

US EPA ARCHIVE DOCUMENT

ZENECA Ag Products
ZENECA Inc.
Wilmington, DE 19850

11123/166
MRID 44901719

VOLUME 21

Study Title

ZA1296: Liquid Chromatographic Determination with Fluorescence Detection of ZA1296 and 4-(methylsulfonyl)-2-nitrobenzoic acid in Crops after Conversion to 2-amino-4-(methylsulfonyl)-benzoic acid - A Modification of TMR0643B

Data Requirement

Guideline Ref.: 860 2340, Supplemental to MRID No.: 44505216

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Study Completed On

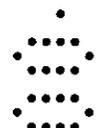
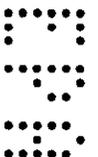
March 31, 1999

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TMR0882B



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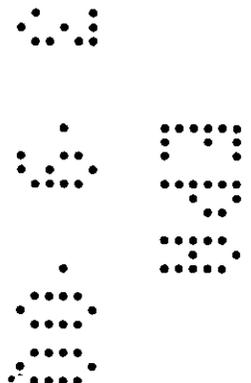
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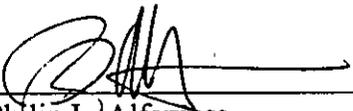
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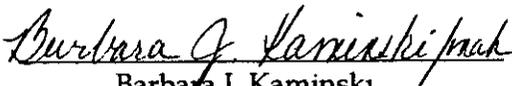
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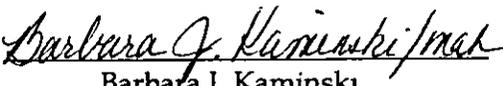


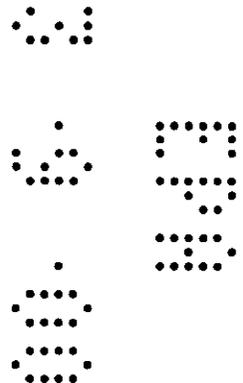
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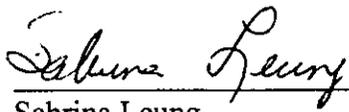


Certificate of Authenticity

I, the undersigned declare that this study was performed under my direction and that this report represents a true and accurate record of the results obtained.

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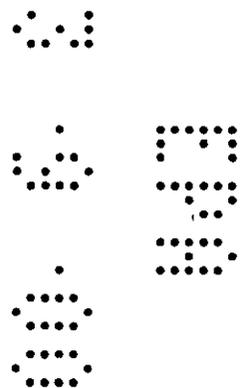


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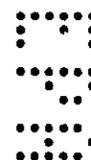
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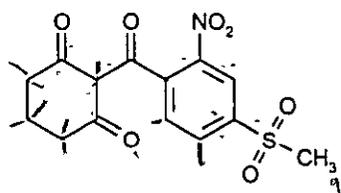
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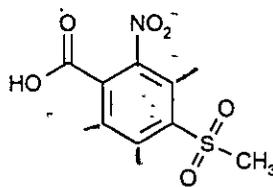
1 Introduction and Summary

This method is intended for the determination of residues of 2-[4-(methylsulfonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione (ZA1296), and 4-(methylsulfonyl)-2-nitrobenzoic acid (MNBA) in corn grain, corn fodder, corn forage, and sugar cane. MNBA is a principal metabolite of ZA1296. Chemical structures and molecular weights (mw) are shown below.

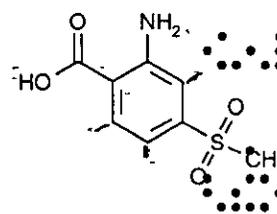
ZA1296 and MNBA residues are extracted from crops with an acetonitrile-water mixture (1:1, v:v). An aliquot of the crude extract is evaporated to remove the acetonitrile. The evaporated extract is acidified and brought to the original volume with water. Cleanup is accomplished by separation by reversed-phase HPLC. 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 SnCl₂ and HCl. After final cleanup by C18 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 AMBA calibration standards to quantitate the AMBA conversion product. The limit of quantitation for ZA1296 and MNBA is 0.01 mg/kg.



