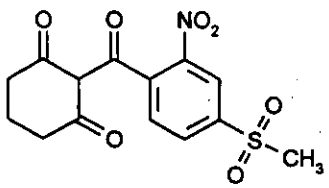
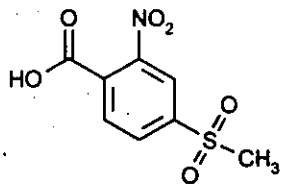
1 Introduction and Summary

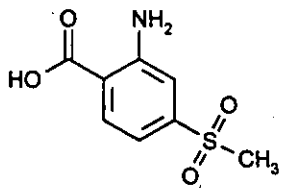
This method is intended for the determination of residues of 2-(4-methylsulfonyl-2-nitrobenzoyl)-1,3-cyclohexanedione (ZA1296), 4-methylsulfonyl-2-nitrobenzoic acid (MNBA), and, 2-amino-4-methylsulfonyl-benzoic acid (AMBA) in soil. MNBA and AMBA are the principal metabolites of ZA1296. Chemical structures and molecular weights (mw) are shown below.



ZA1296
MW = 339 daltons



MNBA
MW = 245 daltons



AMBA
MW = 215 daltons

ZA1296, MNBA, and AMBA residues are extracted from soil with 0.05 N ammonium hydroxide. The crude extract is adjusted to a pH of 3.5-4.0, then filtered and subjected to high performance liquid chromatography (HPLC) analysis for the direct determination of AMBA using fluorescence detection. Concurrently, two eluate fractions corresponding to the elution volumes of ZA1296 and MNBA are collected from the HPLC. The MNBA, now isolated in the first fraction, is chemically reduced to 2-amino-4-methylsulfonyl-benzoic acid (AMBA) using a solution containing stannous chloride in HCl. The ZA1296 residue, isolated in the second fraction, is oxidized to MNBA using hydrogen peroxide. After elimination of excess peroxide with catalase enzyme, the newly formed MNBA is reduced to AMBA using $SnCl_2$ and HCl. After final cleanup by C_{18} solid phase extraction, both the ZA1296 and MNBA fractions are each analyzed for the AMBA conversion product by a reversed-phase HPLC system using fluorescence detection. The external standard method uses ZA1296 and MNBA standards that have been converted to AMBA concurrent with the samples. The limit of quantitation for each analyte is 0.005 mg/kg. The upper limits validated are 0.20 mg/kg for ZA1296, 0.05 mg/kg for MNBA and 0.01 mg/kg for AMBA.

This report is an addendum to Report No. TMR0661B and includes modifications made prior to, and as a result of, the Independent Laboratory Validation (ILV).

2 Materials and Methods

The equipment and reagents described below were used to generate the data and chromatograms presented in this report. Equipment with equivalent performance specifications and reagents of comparable purity can be used.

2.1 Apparatus

2.1.1 High Performance Liquid Chromatograph

Hewlett-Packard (HP) model 1090, equipped with a UV diode-array detector (DAD), HP model 1046 fluorescence detector (FLD), autosampler, 250-µL injection volume option, and column oven. System control and data handling were done using the DOS HPLC 3D ChemStation software, version A.03.02.

2.1.2 Injection Volume Modification for HP1090 HPLC

This modification increases the injection volume to a maximum of 500 µL and requires the following. Two, 250-µL sample loops installed in series (HP # 79846-87613), Hamilton series 1700 gas-tight 500-µL syringe (Hamilton #81230, Fisher #14-815-113). Waste sleeve, 250 µL, modified to fit 500-µL syringe plunger (HP 79846-24502). Note: the HP ChemStation software only recognizes syringes up to 250 µL. Once this modification has been made, all actual injection volumes will be two times the volume specified by the ChemStation software. Due to the larger syringe volume, reduce the syringe draw rate to about 200 µL/minute.

2.1.3 Fraction Collector

Waters fraction collector (#37040), equipped with a 3-way valve (# 37049) for waste diversion, and a vial tray adapter for 4-mL vials (#37044).

2.1.4 Solid-Phase Extraction (SPE) Manifold

24-unit SPE manifold (Baxter #9401-DK), extra 24-hole top plate with 16-mm diameter holes for setting up elution rack (Baxter #9421-DK).

2.1.5 Evaporation Manifold

12-unit evaporation manifold with Dri-Bath and aluminum heating block with 15- or 16-mm tube size (Chemical Research Supplies #201188). Note: this item was used for work conducted for this report. The evaporator manifold has been discontinued by the manufacturer. Item 2.1.6, below (or equivalent) may be substituted.

2.1.6 Evaporator

Techne Sample Concentrator and Dri-Bath Block Heater (Techne Inc.. Model No. DB-3) or N-Vap Evaporator manifold or equivalent evaporator designed to provide a controlled flow of nitrogen to a vial maintained at a precisely controlled temperature.

2.1.7 HPLC Column, HPLC Cleanup and Analytical Determination

Phenomenex Prodigy C_{18}, 5-µm particle size, 250 x 3.2 mm i.d. (Phenomenex #00G-3300-RO) with a guard column (Phenomenex #03A-3300-RO). An equivalent column is the Inertsil ODS-2, 5-µm particle size, 250 x 3.0 mm i.d. (MetaChem #0296-250-X030, or Keystone 255-181-3).

2.1.8 HPLC Column - Confirmatory

Keystone Prism reversed-phase, 5-µm, 250 x 3.2 mm i.d. (Keystone # 255-321-3); guard column (Keystone #864-025-321).

