reagents and analytical standards used in this method.

2.2 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 approved eye protection, gloves and a laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area(s) immediately.

All stock solutions are stored in amber glass bottles in a freezer (ca. -20°C) when not in use. No analyte stability or solubility problems have been observed in the standard solutions used in this study when used within a period of at least six months.

Prepare individual 100 ng/µL stock solutions for mesotrione, AMBA and MNBA by one of two methods. The first method is to weigh exactly 10.0 mg of analyte (corrected for purity) into a weighing dish and quantitatively transfer (using methanol) to a "Class A" 100-mL volumetric flask. Next, add additional methanol to the 100 mL mark on the flask.

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

$$V\ (mL) = \frac{wt.\ (mg) \times P}{C\ (ng/uL)} \times 10^3$$

Where "V" is the volume of methanol needed; "wt." is the weight, in mg, of the solid analytical standard; "P" is the purity, in decimal form, of the analytical standard; "C" is the desired concentration of the final solution, in ng/µL; and 10^3 is a units conversion factor. In this second case, the standard material is weighed directly into the amber glass storage bottle.

Sample fortification solutions are prepared in methanol from the two primary stock solutions. It is recommended that, as a minimum, the following concentrations be prepared: 5 ng/µL, 0.5 ng/µL and 0.02 ng/µL. The preparation of LC calibration standards is discussed in section 3.5.

Fortification standards should be stored in amber glass bottles refrigerated at ≤5°C. An expiration date of three months is recommended unless additional data are generated that show a longer expiration date is appropriate. In which case, the expiration date may be extended to a maximum of six months.

2.3 Safety Precautions and Hazards

Whereas most of the chemicals in this method have not been completely characterized, general laboratory safety precautions are advised (e.g., safety glasses, gloves, etc.). The user(s) should consult the relevant MSDS for commonly used reagents and materials.

3.0 Analytical Procedure

Note: Due to the low detection limit of the method it is important that precautions be taken to avoid cross contamination in the laboratory.
Specifically:

- Where possible disposable glassware/plastic-ware has been specified, new glassware/plastic-ware should be used for each batch of samples.
- Each solvent used in the method should be checked prior to use to verify that it is free from contamination.
- Existing glassware should be solvent (acetone or acetonitrile) rinsed, after washing and before use in the method.

3.1 Sample Preparation

It is important that a homogeneous soil sample be available for analysis. All samples should be prepared using an approved method of preparation for residue analysis prior to analysis.

3.2 Extraction

a) Weigh representative amounts of soil (10 ± 0.1 g) into separate 50-mL disposable plastic centrifuge tubes. At least one untreated control and two control samples fortified with known amounts of mesotrione, AMBA and MNBA in methanol should be analyzed 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 mechanical shaker at a speed that visibly agitates the samples for a minimum of 30 minutes. Tubes should be placed in a flat or horizontal orientation.

b) Centrifuge samples at 6000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for about five minutes. Decant the supernatant liquid into a separate plastic 125-mL screw-cap bottle.

Note: With some soils, particularly those with a 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 50:50 (v/v) 0.05M NH_4OH:acetone. Add extraction solvent to the solid soil remaining in the centrifuge tube from the first extraction at 3.2 (b). Cap and shake by hand or vortex to mix. If shaking cannot break up the compacted soil, use a suitable implement (e.g., 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 should be placed in a flat or horizontal orientation.

d) Centrifuge samples at 6000 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 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 3.2 (d). Cap and shake by hand or vortex to mix. If shaking cannot break up the compacted soil, use a suitable implement (e.g., 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 should be placed in a flat or horizontal orientation.

f) Centrifuge samples at 6000 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. Cap and shake briefly to mix.

g) Centrifuge the combined extracts at 6000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes.

h) Remove a 6-mL aliquot and transfer to a 15-mL disposable, graduated centrifuge tube. Place on an N-Evap unit with a bath temperature of ca. 40°C 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 3-5 minutes, if needed. Centrifuge at 4500 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).

3.3 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 working hour period).

3.4 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 in sealed containers at a temperature of ≤5°C (refrigerated) when the analyses cannot be completed in a single working day.

3.5 Preparation of Calibration Standards for LC/MS/MS

Standards for external calibration should be prepared in 10:90 (v/v) methanol:ultra pure water. It is recommended that the following concentration levels be prepared: 100 pg/µL, 5.0 pg/µL, 2.0 pg/µL, 1.0 pg/µL, 0.5 pg/µL, 0.2 pg/µL and 0.1 pg/µL. The 100 pg/µL is not used for calibration, but as a convenient stock to prepare subsequent dilutions.