ZA1296
MW = 339 daltons



MNBA
MW = 245 daltons



AMBA
MW = 215 daltons

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- μ L 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 (Optional)

This modification increases the injection volume to a maximum of 500 μ L and requires the following. Two, 250- μ L sample loops installed in series (HP # 79846-87613), Hamilton series 1700 gas-tight 500- μ L syringe (Hamilton #81230, Fisher #14-815-113). Waste sleeve, 250 μ L, modified to fit 500- μ L syringe plunger (HP 79846-24502). Note: The HP Chemstation software only recognizes syringes up to 250 μ L. 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 μ L/min.

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

Evaporation manifold with aluminium heating block to fit 4-mL and 8-mL vials (Techne-Dri Block DB-3, with sample concentrator unit)

2.1.6 HPLC Column, HPLC Cleanup

Inertsil ODS-2, 5- μ m particle size, 250 x 4.6 mm i.d. (MetaChem #0296-250-X046, or Keystone 255-181), with a guard column (MetaChem #0296-CS). An equivalent column is the Phenomenex Prodigy ODS-2, 5- μ m particle size, 250 mm x 4.6 mm i.d. (Phenomenex #00G-3300-EO) with a guard column (Phenomenex #03A-3300-EO).

2.1.7 HPLC Column, HPLC Final Determination

Waters Spherisorb ODS-2, 250 mm x 3.0 mm i.d (MetaChem # 0184-250X030). An equivalent column is the MetaSil ODS, 250 mm x 3.0 mm i.d. (MetaChem # 0380-250X30).

2.1.8 HPLC Column -Cleanup, Confirmatory

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

2.1.9 C18 SPE Columns

Varian Bond Elut LRC solid phase extraction columns, C18, 500 mg, 10-mL reservoir volume. (Phenomenex #AHO-1210-3027, Chrom Tech #1211-3027). Bond Elut columns with 500 mg of packing material and a 2.8-mL reservoir volume may be substituted.

2.1.10 Eppendorf Automatic Pipet

Eppendorf Variable volume pipet with disposable tips; 0.1-10 μ L (Brinkman #2244-0004, Fisher #21-381-200), 10-100 μ L (Brinkman #2244-0101, Fisher #21-371-202), 200-1000 μ L (Brinkman #2244-0209, Fisher #21-371-204)

2.1.11 Peroxide Test Strips

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

2.1.12 Syringes, Standard Aliquoting

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

2.1.13. Vials, sample

4-mL screw-top vials, amber glass, silanized (Supelco #2-7216) for fraction collection; 4-mL screw-top vials, clear glass, silanized (Supelco #2-7220) for collection of post-conversion cleanup eluate; 8-mL screw-top vials (Fisher #06-412-3) for crude extract evaporation.

2.1.14. Caps, vial

Teflon-lined caps for 4-mL vials, 13-425 (Fisher # 06-406-40), Teflon-lined caps for 8-mL vials, 15-425 (Fisher # 06-450-44).

2.1.15 Vials, autosampler

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

2.1.16. Glass Pipettes

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

2.1.17 Glass Bottles

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

2.1.18 Tekmar Tissuemizer

Model SDT-25 tissuemizer (Tekmar #10-0103-0180, equipped with a SDT-182EN probe (Tekmar #10-0104-000). Equivalent laboratory homogenizer may be substituted.

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

- 2.2.4 Methanol**
HPLC Grade
- 2.2.5 Ammonium Acetate**
HPLC Grade
- 2.2.6 Stannous Chloride**
ACS Grade
- 2.2.7 Hydrogen Peroxide**
30%, ACS Grade
- 2.2.8 Formic Acid**
88%, ACS Grade
- 2.2.9 Hydrochloric Acid**
Concentrated, ACS Grade
- 2.2.10 Catalase Enzyme**
Approx. 10,000 - 20,000 activity units per mg (Sigma # C40)
- 2.2.11 Acetonitrile : Water Extraction Solution**
Equal volumes of water and acetonitrile.
- 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, 90 mg/mL SnCl₂ in 3 N HCl.**
Add 3.6 g of stannous chloride to 10 mL of concentrated HCl, swirl to dissolve. Stannous chloride should dissolve easily in concentrated HCl. Dilute by adding 30 mL of deionized or distilled water. The solution should be clear, both before and after the addition of the water.

2.2.15 Catalase Reagent, ca 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.

2.2.16 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.

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

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

2.2.18 UV Calibration Solution For Establishing Analyte Retention Times

A solution containing 0.20 µg/mL each of MNBA, AMBA, and ZA1296. Stock standards are serially diluted to this level using 0.2% formic acid in water.