2.1.9 Eppendorf Automatic Pipet

Eppendorf Variable volume pipet with disposable tips; 0.1-10 µL (Brinkman #2244-0004, Fisher #21-381-200), 10-100 µL (Brinkman #2244-0101, Fisher #21-371-202), 200-1000 µL (Brinkman #2244-0209, Fisher #21-371-204), EDP-Plus Electronic Motorized Pipet, 2500 µL (Rainin # EP-2500).

2.1.10 Disposable Syringe Filters

Whatman Anotop inorganic membrane filters, 0.2-micron pore size with Luer hub, 25-mm diameter (Fisher #09-911-2, Whatman #6809-4022).

2.1.11 Disposable Polypropylene Syringe

3-mL syringe with Luer hub.

2.1.12 Peroxide Test Strips

Quantofix brand peroxide test strips, range 0-100 mg/L (Baxter # P1127-10).

2.1.13 pH Test Strips

Range 0-6 pH units (Baxter # P1119-5A).

2.1.14 Syringes, Standard Aliquoting

25-, 100-, 250-µL capacity Gas-Tight syringes (Hamilton 1700 series) for aliquoting calibration and fortification solutions.

2.1.15 Repipet Dispensers

Universal Repipet Dispensers; 1.0-mL for dispensing conversion reagents (Fisher # 13-687-18), 10-mL for dispensing C_{18} SPE mobile phase solutions (Fisher #13-687-20), 100-mL for dispensing extracting solution (Fisher #13-687-23).

2.1.16 Vials, Sample

4-mL screw-top vials, amber glass, silanized (Supelco #2-7216) for HPLC fraction collection; 4-mL screw-top vials, clear glass, silanized (Supelco #2-7220) for collection of post-conversion SPE cleanup eluate.

2.1.17 Caps, Vial

Teflon-lined caps for 4-mL vials, 13-425 (Fisher # 06-406-40).

2.1.18 Vials, Autosampler

2-mL crimp-top, silanized autosampler vial (Supelco # 2-7061).

2.1.19 Glass Pipettes

2-, 5-, and 10-mL disposable glass pipettes for general use.

2.1.20 Glass Bottles

4-oz, wide-mouthed bottles, with lids.

2.1.21 Centrifuge Tubes

Disposable, 50-mL polypropylene centrifuge tubes (Fisher #05-538-60).

2.2 Reagents

2.2.1 High Purity Water

HPLC grade or equivalent, for preparation of HPLC mobile phase.

2.2.2 Water

Distilled or deionized, for all uses other than HPLC mobile phase preparation.

2.2.3 Acetonitrile

HPLC grade or equivalent.

2.2.5 Methanol

High purity for pesticide residue determination.

2.2.6 Ammonium Acetate

HPLC grade.

2.2.7 Stannous Chloride

ACS grade.

2.2.8 Hydrogen Peroxide

30%, ACS grade.

2.2.9 Formic Acid

88%, ACS grade.

2.2.10 Hydrochloric Acid

Concentrated, ACS grade.

2.2.11 Catalase Enzyme

Sigma # C40.

2.2.12 0.2 % Formic Acid in Water

Add 2 mL of formic acid to 1000 mL of deionized or distilled water.

2.2.13 0.2 % Formic Acid in Acetonitrile

Add 2 mL of formic acid to 1000 mL of HPLC grade acetonitrile.

2.2.14 Stannous Chloride Reagent, 60 mg/mL $SnCl_2$ in 2 N HCl

Add 3.6 g of stannous chloride to 10 mL of concentrated HCL, swirl to dissolve. Dilute by adding 50 mL of deionized or distilled water.

2.2.15 Hydrogen Peroxide Reagent, 6% H_2O_2

Dilute 60 mL of 30% hydrogen peroxide to 300 mL with deionized or distilled water.

2.2.16 Catalase Reagent, ~ 20,000 units per mL

Dissolve 10 mg (equivalent to 20,000 activity units/per mg) of catalase enzyme in 10 mL of deionized or distilled water.

2.2.17 HPLC Mobile Phase A, 9 mm ammonium acetate and 0.25% formic acid

Add 2.76 g of ammonium acetate to a 4-L bottle of HPLC grade water, add 10 mL of formic acid, shake well to dissolve.

2.2.18 HPLC Mobile Phase B, acetonitrile:water (95:5)

Add 50 mL of HPLC grade water to 950 mL of HPLC grade acetonitrile.

2.2.19 UV Calibration Solution For Establishing Analyte Retention Times

A solution containing 0.05 µg/mL each of MNBA, AMBA, and ZA1296. Stock standards are serially diluted to this level using methanol:0.2% formic acid in water (7:93, v:v).

2.2.20 Acetate Buffer

0.1 M acetate buffer, pH 4.7. Prepare by combining 0.77 g of ammonium acetate, 0.58 mL of glacial acetic acid and 200 mL of distilled or deionized water.

2.2.21 Acetic Acid

Glacial acetic acid, ACS grade.

2.2.22 Solid Phase Extraction (SPE) Columns

500 mg, C18 packing material, 10 mL eluant reservoir . Varian Bond Elut LRC columns (Varian # 1211-3027) or equivalent.

2.2.23 Soil Extracting Solution, 0.05 M Ammonium Hydroxide

Combine 3.4 mL concentrated ammonium hydroxide and 1 liter of deionized water.

2.3 Reference Materials

2.3.1 ZA1296, MNBA, and AMBA Reference Standards

Available from Zeneca Inc., 1200 South 47th Street, Box Number 4023, Richmond CA 94804-4023; Attention C. Doss, Product Development Department. The ZA1296 was of 99.7% purity and had the reference number ASW-1662R 1995-I15. The MNBA was of 99% purity and had the reference number ASW-1580A 1995-I15. The AMBA was of 99% purity and had the reference number ASW-1664R 1995-I15.