LC calibration standards should be stored in amber glass bottles refrigerated at ≤5°C. An expiration date of three months is recommended unless additional study data are generated that show a longer expiration date is appropriate. In which case, the expiration date may be extended to a maximum of six months.

4.0 Final Determination by LC/MS/MS

Two different LC systems are recommended for the analysis of mesotrione, AMBA and MNBA. Mesotrione has a tendency to exhibit binding and severe chromatographic peak tailing on most silica-based LC column materials. Polymeric columns appear to be the best choice for LC of mesotrione. Although MNBA and AMBA can be retained on a polymeric column, better retention and peak shape are obtained using the Synergi™ column. In addition, in order to analyze all three analytes on the same polymer column, the starting mobile phase must be made very weak (98% aqueous), which is believed to increase the likelihood of mesotrione carryover in the LC system.

The following instruments and conditions have been found to be suitable for this analysis in this laboratory. Other instruments may also be used, however optimization may be required to achieve the desired separation and sensitivity. Operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 LC System Description and Operating Conditions: System 1

LC Instrumentation: Waters Alliance Model 2695 LC System

System 1: For mesotrione only.

Column Temperature: 30-35°C Injection Volume: 20 µL
Sample Compartment Temp.: ca. 5-10°C (if possible)

Column: Polymer Laboratories PLRP-S, 100Å, 50 x 4.6 mm id (cat. no. 1512-1500)
Guard: Polymer Laboratories PLRP-S, 5 x 3 mm (cat no. 1612-1801) with holder (cat. no. 1310-0016), recommended
Mobile Phase A: 0.1% glacial acetic acid in HPLC grade water
Mobile Phase B: 0.1% glacial acetic acid in acetonitrile

Mobile Phase Program:

Time, min.	%A	%B	Flow, mL/min	Curve*
0.0	85	15	0.8	1
1.0	85	15	0.8	1
3.5	5	95	0.8	6
6.0	5	95	0.8	6
6.5	85	15	1.0	6
8.5	85	15	1.0	1

* 6 = linear, 1 = step immediately to final conditions

Typical Mesotrione Retention Time: ca. 4.2 minutes

Note: To help minimize ion source contamination, it is recommended that a timed event controlled switching valve be used to divert the LC stream to waste during periods of no data collection (e.g., from injection to 3.2 minutes and 5.1 minutes to run completion).

4.2 LC System Description and Operating Conditions: System 2

LC Instrumentation: Waters Alliance Model 2695 LC System

System 2: For AMBA and MNBA only.

These conditions are not suitable for the determination of mesotrione due to significant peak tailing. Keep injection volume at 10 µL, if possible, to minimize matrix effects.

Column Temperature: 30-35°C Injection Volume: 10 µL
Sample Compartment Temp.: ca. 5-10°C (if possible)
Column: Phenomenex Synergi™ 4µ Fusion-RP, 80Å, 75 x 4.6 mm id (cat. no. 00C-4424-E0)
Guard: Phenomenex Fusion-RP guard cartridges, 4 x 3 mm, (cat no. AJO-7557) with holder (cat. no. KJO-4282), recommended
Mobile Phase A: 0.1% glacial acetic acid in HPLC grade water
Mobile Phase B: 0.1% glacial acetic acid in acetonitrile

Mobile Phase Program:

Time, min.	%A	%B	Flow, mL/min	Curve*
0.0	95	5	0.8	1
1.0	95	5	0.8	1
5.0	5	95	0.8	6
8.0	5	95	0.8	6
9.5	95	5	1.0	1

* 6 = linear, 1 = step immediately to final conditions

Typical Analyte LC Retention Times:

Analyte	Approx. Retention time, min.
AMBA	4.8
MNBA	7.6

Note: To help minimize ion source contamination, it is recommended that a timed event controlled switching valve be used to divert the LC stream to waste during periods of no data collection (e.g., from injection to 4.0 minutes and 8.5 minutes to run completion).

4.3 Mass Spectrometer System Description and Operating Conditions

Mass Spectrometer System:

Micromass Quattro Ultima
Instrument Control, Data Collection and Quantitation: Masslynx 4.0

General Operating Conditions:

Source Polarity: Electrospray negative ionization

Capillary (kV)	-2.00
Cone (V)	21.0
Hex 1 (V)	0.0
Aperture (V)	0.0
Hex 2 (V)	0.7
Source Temp (°C)	120
Desolvation Temp (°C)	425
Cone Gas (L/Hr)	ca. 50
Desolvation Gas (L/Hr)	ca. 900

LM 1 Resolution	12.5
HM 1 Resolution	12.5
Ion Energy 1	0.3
Entrance	0.0
Collision	8
Exit	1.0
LM 2 Resolution	12.0
HM 2 Resolution	12.5
Ion Energy 2	1.5
Multiplier (V)	650

Adjust collision cell gas pressure while infusing the analytes to yield maximum product ion response. Analyte specific MRM settings are shown below.