2.2.19 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.

2.2.20 Acetic Acid

Glacial acetic acid, ACS grade

2.3 Reference Materials

2.3.1 ZA1296, MNBA, and AMBA Reference Standards

Available from Zeneca Inc., 1200 South 47th Street, Richmond CA 94804-4016. The ZA1296 was of 99.7% purity and had the reference number ASW-1662R -01R. The MNBA was of 99% purity and had the reference number ASW-1714-01A. The AMBA was of 99% purity and had the reference number ASW-1664R-01R.

2.3.2

Stock Fortification Solutions

Stock ZA1296 and MNBA solutions are prepared in methanol. Fortification solutions are used to fortify untreated (control) samples and demonstrate recovery. 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) = 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 ug/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 ($\mu\text{g/mL}$)

C(ss) = concentration of analyte in the stock fortification solution ($\mu\text{g/mL}$)

$W(ss)$ = 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 at concentrations of 10, 1.0 and 0.10 $\mu\text{g/mL}$ (all in methanol). To increase flexibility in the fortification schemes, fortification solutions with one analyte per solution are recommended.

2.3.4 Stock AMBA Calibration Solutions

A stock AMBA calibration solution is prepared in a manner similar to that of the fortification solutions described in section 2.3.3.

2.3.5 AMBA Calibration Solutions

AMBA calibration solutions are required to determine the amount of the AMBA conversion product present in the final extracts. The stock solution is prepared as described in 2.3.4. A dilution from the stock solution (1000 $\mu\text{g/mL}$) to an intermediate calibration solution at 100 $\mu\text{g/mL}$ is made by volume. Using a volumetric pipet, add 10.0 mL of stock solution to a 100 mL volumetric flask. Dilute to volume with HPLC grade water. The remaining dilutions can be made by weight, using HPLC grade water as the diluant. Prepare the following intermediate concentrations: 10 $\mu\text{g/mL}$, 1.0 $\mu\text{g/mL}$. From the 1.0 $\mu\text{g/mL}$ intermediate calibration standard, prepare the following working calibration standards: 100, 10, 1.0, and 0.12 ng/mL .

2.3.6 UV Calibration Solution

A uv calibration solution is used to establish analyte retention times so the appropriate eluate fractions are collected. The intermediate solution is prepared at a concentration of 10 $\mu\text{g/mL}$, 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 $\mu\text{g/mL}$). Transfer 1.0 mL of the combined intermediate calibration solution to a 2-oz, narrow-mouthed bottle. Evaporate the methanol under a stream of nitrogen. Dilute with 50 mL of HPLC grade water containing 0.2% formic acid to produce a working uv calibration solution with a concentration of 0.20 $\mu\text{g/mL}$.

3 Analytical Procedure

3.1 Crop Extraction

Place a 10-g subsample of a homogeneous crop sample into a 8-oz, wide-mouthed jar. After taking into account the amount of water present in the sample, add an amount of the acetonitrile : water (1:1, v:v) extraction solution that brings the total aqueous volume to 125 mL. For example, a crop sample that contains 40% water (4 mL water) would require that 121 mL of extraction solution be added. Add 1.0 g of NaCl. Macerate at a medium to high speed (>10000 rpm) for three minutes using a tissuemizer. Centrifuge for 10 minutes at about 2000 rpm.

3.2 Pre-Cleanup Evaporation

Use a 10-mL disposable glass pipet to transfer an 8.0-mL aliquot of the supernatant to an 8-mL glass vial. Evaporate, under a stream of nitrogen at a temperature of 60° C, to approx. 2.3-2.7 mL volume, swirling the contents of the vial occasionally (2-3 times) during the evaporation. Add 2 mL of water containing 1.0 % formic acid. Bring to a total volume of 8 mL. The 8 mL volume may be determined by pre-weighing the empty vial and recording its weight. After addition of the 2 mL of 1% formic acid, place the vial on the balance and add HPLC grade water until a net-gain of 8 g over the initial weight of the vial is obtained. Cap the vials and place on aluminium heating block maintained at 65° C. for 10 min. Sonicate for 10 min. Centrifuge for 20 min at 3500 rpm. Transfer 1 mL to an silanized autosampler vial.