2.3.2 Stock Calibration and Fortification Solutions

Two stock ZA1296 solutions are prepared in methanol. Each solution is prepared independently from a separate weighing of ZA1296. One is designated as a stock fortification solution. The other is designated as a stock calibration solution. Two stock MNBA solutions, and two stock AMBA solutions are prepared in a similar manner. Fortification solutions are used to fortify untreated (control) samples and demonstrate recovery. The calibration solutions are used to calibrate the instrument. To prepare each of these solutions, at a nominal concentration of 1.0 mg/mL, place a known quantity (± 0.1 mg) of approximately 50 mg active ingredient into a 4-oz, narrow-mouthed bottle. Add to the bottle a known amount of methanol, to produce a solution of approximately 1.0 mg active ingredient/mL. Calculate the amount of methanol needed to produce a 1.0-mg/mL solution as follows:

$$A = \frac{(W \times P \times D)}{C(ss)}$$

Where:

C(ss) = Target concentration of the analyte in the final stock solution (mg/mL)

W = Weight of primary standard (mg)

P = Purity of primary standard (100% = 1.00)

D = Density of methanol (g/mL)

A = Weight of methanol

2.3.3 Working Fortification Solutions

Prepare working fortification solutions by diluting aliquots of the fortification stock solutions with methanol. For example, to prepare a 100 µg/mL working solution, place 5.0 g of ZA1296 stock fortification solution (1.0 mg/mL) in a 4-oz, narrow-mouthed bottle. Dilute with methanol to a total weight of 50 g. Although dilution by weight is described here, dilution by volume is not precluded. The concentration of the analyte in this solution is calculated as follows:

$$C(ws) = \frac{C(ss) \times W(ss)}{W(ws)}$$

Where:

C(ws) = Concentration of analyte in the working fortification solution (µg/mL)

C(ss) = Concentration of analyte in the stock fortification solution (µg/mL)

W(ss) = Weight of stock fortification solution added (g)

W(ws)=Final weight of working fortification solution (stock solution and diluant, g)

Serially dilute the above working solution to obtain other working fortification solutions as needed. Prepare similar MNBA and AMBA working fortification solutions. To increase flexibility in the fortification schemes, fortification solutions with one analyte per solution are recommended.

2.3.4 Intermediate Calibration Standard Solutions

Intermediate calibration standard solutions are prepared by serially diluting stock standard solutions as described in Section 2.3.3, using methanol as the diluant. To quantitate a sample which has an analyte concentration ranging from 0.005 to 0.20 mg/kg, and a final extract concentration equivalent to extractives from 0.09 g of soil per mL of extract, intermediate standard solutions of 0.01 and 0.10 µg/mL are needed for each analyte. The intermediate standard solutions will be aliquoted and converted to AMBA to give the final calibration solutions described in Section 3.7. Since this method involves the separate conversion of each analyte to a common moiety, calibration solutions are required to contain only one analyte per solution.

2.3.5 AMBA Calibration Solutions

AMBA calibration solutions are required for quantitation of AMBA residues during the fraction collection runs. They are also used for monitoring the ZA1296 to AMBA, and the MNBA to AMBA conversion efficiencies. A stock solution is prepared in methanol, as described in 2.3.2. Serial dilutions are made using water as a diluent. Due to the differing densities of methanol and water, the first dilution from the stock solution should be made by volume. Once the methanol concentration in the diluted standard becomes negligible, further dilutions can be made by weight, as described in 2.3.3. AMBA calibration solutions with concentrations of 1.0 and 10 ng/mL are for quantitation of AMBA residues during the fraction collection.

2.3.6 UV Calibration Solution

A UV calibration solution containing all the analytes combined is used to establish analyte retention times so that the appropriate HPLC eluate fractions are collected. The intermediate solution is prepared at a concentration of 10 µg/mL, each analyte. To prepare, transfer 0.25 g of each of the three, 1.0 mg/mL stock solutions (ZA1296, MNBA, and AMBA) to a 2-oz. narrow mouthed bottle. Dilute with methanol to a total weight of 25 g (concentration of each analyte = 10 µg/mL). Prepare a diluting solution by combining 7 mL methanol and 93 mL of water containing 0.2% formic acid. Transfer 0.25 mL of the combined intermediate calibration solution to a 2-oz, narrow-mouthed bottle. Dilute with 50 mL of the diluting solution to produce a working UV calibration solution with a concentration of about 0.05 µg/mL. The final calibration solution will contain approximately 7.5% methanol.

3 Analytical Procedure

3.1 Soil Extraction

Place a 10-g subsample of a homogeneous soil sample into a 4-oz, wide-mouthed jar equipped with a PTFE liner. Add 50 mL of 0.05 M ammonium hydroxide extraction solution. Place on a mechanical shaker and shake for 30 minutes Centrifuge for 15 minutes at about 2200 rpm.

3.2 pH Adjustment and Filtration

Transfer approximately 30 mL of the supernatant to a 50-mL polypropylene centrifuge tube. Adjust the pH of the aliquot to approximately 3.5 - 4.0 with formic acid. The amount of acid required may vary by soil type. Most soils require 50 µL, while some may require up to 100 µL. Add an initial amount, then cap and shake the tube. Check the pH using the appropriate pH indicating strip. Add more formic acid if needed. Repeat the pH measurement. Centrifuge for

10 min at about 2000 RPM. Allow to stand for 15-30 min, then filter through a 0.2-µm or 0.45-µm syringe filter unit. If the solution looks cloudy prior to the HPLC cleanup step, refilter. Reducing the pH to 3.0 or less may cause adsorption of AMBA onto precipitated material. Losses of AMBA may also occur in the filtration step at a pH of 3.0 or less.