MRM Operating Conditions:

MS/MS Transitions

Analyte	MW (exact)	MS/MS Transition
Mesotrione	339.04	338.2 → 291.0
AMBA	215.03	214.0 → 169.9
MNBA	245.00	244.0 → 199.8

MRM Experiment Settings

	Dwell (s)	Cone (V)	Coll (eV)	Delay (s)
Mesotrione	0.40	30.00	10.00	0.10
AMBA	0.30	59.00	17.00	0.10
MNBA	0.30	20.00	7.00	0.10

Final determination by LC/MS/MS is considered to be highly specific; therefore no confirmatory conditions are included. MS/MS product ion scans showing the fragmentation of Mesotrione, AMBA and MNBA are shown in Figure 2. Representative MS/MS chromatograms of external standards and samples are shown in Figures 3 through 8.

Note: These settings are specific for the Micromass Quattro Ultima used for method development and validation. These settings will need to be optimized for the user's specific instrument and operating conditions.

5.0 Calculation of Results

Determination of Residues in Samples:

Inject the sample extract from 3.2(j) into the analysis system. The sample solution must be diluted if the analyte response exceeds the linear range of the calibration curve. Quantitation is achieved using a linear least squares curve fit to the external standards. Acceptable calibration curve fits include linear, linear forced through zero, or linear weighted 1/x, as appropriate.

Determination of Residues in Fortified Samples:

Validate the method performance for each set of samples analyzed by including a control sample and two or more control samples fortified with known amounts of mesotrione,

AMBA and MNBA prior to the extraction procedure. The fortification levels for external recoveries should approximate the expected residue levels in the study samples.

Recovery data are generally considered acceptable when the mean values are between 70% and 120% with a relative standard deviation of ≤20%.

Calculations:

Calculations may be performed by computer program (preferred) or manually as shown below.

Calculate the analyte concentration (in ppb) for field-incurred residues using the equation:

$$\text{RES(ppb)} = \frac{\text{Analyte found (ng)}}{\text{SWI (mg)}} \times 1000$$

where RES is the residue value in ppb, analyte found (ng) is calculated from the standard calibration curve, and SWI is the sample weight injected (mg). NOTE: If the analyte found is calculated in units of pg (instead of ng), the 1000 multiplication factor is not needed.

The amount, in milligrams, of sample weight injected (SWI) can be calculated using the equation:

$$\text{SWI(mg)} = \frac{\text{FW(g)} \times \text{IV}(\mu\text{L})}{\text{FV(mL)}}$$

where FW = final sample weight (g), IV = LC injection volume (µL) and FV = final volume in which sample is dissolved (mL).

The final sample weight (FW) is calculated by the equation:

$$\text{FW(g)} = \left[\frac{\text{SWE(g)} \times \text{A1(mL)}}{\text{EV(mL)} + \{\text{SWE(g)} \times \text{M(\%)}/100\}}\right] \times \left[\frac{\text{A2(mL)}}{\text{INV(mL)}}\right]$$

where FW = final weight (g), SWE = sample weight extracted (g), A1 = aliquot 1 volume (mL), EV = total extraction solvent volume (mL), M = sample moisture in percent, A2 = aliquot 2 volume (mL), if needed, INV = interim volume (mL) is the total volume from which the 2nd aliquot is taken.

NOTES: Either actual or nominal sample weights may be used in the calculations. All of the calculations performed in this report used the 10.0 g nominal sample weight. For method performance (recovery) samples, the M% (moisture) value is set to zero since the fortifications are based upon their wet weights. If no sample dilutions are performed, the second term in the equation (i.e., A2/INV) is equal to one.

Corrections may be made to the residue value (RES) calculated above. At the discretion of the study director, this value may be corrected to account for the average recovery and/or

sample moisture.

The recovery factor, expressed as a percentage (R%), is calculated using the following equation.

$$R\% = \frac{\text{RES fortified (ppb)} - \text{RES control (ppb)}}{\text{ppb analyte added}} \times 100$$

To correct a residue value to its dry weight value, the following equation may be used:

$$SDW(ppb) = \left[\frac{CR(ppb)}{\frac{(100 - M(\%))}{100}} \right]$$

where SDW = soil dry weight residue (ppb), CR = corrected soil residue, and M = soil moisture (%). For study samples, soil moistures should be determined following the appropriate SOP.