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 be determined. This delay is defined as the amount of 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 flow rate. (Note: 0.02" 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 be determined prior to isolation by HPLC fraction collection. See section 4.1.1 for HPLC parameters. Prior to the start of the fraction collection run a uv calibration solution is injected. This solution contains MNBA, AMBA, and ZA1296; each at a concentration of 0.20 ug/mL. Make three, 250-uL 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.

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 follows: For MNBA, the fraction collector elution time \pm 0.4 minutes. For ZA1296, the fraction collector elution time \pm 0.6 min. 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. The MNBA window is narrower due to the increased amount of co-extractives eluting in this portion of the chromatographic run. 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

Make a single, 250-uL injection (equal to 0.02 g of crop) of each crop 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 3.3.3. Collect the eluate fractions in 4-mL silanized, amber glass vials (Be sure that these vials are devoid of any paper or cardboard fibers). Prior to injection of the extracts 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). At the end of the fraction collection run, inject the UV calibration solution to confirm that the retention times of the analytes have not drifted significantly.

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 62-68° 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. Therefore, remove each vial from the heating block as soon as the level of liquid approaches the bottom of the vial (approx. 100 µL remaining). Once all vials are removed, place them in a block maintained at room temperature and continue evaporation under a stream of nitrogen until dry.

3.4 Conversion of ZA1296 Residues to AMBA

3.4.1 Oxidation of ZA1296 Residues to MNBA

Add 300 µL of a 30% hydrogen peroxide solution to each of the vials (fraction B) containing ZA1296 residues. Heat at 83-87° C for 20 min. Vortex every 5 min during heating. Remove from heating block and cool to room temperature. Centrifuge vial prior to opening (see section 8.12).

3.4.2 Elimination of Excess Hydrogen Peroxide

Using an Eppendorf pipet add 650 µL of HPLC grade water to each vial. Cap and swirl gently to mix. Place all vials in an aluminium block. Place block in contact with dry ice to facilitate freezing of vials. Once frozen, uncap vials and, using an Eppendorf pipet, carefully add 50 µL of a solution containing ca 20,000 units/mL of catalase enzyme. As the contents of the vial thaws the rate of the enzyme reaction will increase. (Addition of the enzyme solution to at room temperature or unfrozen vials will cause vigorous bubbling, foaming, and aerosol formation.)

After approximately 10 minutes, swirl the contents of each vial. Tiny bubbles will form in the solution. Sustained bubble formation (without swirling) indicates the reaction is not yet near completion. The amount of bubbles formed when the solution is swirled decreases as the reaction is nearing completion. Check bubble formation every 3-5 min. Once bubble formation is at a minimum, the vial may be capped.

Some reactions may occur very slowly. If the reaction is not complete within 30 min, add another 50-µL aliquot of catalase solution. Continue to monitor, by swirling every 3-5 min. Excessively slow reactions may be speeded up by placing on a heating block for 5 min at 62-68° C. Once all vials have been capped, place vials on a mechanical shaker for 20 min.

Important: Capping the vials too early in the reaction process will lead to a build up of pressure in the vial and may cause loss of material upon opening. Opening of capped vials should only be done after centrifuging.

3.4.3 Determination of Residual Peroxide

The elimination of peroxide must be confirmed before proceeding with the following reduction reaction. Centrifuge vial prior to opening. Using an Eppendorf pipet, transfer 1 μ L 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 10 min before retesting. If the test is still positive add an additional 50 μ L of the catalase reagent, shake for 10 min, then retest. Note: 2-4 tests can be performed using each test strip. Do not dip the test strips in the solutions; excessive losses will occur.

3.4.4 Reduction of MNBA to AMBA (ZA1296 residue)

Add 500 μ L of the stannous chloride reagent (90 mg SnCl_2 / mL of 3 N HCl) to each vial. Tightly cap the vial and heat at 63-67° C for 20 min. Shake vials every 5 min. Remove and allow to cool to room temperature. These samples will next under go cleanup in section 3.6.

3.5 Reduction of MNBA Residues to AMBA

Add 1.0 mL of HPLC grade water to each vial (fraction A) containing MBNA residues. Add 500 μ L of the stannous chloride reagent (90 mg SnCl_2 / mL of 3 N HCl) to each vial. Tightly cap the vial and heat at 63-67° C for 20 min. Shake vials every 5 min. Remove and allow to cool to room temperature. These samples will next under go cleanup in section 3.6.