3.3 HPLC Cleanup

3.3.1 Determination of the Fraction Collector (FC) Delay

To set accurate fraction collection windows, the fraction collector delay must first be determined. This delay is defined as the amount to time required for an analyte to travel, via connecting tubing from the UV diode array detector (DAD), as measured by the DAD retention time, to the outlet of the fraction collector. The normal instrument configuration places the DAD, fluorescence detector (FLD) and fraction collector (FC) in series after the HPLC column. The DAD-to-FLD delay can be measured by making an injection of an AMBA standard and subtracting the AMBA retention time measure by the FLD from the retention time measured by the DAD. The FLD-to-FC delay can be estimated by measuring the volume of the tubing connecting the FLD to the FC and dividing by the HPLC flowrate. (Note: 0.02 in. i.d. Teflon tubing is recommended for this connection.) Adding the DAD-to-FLD delay to the FLD-to-FC delay results in the total DAD-to-FC delay. See Appendix A for an example.

3.3.2 UV Retention Time Calibration

The retention behavior of the analytes MNBA and ZA1296 must first be determined prior to isolation by HPLC fraction collection. See Section 4.1.1 for HPLC parameters. Prior to each fraction collection run a UV calibration solution is injected. This solution contains MNBA, AMBA, and ZA1296, each at a concentration of 0.05 µg/mL. Make three, 500-µL injections of this standard. Monitor the calibration by UV detection. If needed, make additional injections until retention times have stabilized. Following the last injection of the UV calibration standard make 2 injections of a water blank to minimize the chance of analyte carry-over. See Figure 1 for chromatograms of UV calibration solution and crude soil extract.

3.3.3. Fraction Collection Window Determination

The fraction collector delay time determined in Section 3.3.1 is added to each of the UV retention times determined in Section 3.3.2., resulting in the fraction collector elution time for each analyte. Fraction collection windows for each analyte are nominally defined as the analyte fraction collector elution time ± 0.6 minutes. This window may be extended if the UV calibration indicates tailing analyte peaks. If needed, the window may be narrowed in an effort to reduce interfering co-extractives. Windows should also be modified to avoid collection of any AMBA residue that may be present. An overview of the fraction collection window determination procedure can be found in Appendix A.

3.3.4 Analyte Isolation and Fraction Collection; Concurrent Determination of AMBA

Make a single, 500-µL injection (equal to 0.10 g of soil) of each soil extract into the HPLC system. Collect eluate fractions, corresponding to the fraction collection windows determined for MNBA (referred to as fraction A) and ZA1296 (fraction B), as determined in Section 3.4.3. Collect the eluate fractions in clean 4-mL silanized, amber glass vials (see Appendix D). Also make a single injection of a water blank and collect fractions. Fractions resulting from this water blank are referred to as the HPLC reagent blank (HRB). In addition, inject 1.0 and 10.0 ng/mL AMBA calibration solutions for quantitation of AMBA. After every 4-6 extract injections reinject the 1.0 ng/mL AMBA calibration standard. Do not collect fractions during of the analysis of the AMBA standards. Monitoring the retention time of AMBA also helps to verify that the retention times remain constant and ensure that any residues elute in the collected fractions.

3.3.5 Evaporation of Extract Fractions

After collection, evaporate eluate fractions to dryness under a stream of nitrogen. Vials may be placed in a heating block maintained at a temperature of 68-72°C to facilitate evaporation. It is critical to completely dry the fractions since small amounts of acetonitrile can reduce the effectiveness of the catalase reaction in Section 3.4.2. However, care should be taken to prevent over-drying and potential loss of analyte. Remove each vial from the heating block as soon as it appears dry. Once all vials are removed, place them in a heating block maintained at room temperature and continue drying with a gentle stream of nitrogen for an additional 5 minutes.

3.4 Conversion of ZA1296 Residues to AMBA

3.4.1 Oxidation of ZA1296 Residues to MNBA

Add 500 µL of a 6% hydrogen peroxide solution to each of the vials (fraction B) containing ZA1296 residues. Tightly cap and heat the vials at 68-72°C for 35 minutes. Shake every 5-8 minuets during heating. Remove from heating block and allow to cool to room temperature.

3.4.2 Elimination of Excess Hydrogen Peroxide

Using an Eppendorf pipet, carefully add 30 µL of a solution containing ca 20,000 units/mL of catalase enzyme. Addition of the enzyme will cause bubbling and foaming due to the rapid generation of oxygen. As a result, move the vial away from other open vials. Due to the vigorous bubbling and aerosol formation, a new Eppendorf pipet tip should be used for the addition of the catalase to each vial. This will avoid contamination of the catalase reagent and other sample vials. (If the reaction is too vigorous, add the catalase very slowly, allowing the reagent to slowly flow down the side of the vial.) Swirl the contents gently until the vigorous bubbling subsides, then cap the vial. Shake well, allow contents to drain to bottom of the vial, then carefully vent the cap. Tighten the caps and place vials in a rack and place the rack on a mechanical shaker for 5-10 minutes.