The recovery corrected soil residue can be determined by the equation:

$$CR(ppb) = \left[\frac{RES(ppb)}{AR(\%)} \right] x100$$

where CR = recovery corrected residue (ppb), RES = residue found (ppb) and AR = average recovery (%).

When the average percentage recovery is greater than 100%, the sample residue values should not be corrected.

6.0 Interferences and Confirmation

Due to the high selectivity of the detection technique, interference arising from the sample matrix has not been observed. Final determination by LC/MS/MS is considered to be highly specific; therefore no confirmatory conditions are included.

During method development, matrix suppression of the signal for AMBA was observed at higher injection volumes (20 µL or more). To minimize this effect, the injection volume for this analysis should be set to 10µL, if possible. Matrix effects are variable by soil type and location and should be periodically evaluated if recovery issues are encountered.

There is a likelihood that a small amount of mesotrione (parent) carryover may be observed from the LC system, especially after high level standards or residue detections. As a precaution, analyze controls, low level recoveries and lower standards at the beginning of the injection sequence. In addition, it may also be prudent to inject 10:90 MeOH:water blanks after high residues to help clean out the system.

It is recommended that each batch of solvent or reagents be checked for potential contamination prior to use. This method uses disposable labware, where possible. All reusable glassware should be detergent washed then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

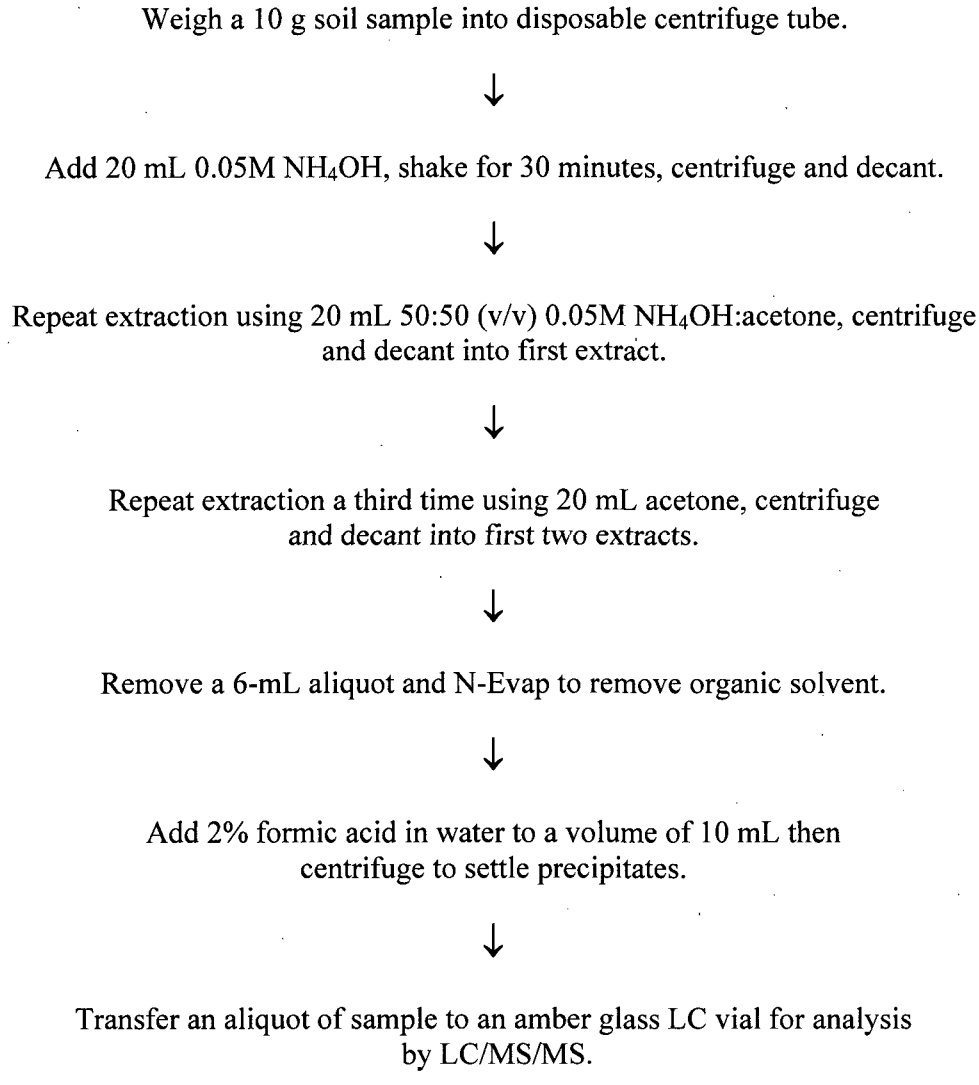
7.0 Modifications and Potential Problems

It is possible that contaminants from chemicals, solvents, glassware, etc. may interfere with the analysis and give a false positive result. It is recommended that reagent blank samples be included in a sample set if contamination is suspected. In addition, some carryover of mesotrione in the LC system may occur as discussed above.