3.6 Post-Conversion Cleanup of All Fractions

3.6.1 Condition a 500 mg, C18 solid phase extraction column with 5 mL of acetonitrile containing 0.2% formic acid. Follow with 6 mL of HPLC grade water containing 0.2% formic acid. Elute using vacuum at a rate of 0.5 - 1.0 mL/min. Stop elution prior to the level of eluant reaching the column bed. Continue using gravity elution until all the level of liquid reaches the column bed, where upon the gravity elution should stop without the column bed going dry.

3.6.2 Place the stopcocks on the SPE manifold in the off position. Centrifuge sample vials prior to opening. Using an Eppendorf pipet, transfer the contents of the first vial to the column reservoir, do not yet discard the pipet tip. Using a second pipet or other liquid handling device, add 0.5 mL of HPLC grade water to the sample vial, cap and swirl. Using the first Eppendorf pipet, transfer the wash solution from the vial to the SPE column. Proceed with the remaining vials in the same manner. Once the contents of all the sample vials have been transferred, open the stopcocks and turn on the vacuum to allow elution at a rate of 0.5 - 1.0 mL/min. Stop elution prior to the level of eluant reaching the column bed. Continue using gravity elution until all the level of liquid reaches the column bed, where upon the gravity elution should stop without the column bed going dry.

3.6.3 Wash columns with a 1.5 mL aliquot of 0.2% formic acid in water. Use the same method of vacuum elution followed by gravity elution. Wash the column with a second 1.5-mL aliquot of 0.2% formic acid in water. Continue the vacuum elution until the column goes dry. Increase the vacuum to 5-10" Hg to further dry the column for an additional 30 seconds.

3.6.4 Set-up the elution rack outside of the SPE manifold. Use a top plate with 16-mm holes to hold the SPE columns. A second plate with 16-mm holes is used to hold the 4-mL, silanized, glass collection vials. The 4-mL vials should be pre-weighed to allow for final volume determination.

3.6.5 Transfer the C18 SPE columns from the manifold to the elution rack and elute columns with 3.0 mL of acetonitrile containing 0.2% formic acid. Collect eluate in 4-mL, silanized glass vials. Start gravity flow after addition of the acetonitrile by applying a small amount of positive pressure to the column reservoir using a small pipet bulb. Once gravity elution has completed, use the small pipet bulb to purge the column of eluant.

3.6.6 Start evaporation of eluate under a stream of nitrogen. Vials may be placed in a heating block maintained at 63-67° 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 uL. Note: At this point the remaining extract is predominantly water.

3.6.7 When all vials have been removed from the heating block add acetate buffer to bring the total volume in each sample vial to 1.00 mL. Based on the initial weight of the vials obtained in section 3.6.4, add the required volume of buffer to obtain a net weight gain of 1.00 g. Assume the density of the buffer is 1.0 g/mL. Place the capped vials in a heating block maintained at 63-67° C for 2-3 min. Vortex for 20 sec., then sonicate for 2-3 min. Transfer to silanized autosampler vials for analysis.

3.7 Determination of ZA1296 and MNBA Conversion Efficiency

3.7.1 Discussion

Prior to use of this method for determination of residues, the efficiency of the conversion reactions (ZA1296 → MNBA → AMBA, and MNBA → AMBA) must be established. Since the method relies on the quantitation of the conversion product (AMBA) using AMBA calibration solutions, any unexpected decrease in conversion efficiency will cause a significant reduction of the final recoveries. As shown in the recovery values shown in Table 1, recoveries of both analytes range from 79-85%. A significant portion of the losses involved are due to losses incurred in the conversion process, as neither reaction is 100% complete. Conversion losses of up to 10% are normal. Losses also occur at the HPLC cleanup stage and the initial evaporation step. Additional losses at the conversion steps will lower recoveries further and average recoveries will approach or fall below the required 70% specification. The following procedure is used to determine the conversion rate for each reaction. In this procedure known amounts of ZA1296 and MNBA are reacted to form AMBA. The AMBA is then quantitated using AMBA calibration solutions. The conversion efficiency is then calculated. **If the analyst is unfamiliar with the method, this procedure should be performed on four replicates of a known amount of ZA1296 and on four replicates of a known amount of MNBA, prior to running residue determinations.** It should also be performed if it has been some time since the analyst performed the method, or if it is being run on new or different instrumentation and/or reagents. It can also be performed on two replicates of each analyte, concurrent with the residue determination, for diagnostic purposes.