3.4.3 Determination of Residual Peroxide

The elimination of peroxide must be confirmed before proceeding with the following reduction reaction. Using an Eppendorf pipet, transfer 1 µL of the contents of each vial to a peroxide-indicating test strip. An immediate (3-5 sec.) blue color indicates the presence of peroxide. If peroxide is indicated, cap the vial and shake for 5 minutes before retesting. If the test is still positive add an additional 20 µL of the catalase reagent, shake for 5 minutes, then retest. Note: 2-4 tests can be performed using each test strip.

3.4.4 Reduction of MNBA to AMBA

Add 500 µL of the stannous chloride reagent (60 mg $SnCl_2$ / mL of 2 N HCl) to each vial. Tightly cap the vial and heat at 68-72°C for 20 minutes. Shake vials every 5 minutes. Remove and allow to cool to room temperature. Add 970 µL of distilled or deionized water to each vial, cap and shake. If additional catalase reagent was added in step 3.5.3, reduce the amount of water added by the same amount. The total volume of all reagents added should be 2.0 mL. These samples will next undergo cleanup in Section 3.6.

3.5 Reduction of MNBA Residues to AMBA

Add 500 µL of distilled or deionized water to each vial (fraction A) containing MNBA residues. Add 500 µL of the stannous chloride reagent (60 mg $SnCl_2$ / mL of 2 N HCl) to each vial. Tightly cap the vial and heat at 68-72°C for 20 minutes. Shake vials every 5 minutes. Remove and allow to cool to room temperature. Add 1000 µL of distilled or deionized water to each vial, cap and shake. The total volume of all reagents added should be 2.0 mL.

3.6 Post-Conversion Cleanup of All Fractions

1. Condition a 500 mg, C_{18} SPE column with 5 mL of acetonitrile containing 0.2% formic acid. Follow with 8 mL of distilled or deionized water containing 0.2% formic acid. Elution should be by gravity flow (except where noted). Gravity flow will stop without analyst intervention when reservoir is empty without draining column bed.

2. Using an Eppendorf pipet, transfer the entire contents of the vial to the column reservoir and allow to elute to waste.

3. Rinse the vial with 1.0 mL of water containing 0.2% formic acid, cap and shake. After the sample has eluted, add the rinse portion to the SPE column reservoir, elute to waste. Rinse the SPE column with 2 additional, 1.5 mL aliquots of water containing 0.2% formic acid. Dry the columns by applying a vacuum to the SPE manifold, allowing air to be drawn through the columns. Run the vacuum for 4-5 minutes.

4. Set up the SPE elution rack outside the SPE manifold. Use top plates with 16-mm holes to hold the 4-mL eluate collection vials. A second plate with 16-mm holes is used to hold SPE columns. Adjust the height of this rack so that the tip of the SPE column fits inside the rim of the 4-mL vial. Refer to Appendix B for a discussion on obtaining an initial weight for the vials.

5. Transfer the C_{18} SPE columns from the manifold to the elution rack and elute each column with 3.0 mL of acetonitrile containing 0.2% formic acid. Collect eluate in 4-mL silanized glass vials. After addition of the acetonitrile, start gravity flow by applying a small amount of positive pressure to the column reservoir using a small pipet bulb.

6. Start evaporation of eluate under a stream of nitrogen. Vials may be placed in a heating block maintained at 68-72°C to facilitate evaporation. After about 1 mL has been evaporated, add 0.6 mL of a 0.1 M, pH 4.7, acetate buffer. Continue evaporation under a stream of nitrogen. Remove each vial from the heating block when the volume reaches approximately 200-250 µL. Note: at this point the remaining extract is predominantly water.

7. When all vials have been removed from the heating block add acetate buffer to bring the total volume in each sample vial to 1.0 mL. Standard vials are brought to a volume of 1.5 mL. See Appendix B for a discussion on various methods that can be used to accurately determine this volume. Place the capped vials in a heating block maintained at 68-72°C for 2-3 minutes. Vortex for 20 seconds, then sonicate for 2-3 minutes. Transfer to silanized autosampler vials for analysis.

3.7 Preparation of ZA1296 and MNBA Calibration Solutions

3.7.1 Evaporation of Intermediate ZA1296 and MNBA Standard Solutions

Using an appropriate sized syringe (250- and 100-µL gas-tight syringes for the example below), transfer an aliquot of intermediate standard solution (prepared in Section 2.3.4) to a 4-mL, silanized vial. Evaporate the aliquot to dryness under a stream of nitrogen. ***Do not use heat for this evaporation.*** Remove from stream of nitrogen as soon as all traces of solvent have been evaporated. Do not over-dry. To quantitate samples where the limit of quantitation is 0.005 mg/kg, and final extracts are at a concentration of 0.10 g of soil per mL of extract, prepare standards as shown in the following table:

Analyte	Intermediate Standard Concentration (µg/mL)	Aliquot Volume (µL)	Final Volume (mL)	Final Concentration (ng/mL)
ZA1296	0.10	300	1.5	20.0
	0.10	60	1.5	4.0
	0.01	75	1.5	0.50
MNBA	0.10	300	1.5	20.0
	0.10	60	1.5	4.0
	0.01	75	1.5	0.50

3.7.2 Conversion Reactions

Convert the evaporated ZA1296 standards to AMBA per the reactions described in Section 3.4. In a similar manner, convert the evaporated MNBA standards to AMBA per the reactions described in Section 3.5. Perform the post-conversion cleanup (Section 3.6) on all standards. It is important that all standards and samples be subjected to identical conditions during the conversions and post-conversion cleanup. After the sample eluate fractions and the standard aliquots are evaporated (just prior to Section 3.4), all ZA1296 standards and samples should be processed together as a single batch. Likewise, all MNBA standards and samples should be process as a batch starting with Section 3.5.