Any modifications to this method must be documented in the study raw data.

12.0 Figures

Figure 1. Method 1200-03 Flow Diagram



13.0 Appendices

Appendix 1. Apparatus

General laboratory glassware (beakers, graduated cylinders, disposable pipets, pipet bulbs, etc.) available from a general laboratory supply company.

Balance, analytical (Sartorius R160P), or equivalent. Electronic display of 0.01 mg, for weighing in preparation of the stock standard solutions.

Balance, laboratory (Mettler model BB1300), or equivalent. Electronic display of 0.01 g, for weighing soil samples.

Bottles, amber Boston round, 2 oz. and 4 oz., with Polyseal-lined cap (Fisher Scientific cat. nos. 03-320-4A and 03-320-4B) or equivalent.

Mixer, Vortex-Genie 2 (Fisher Scientific cat. no. 12-812) or equivalent.

Pipets, glass, Class A certified, assorted volumes. (These pipets should be used for sample fortification and standard solution preparations).

Pipetter, Eppendorf Repeater, 100 – 1000 µL variable volume range (VWR cat. no. 53511-582) and 500-5000 µL variable volume range (VWR cat. no. 53513-412), or equivalent.

Mechanical reciprocating shaker, IKA Labrotechnik Model HS501 digital, 300 rpm, or equivalent.

Centrifuge, Sorval Super T21, Kendro Laboratory Products, or equivalent.

Centrifuge, Centrific Model 225 (Fisher Cat. No. 04-978-50), or equivalent.

N-Evap Model 111 nitrogen evaporator (Organomation Associates, Berlin, MA), or equivalent.

Tubes, disposable centrifuge, polypropylene, 50-mL (VWR cat. # 21008-240) and 15-mL (VWR cat. # 21008-214) graduated with plastic screw cap, or equivalent.

Ultrasonic bath, Cole-Parmer Model 8893, or equivalent.

LC Vials, amber snap cap ID, 1.5 mL (National Scientific, Inc. cat no. C4001-6W) and pre-slit snap-it™ caps (National Scientific, Inc. cat no. C40011-55), or equivalent.

Appendix 2. Reagents and Analytical Standards

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. All reagents and polypropylene glycols are stored at room temperature. Solid analytical standards are stored in a freezer (temperature < -10°C) unless specified otherwise on the sample shipment paperwork.

Mesotrione, obtained from the Analytical and Product Chemistry Department, Syngenta Crop Protection, Inc., Greensboro, North Carolina.

AMBA and MNBA, obtained from the Technology Support/Chemistry Group, Syngenta Crop Protection, Inc., Greensboro, North Carolina.

Acetone, HPLC grade (VWR cat. no. JT9002-33), or equivalent.

Acetonitrile, HPLC grade (VWR cat. no. EM-AX0145-1), or equivalent.

Methanol, HPLC grade (Fisher cat. no. A452SK-1), or equivalent.

Acetic acid, glacial, HPLC grade or better (VWR cat. no. EM-AX0074-6), or equivalent.

Formic acid, 88%, certified ACS (Fisher cat. no. A118P-100), or equivalent.

Water, ultra pure or HPLC grade (Fisher cat. no. W5SK-4), purified in-house with a HYDRO® purification system or equivalent.

Ammonium hydroxide, certified ACS plus (Fisher cat. no. A669-212), or equivalent.

0.05M ammonium hydroxide extraction solution. To prepare one liter, add 3.4 mL ammonium hydroxide to ca. 900 mL purified water. Dilute to 1 liter with purified water, mix well and cap tightly when not in use. Store at room temperature.

2% formic acid solution: Add 2.0 mL of concentrated formic acid to 98 mL purified water. Mix well and store at room temperature. (Recognize that the actual assay amount of formic acid is slightly less than 2%.)

LC Mobile Phase A: 0.1% glacial acetic acid in water. To prepare one liter, add 1 mL of HPLC grade glacial acetic acid to one liter of HPLC grade water. Mix well and store at room temperature.