3.7.2 Evaporation of Working Fortification Solutions

Using an appropriate sized syringe, transfer an 100-uL aliquot of a 0.10 ug/mL working fortification solution (section 2.2.3) to a 4-mL, silanized vials. Prepare 4 replicates of ZA1296 and 4 separate replicates of MNBA. Evaporate the aliquot to dryness under a stream of nitrogen. **Do not use**

heat for this evaporation. Do not overdry. Remove from stream of nitrogen just after dryness is obtained.

3.7.3 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 vials.

3.7.4 Determination of AMBA Conversion Product

Using the conditions found in section 4.1.1 (under Analytical Determination) determine the response of the AMBA conversion product present in each replicate. Compare the response of the AMBA conversion product in each replicate to that of a 10 ng/mL AMBA standard. Calculate the response factors as follows:

$$\text{RF (AMBA)} = \frac{\text{Concentration of AMBA}}{\text{Average response of the AMBA standard}}$$

$$\text{RF (ZA1296)} = \frac{\text{Concentration of ZA1296}}{\text{Response of AMBA conversion product in ZA1296 fraction}}$$

$$\text{RF (MNBA)} = \frac{\text{Concentration of MNBA}}{\text{Response of AMBA conversion product in the MNBA fraction}}$$

3.7.5 Calculation of Conversion Efficiency

Conversion efficiency (CE) of the ZA1296 reactions is calculated 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 AMBA as follows:

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

3.7.6 Expected Conversion Efficiencies

The expected conversion efficiency for the ZA1296→MNBA→AMBA reaction scheme is 85-90% with a relative standard deviation (RSD) of 2-4%. The expected conversion efficiency for MNBA→AMBA is 90-95% with an RSD of 2-4%. Conversion rates less than the rates reported here may result in recoveries less than 70%, and should be investigated prior to conducting residue determinations. RSDs of >4% should also be cause for concern with respect to residue analysis.

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).
Cleanup Column:	Inertsil ODS-2, (or Phenomenex Prodigy ODS-2) 250 mm x 4.6 mm i.d., 5-µm particle size. With appropriate guard column. Column maintained at 50° C
Analytical Determination Column	Waters Spherisorb ODS-2 (or Metachem MetaSil) 250 mm x 3.0 mm i.d. 5-µm particle size. Column maintained at 50° C
Mobile Phase:	A: 9 mM ammonium acetate, 0.25% formic acid in water B: acetonitrile : water, 95:5, (v:v)

Gradient: HPLC CleanupAnalytical Determination

<u>Time</u>	<u>%B</u>	<u>Time</u>	<u>%B</u>
0.0	7	0.0	16
5.0	45	5.0	16
9.0	45	7.0	80
10.0	90	9.0	80
12.0	90	10.0	16
12.5	7	12.0	16
14.0	7		

Post Run Time: 3 min

Flow Rate: 1.5 mL/min for HPLC cleanup, 0.75 mL/min for analytical determination

Injection Vol.: 250 µL for HPLC cleanup, 200 µL for analytical determination

FLD Parameters: Excitation - 227 nm, Emission 424 nm
PMT gain - as needed
Lamp - 3 (220 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 3.4.3 or Appendix A

4.2 Injection Volume Modification for HP1090 HPLC

The final determination of the AMBA conversion product may require an injection volume in excess of 250 μL . Depending on the response of the fluorescence detector these larger injection volumes may be required. 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" ss tubing with a volume of 250 μL) between the existing loop and the auto-injector needle.

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

4.3 Calibration of Injection Volume

4.3.1 Calibration Procedure

The nominal injection volume as given by the HPLC system must be verified prior to use for sample cleanup (section 3.3). The volume injected is used to directly calculate the final crop:solvent ratio (CS, section 5.1.3), therefore the accuracy of this measurement is critical. The volume delivered can be measured by filling a series of autosampler vials with deionized water, and then weighing before and after injection. Take a

minimum of 5 measurements and determine the average weight (volume) removed. If the average volume injected differs from the nominal volume, use the experimentally obtained volume for calculation purposes. Due to the nature of the method and the recovery values indicated in Table 1, the injection volume should be corrected if it is determined that the actual volume deviates from the nominal value by more than 2%.