4 Instrumentation

4.1 Operating Parameters Outline

4.1.1 High Performance Liquid Chromatograph

Model: Hewlett Packard Model 1090A Liquid Chromatograph, equipped with a model 1040A UV diode array detector (DAD) and a model 1046 fluorescence detector (FLD). Modified for 500 µL injections.

Column: Phenomenex Prodigy C_{18} (or Inertsil ODS-2), 250 x 3.2 mm i.d., 5 µm particle size

Guard Column: Phenomenex Prodigy C_{18} (or Inertsil ODS-2), 30 x 3.2 mm i.d., 5 µm particle size

Column Temp: 50°C

Mobile Phase: A: 9 mM ammonium acetate, 0.25% formic acid in water

B: acetonitrile:water, 95:5, (v:v)

Gradient:

HPLC Cleanup		Analytical Determination	
Time	%B	Time	%B
0.0	7	0.0	5
5.0	45	3.0	15
9.0	45	6.0	15
10.0	90	10.0	40
12.0	90	12.0	90
12.5	7	13.0	90
14.0	7	14.0	5
		15.0	5

Post Run Time: 3 min

Flow Rate: 0.75 mL/min

Injection Vol.: 500 µL for fraction collection runs, 250 µL for analytical runs

FLD Parameters:	Excitation - 227 nm	Emission - 424 nm
	PMT gain:	9, 10, or 11
	Lamp:	1 (55 Hz)
	Delay off, Gate off	
	Response Time:	2.0 sec
DAD Parameters:	254 nm	
	Peak width:	0.02 min
	Sampling interval:	0.32 sec
	Autobalance:	on

Contact Closure to start Fraction Collector: Contact no. 4, on at 0.01 min, off at 0.02 min

4.1.2 Fraction Collector

Model: Waters Associates model 3500. Equipped with 3-way waste valve and WISP vial rack for holding 4-mL vials

Mode: No. 3, timed window

Collection Windows: As determined in Section 3.3.3 or Appendix A

4.2 Injection Volume Modification for HP1090 HPLC

The following modifications are required to allow injections up to 500 µL. These modifications assume that the HP 1090 is equipped with the 250 µL injection option. Other instruments may require different (or no) modifications.

1. Replace 250-µL syringe with a 500-µL Hamilton Gas-Tight syringe (1700 series). The waste sleeve hole for the 250-µL syringe plunger will have to be enlarged to accommodate the larger diameter of the 500-µL syringe plunger.

2. Increase the total volume of the injection loop to at least 500 µL. This can be done by adding an additional 250-µL loop (or any 1/16" stainless steel; tubing with a volume of 250 µL) between the existing loop and the auto-injector needle.

NOTE: IT IS EXTREMELY IMPORTANT TO REMEMBER THE HP-CHEMSTATION SOFTWARE CONTROLLING THE HPLC DOES NOT RECOGNIZE INJECTION VOLUMES > 250 µL. AFTER THIS MODIFICATION IS MADE, ALL INJECTION VOLUMES ARE ACTUALLY TWO TIMES (2X) THE VOLUME ENTERED IN THE CHEMSTATION METHOD.

4.3 Fluorescence Detector Linearity

The linear operating range of individual detectors should be determined prior to the quantitation of samples. The HP 1046 fluorescence detector utilizes a PMT (photomultiplier tube) gain setting that can directly affect the linear range. A series of AMBA standards (prepared in Section 2.3.5) should be run over the concentration range of 0.05 to 20 ng/mL. This series of standards should be repeated using different PMT gain settings. PMT gain settings of 8, 9, 10, and 11 should be tested. Select the PMT setting that provides the most linear response, and adequate sensitivity at the lower concentrations. Signal-to-noise ratio can also be a consideration when selecting the proper PMT gain setting.

Note: The linear range should extend to approximately 1/5 to 1/10 of the equivalent analyte level determined at the LOQ level (equivalent to approximately 0.05 ng/mL of AMBA). This will allow the background found in controls to be determined and used for correcting fortified sample recoveries (if needed).

4.4 Calibration and Analysis - Fraction Collection Run

Determine the retention time of the analytes using the UV calibration standard prepared in Section 2.3.6. After the proper fraction collection windows discussed in Section 3.3.3 have been established proceed with the collecting of sample fractions, and the simultaneous determination

of AMBA. For the quantitation of AMBA residues, inject the 1.0-ng/mL and 10-ng/mL AMBA calibration standards prior to the injection of the sample extracts. Inject the 1.0 ng/mL AMBA standard solution after every 4-6 sample extract injections. Monitor the retention time of AMBA to ensure that retention times are not changing over the course of the fraction collection run. It is suggested that three individual instrument methods be used for control of the HPLC during the fraction collection run. The methods are:

1) a UV- monitored method for analysis of the UV calibration standard
2) a FLD-monitored method for analysis of the water blanks and AMBA standard
3) a FLD-monitored method with timed events for fraction collector control for later analysis of sample extracts.

All instrumental parameters, except for timed events and mode of detection, must be identical for each method.

4.4.1 Analytical Scheme

A suggested analytical scheme could include injections in the following order:

1. UV calibration standard, three injections (or until retention times are stable)
2. 2-4 injections of a water blank (to eliminate carry-over)
3. AMBA calibration standards at 1.0 and 10.0 ng/mL (for quantitation of AMBA residues)
4. Reagent blank, water (to monitor background through the rest of the method)
5. Sample extracts
6. AMBA calibration standard at 1.0 ng/mL
7. Sample extracts
8. AMBA calibration standards at 1.0 and 10.0 ng/mL

4.5 Calibration and Analysis - ZA1296 and MNBA Analytical Run

Calibrate the HPLC using the calibration standards prepared in Section 3.7.1. For soil extracts equivalent to extractives from 0.09 g soil per mL of extract and samples potentially containing residues at 0.005 to 0.20 mg/kg, calibration standards at 0.50, 4.0, and 20.0 ng/mL are suggested.