4.3.2 Hints For Obtaining Accurate Injection Volume

If initial injection volumes are low or inconsistent, consider the following points. Tightly sealed vials make the accurate withdrawal of sample difficult due to formation of a vacuum in the sealed vial. Since the samples are aqueous, there is no need for a tightly sealed vial. A loosely sealed vial will allow air to enter and displace the liquid as it is being withdrawn. Do not fill the vial completely. A filled vial is more susceptible to this problem than one with some headspace. Additionally, if the autosampler has a programmable withdrawal rate, choose a slower rate to allow more time for air to enter the vial.

4.4 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 should be run over the concentration range of 0.02 to 10 ng/mL. This series of standards should be repeated using different PMT gain settings. 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 0.01 -0.02 ng/mL of AMBA) This will allow the background found in controls to be accurately determined and corrected.

4.5 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. It is suggested that two individual instrument methods be used for control of the HPLC during the fraction collection run. These are, 1) a uv monitored method for analysis of the uv calibration standard and the running of the water blanks, and 2) a FLD monitored method with timed events for fraction collector control for

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

4.5.1 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. Two injections of a water blank to eliminate carryover
Reagent blank consisting of water used to monitor background, (separate vial from 2, above)
4. Sample extracts
5. UV calibration standard to ensure stability of analyte retention times

4.6 Calibration and Analysis - Analytical Run

Calibrate the HPLC using the calibration standards prepared in section 2.3.5. For crop extracts equivalent to extractives from 0.02 g crop per mL of extract and samples potentially containing residues at 0.01 to 0.10 $\mu\text{g/g}$, calibration standards at 0.12, 1.0, and 10 ng/mL are suggested.

4.6.1 Analytical Scheme

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

1. One injection of each AMBA calibration standard (0.12, 1.0, and 10 ng/mL)
2. Reaction blank, reagent blank, and/or control extract (ZA1296, fraction B)
3. Fraction B, ZA1296 sample extracts
4. One injection of each AMBA calibration standard (0.12, 1.0, and 10 ng/mL)
5. Reaction blank, reagent blank, and/or control extract (MNBA, fraction A)

- 6 Fraction A, MNBA sample extracts
7. One injection of each AMBA calibration standard (0.12, 1.0, and 10 ng/mL)

4.7 Fortification

If available, analyze fortified- and unfortified-control samples with each sample set to demonstrate method recovery. For example, add 100 μ L of a working fortification solution (1.0 μ g/mL) to a control sample (10 g) to produce a fortification level of 0.01 μ g/g. Extract as detailed in section 3.1. If control samples are available it is recommended that one unfortified and 2 fortified samples be analyzed with each set of 8-10 field samples. One of the two fortified samples should be fortified at the method's limit of quantitation (LOQ, 0.01 μ g/g).

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., 10, 1.0, and 0.12 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 AMBA calibration solution as follows:

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

Where,

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

C = concentration of calibration solution (ng/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:

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

Where,

RF(avg) = Average Response Factor

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

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

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

5.1.3 Crop in Extract

Calculate the concentration of the crop: i.e., the amount of crop the final extract represents, as follows:

$$CS = \frac{W(\text{sample})}{V(\text{extract})} \times \frac{V(\text{injected})}{V(\text{final})}$$

Where,

CS = concentration of crop in final extract (g/mL)

W(sample) = weight of sample (g)

V(extract) = total volume of crude extract (mL)

V(injected) = volume injected on HPLC for fraction collection (mL)

V(final) = final volume, after dissolution of residuum from C18 SPE cleanup (mL)

5.1.4 Concentration of Converted AMBA in Final Extracts

Calculate the amount of AMBA, $A_{Z \text{ or } M}$, in the final extract:

$$A_{Z \text{ or } M} = R_{Z \text{ or } M} \times \text{RF}(\text{avg})$$

Where,

$$A_Z \text{ or } A_M = \text{conc. of converted AMBA in final fraction (ng/mL)}$$

$$\text{RF}_{\text{avg}} = \text{average response factor (ng/mL/peak area)}$$

$$R_Z \text{ or } R_M = \text{Sample response (peak area) from detector}$$

Subscript Z refers to ZA1296 (fraction B), while subscript M refers to MNBA (fraction A)

5.1.5 Concentration of Analyte in Original Sample

From the amount of AMBA found in the final extract, an equivalent amount analyte (ZA1296 or MNBA) is calculated.