4.5.1 Analytical Scheme

A suggested analytical scheme could include injections in the following order:

1. Replicate injections (2-3) of an AMBA standard to equilibrate the HPLC.
2. One injection of each ZA1296 calibration standard (0.50, 4.0, and 20 ng/mL)
3. Reaction blank, reagent blank, and/or control extract (ZA1296, fraction B)
4. Fraction B, ZA1296 sample extracts
5. ZA1296 calibration standard 4.0 ng/mL (after every 4-6 sample extract injections)
6. Fraction B, ZA1296 sample extracts
7. ZA1296 calibration standard, 3.6 ng/mL
8. One injection of each MNBA calibration standard (0.50, 4.0, and 20 ng/mL)
9. Reaction blank, reagent blank, and/or control extract (MNBA, fraction A)
10. Fraction A, MNBA sample extracts
11. MNBA calibration standard, 4.0 ng/mL
12. Fraction A, MNBA sample extracts
13. MNBA calibration standard, 4.0 ng/mL

4.5.2 Response Factors

Once a linear operating range has been established for the fluorescence detector (see Section 4.3), subsequent analyses should provide a linear, stable response. The relationship between response vs. amount of analyte injected is generally quite stable and consistent. However, other factors come into play and are addressed during the calibration procedure. These include the linearity, and precision of the processes involved in converting ZA1296 and MNBA to AMBA. In order to show that the percent of conversions are the consistent throughout the calibration range, individual response factors are calculated for each calibration level. In order to offset any variability in the percent of conversions, the response factors obtained for each of three calibration level are averaged, and this average response factor is then used for sample calculations.

4.6 Fortification

While not required for the successful use of this method, if possible, analyze fortified and unfortified control samples with each sample set to demonstrate method recovery. For example, add 50 µL of a working fortification solution (1.0 µg/mL) to a control sample (10 g) to produce a fortification level of 0.005 mg/kg. Extract as detailed in Section 3.1.

5 Calculations

The concentration of the analyte in the original sample is calculated by using the external standard method; i.e., the response obtained for the analyte in the sample extract is compared to the response obtained for separate injections of a known amount of analyte (calibration solution). To use the calculations below, the injection volumes for all calibration solutions and sample extracts must be fixed at the same volume.

5.1 Linear Response Method

All responses obtained from each calibration solution (i.e., 20.0, 4.0, and 0.50 ng/mL) are averaged and a response factor for that level is calculated as shown in Section 5.1.1. Then the response factors for all three calibration levels are averaged to obtain an average response factor as shown in Section 5.1.2.

5.1.1 Response Factor at Each Calibration Level

Calculate the response factor, RF, resulting from all injections at each level of calibration solution as follows:

$$RF = \frac{C}{R}$$

Where:

RF = response factor (µg/mL/peak area)

C = concentration of calibration solution (µg/mL)

R = average response units (e.g., peak area) from detector for all injections of the calibration solution

5.1.2 Average Response Factor

Calculate the average response factor as follows:

$$RF(avg) = \frac{RF(high) + RF(mid) + RF(low)}{3}$$

Where:

RF(avg) = Average response factor

RF(high) = Response factor of the high-level calibration standard

RF(mid) = Response factor of the mid-level calibration standard

RF(low) = Response factor of the low-level calibration standard

5.1.3 Soil In Extract, AMBA Determination

For the determination of AMBA residues, calculate the concentration of the soil: i.e., the amount of soil the extract represents, as follows:

$$C = \frac{W(sample)}{V(extract)}$$

Where:

C = Concentration of soil in extract (g/mL)

W(sample) = Weight of sample (g)

V(extract) = Total volume of extract (mL)

5.1.3 Soil In Extract, ZA1296 and MNBA Determination

For determination of ZA1296 and MNBA residues, calculate the concentration of the soil; i.e., the amount of soil the extract represents, as follows:

$$C = \frac{W(sample)}{V(extract)} \times \frac{V(injected)}{V(converted)} \times \frac{V(cleanup)}{V(final)}$$

Where:

C	=	Concentration of soil in extract (g/mL)
W(sample)	=	Weight of sample (e.g., 10 g)
V(extract)	=	Total volume of extract (e.g., 50 mL)
V(injected)	=	Volume injected on HPLC for fraction collection (e.g., 0.50 mL)
V(converted)	=	Final volume after conversion reactions, prior to C_{18} SPE cleanup (e.g., 2.0 mL)
V(cleanup)	=	Volume subjected to C_{18} SPE cleanup (e.g., 2.0 mL)
V(final)	=	Final volume, after dissolution of residuum from C_{18} SPE cleanup (e.g., 1.0 mL)

5.1.4 Analyte in Sample

Calculate the analyte concentration, A in the original sample as follows:

$$A = \frac{R \times RF(avg)}{C}$$

Where:

A	=	Concentration of analyte in original sample (mg/kg or ppm)
RF(avg)	=	Average response factor (µg/mL/peak area)
R	=	Response (peak area) from detector for sample
C	=	Concentration of soil in final extract (e.g., 0.10 g/mL)

5.2 Conversion Efficiency

It is useful for troubleshooting purposes to monitor the performance of the reactions involved in the converting ZA1296 to AMBA by calculating the conversion efficiency (CE) as follows:

$$\text{CE (ZA1296)} = \frac{\text{RF (AMBA)}}{\text{RF (ZA1296)}} \times \frac{\text{Molecular Weight ZA1296}}{\text{Molecular Weight AMBA}} \times 100$$

In a similar manner calculate the conversion efficiency of the reaction converting MNBA to MBA as follows:

$$\text{CE (MNBA)} = \frac{\text{RF (AMBA)}}{\text{RF (MNBA)}} \times \frac{\text{Molecular Weight MNBA}}{\text{Molecular Weight AMBA}} \times 100$$

6 Interferences

6.1 Reagent Interferences

A more common source of interferences are the reagents and potential cross-contamination from the analytical standards. The majority of peaks in the background chromatographic profile can be attributed to the reagents. Contamination from analytical standards is difficult to trace due to the conversion reactions required to detect individual analytes. Consider the following two examples.

1. A sample (ZA1296, fraction B) upon analysis has a significant unexpected peak that co-elutes with AMBA. This unexpected result could have been due to
   (a) contamination with ZA1296 prior to the fraction collection process;
   (b) contamination with MNBA or ZA1296 after the fraction collection process, but not after the conversion process; or
   (c) contamination with AMBA after fraction collection.

2. In a similar manner, a contaminated sample (MNBA fraction) could have been due to

   (a) contamination with MNBA before fraction collection;
   (b) contamination with MNBA after fraction collection but before conversion to AMBA; or
   (c) contamination with AMBA after fraction collection.

   This last sample could not have been contaminated with ZA1296 since no oxidation step is performed to generate MNBA before the reduction to AMBA.

6.2 HPLC Reagent Blank

The HPLC Reagent Blank (HRB) consists of a blank water sample that is injected during the fraction collection run. It is treated the same as a sample extract; i.e., fractions are collected and conversions are performed. This blank is used to show any HPLC system, or fraction collector contamination, including injector carry-over from previous high-level injections. Reagents used prior to fraction collection can be tested by starting the reagent blank earlier in the process. For example, a reagent blank can be initiated acidifying an aliquot of the soil extracting solutions, then continuing that sample through the method.

6.3 Reaction Blank

A simple, yet effective diagnostic tool is a reaction blank. This is a type of reagent blank that is initiated at the beginning of the conversion reaction process. The ZA1296 and MNBA reaction blanks are prepared by subjecting one empty vial to the ZA1296 oxidation and reduction reactions and a second vial to the MNBA reduction reaction. These blanks will indicate contamination from the conversion reagents. In some cases it is possible to determine the source of the contamination. If, for example, only the ZA1296 reaction blank is contaminated, then the source is most likely the catalase reagent or the hydrogen peroxide. If both the ZA1296 and

MNBA blanks are contaminated, then the possible sources are greater in number but tend to eliminate the oxidization reagents. Analysis of individual reagent solutions is usually not effective for exposing sources of contamination since a complete reaction sequence is required for AMBA formation, or for the formation of a non-AMBA contaminant. In most cases the most time-efficient remedy is to carefully remake all reagents.

7 Confirmatory Techniques

Unexpected positive results can be confirmed through the use of an alternate column for the fraction collection process. Due to the ionic nature of analytes, their retention characteristics can be further modified by altering the HPLC mobile phase.

7.1 Alternate HPLC Mobile Phase

Figure 3 shows the effect of changing the ammonium acetate and formic acid concentrations has on the chromatographic profile. This can be an effective technique for confirming the presence of MNBA. MNBA is highly sensitive to changes in pH and the ionic strength of the mobile phase compared to ZA1296 and AMBA.

7.2 Alternate Column

Figure 4 shows chromatograms obtained with the alternate column, a Prism-RP. The difference in the selectivity of this stationary phase compared to the Prodigy column, in conjunction with alternate mobile phases, can provide confirmation of AMBA, MNBA and ZA1296 residues. For confirmatory purposes, this column should be used during the HPLC cleanup. The HPLC mobile phase A solution (section 2.2.17) should be 15 mM ammonium acetate and 0.6% formic acid. The column temperature should be raised to 80°C.

Appendix E. Method Modifications

The following modifications were made to TMR0661B prior to, and as a result of, the independent laboratory validation study. While data presented in Table 1 were obtained using the original method, the modifications discussed below are considered minor in nature, and do not impact the validity of data.

Section 3.1

The correction for the amount of moisture present in the soil subsample was eliminated. The amount of extraction solvent added is 50 mL for all samples.

Section 3.6.2, 3.6.3

The amount of post-conversion sample subjected to the C18 SPE cleanup was originally 1.8 mL out of a total of 2.0 mL. This resulted in a final crop:solvent ratio of 0.09 g/mL for the ZA1296 and MNBA analyses. This modification calls for the entire 2.0 mL volume to be transferred to the SPE column. Also included in the modification is a vial rinse and transfer of 1.0 mL to the SPE column, and two 1.5 mL column washes. As a result of this change the final crop:solvent ratio is 0.10 g/mL, and the nominal concentrations of the calibration standards are increased from 0.45, 3.6, and 18.0 ng/mL to 0.50, 4.0, and 20.0 ng/mL

This clean-up of the entire post-conversion volume was implemented to reduce the effect of minor volume variations throughout the conversion process.

Section 3.6.4

The volume of acetonitrile eluant was reduced from 4 to 3 mL. This reduces the time required for evaporation.

Section 3.6.7

The repeating of the heat, vortex, sonicate cycle was eliminated

Other modifications included additions to the materials and reagents lists, clarifications on standard solution diluants and expiration dates.