$$\text{Conc. ZA296 (mg/kg)} = A_Z \text{ (ng/mL)} \times \frac{\text{MW of ZA1296}}{\text{MW of AMBA}} \times \frac{1}{\text{CS}} \times \frac{1}{1000}$$

$$\text{Conc. MNBA (mg/kg)} = A_M \text{ (ng/mL)} \times \frac{\text{MW of MNBA}}{\text{MW of AMBA}} \times \frac{1}{\text{CS}} \times \frac{1}{1000}$$

MW = Molecular Weight

6 Interferences

6.1 Interfering Co-Extractives

Due to the nature of the method, significant interferences arising from the crop matrix have been rare. High selectivity can be attributed to the method's use of fraction collection, conversion to a common moiety, then re-injection with elution of the analyte in a chromatographic region excluded from the original collected fraction. In order for a co-extractive to interfere with the final quantitation that compound would have to, 1) elute near the analyte of interest during the fraction collection and be collected, 2) chemically convert to another species, 3) have that converted species co-elute with AMBA, and 4) exhibit fluorescence using the excitation and emission wavelengths specified in the method. For work conducted for this report, interference levels were less than 20% of the LOQ of 0.01 mg/kg.

6.2 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. A control sample (ZA1296 fraction) upon analysis has a significant peak that co-elutes with AMBA. This unexpected result could have been due to 1) contamination with ZA1296 prior to the fraction collection process; 2) contamination with MNBA or ZA1296 after the fraction collection process, but not after the conversion process; or 3) contamination with AMBA after fraction collection. In a similar manner a contaminated control sample (MNBA fraction) could have been due to 1) contamination with MNBA before fraction collection; 2) contamination with MNBA after fraction collection but before conversion to AMBA; or 3) contamination with AMBA after fraction collection. This last control sample could not have been contaminated with ZA1296 since no oxidation step is performed to generate MNBA before the reduction to AMBA.

6.3 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.

6.4 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 MBNA reduction reaction. This blank will indicate contamination of 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. 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

The basis for the selectivity of the method is discussed in section 6.1. 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 Column

A Keystone Prism-RP column can be used as an alternative column for the HPLC cleanup. Variations in the selectivity of this stationary phase, in conjunction with alternate mobile phases can provide confirmation of MNBA and ZA1296 residues. For confirmatory purposes this column should be used during the HPLC cleanup. The HPLC mobile phase A solution (section 2.2.16) should be 15 mM ammonium acetate and 0.6 % formic acid. The column temperature should be raised to 80° C.

8 Results and Discussion

8.1 Accuracy and Precision

Fortified crop samples were prepared as described under section 4.7, and analyzed according to this method to establish recovery. Recoveries of ZA1296 fortified at 0.01 and 0.10 mg/kg ranged from 70 to 99 percent, with a mean of 79% (n=37) and coefficient of variation of 7%. Recoveries of MNBA fortified at 0.01 and 0.10 mg/kg ranged from 69 to 92% with a mean of 84% (n=35) and coefficient of variation of 6%. Tables 1 through 4 list the recoveries obtained from corn forage, corn fodder, corn grain, and sugarcane.

The precision of the method depends on variations in extraction, cleanup, conversion reactions, and instrumental analysis. These variations can be evaluated from the data obtained during the analysis of fortified samples. The coefficients of variation given in Table 1 through 5 are a measure of the precision of the method.

8.2 Calculated Limits of Detection and Quantitation

Following proposed guidelines, limits of quantitation (LOQ) and limits of detection (LOD) were calculated using the standard deviation of the 0.01 mg/kg recovery results. The LOQ was calculated as 10 times the standard deviation and the LOD was calculated as three times the standard deviation.

The standard deviation for ZA1296 obtained for all 0.01 mg/kg determinations was 0.00069 mg/kg. The 10X values and 3X values were 0.0069 and 0.0023 mg/kg, respectively. Due to the persistence of a low level of background in the corn commodities, averaging 0.0016 mg/kg, this background value was then added to the 10X and 3X values resulting in a calculated LOQ of 0.008 mg/kg, and a calculated LOD of 0.004 mg/kg.

The standard deviation for MNBA obtained for all the 0.01 mg/kg fortifications was 0.00016 mg/kg. The 10X and 3X values are 0.0016 mg/kg and 0.00047 mg/kg, respectively. The amount of background found in the controls of corn commodities ranged from zero to 0.0016 mg/kg. For a worst case estimate, the 0.0016 mg/kg value was then added to the 10X and 3X values. This results in calculated LOQ of 0.003 mg/kg and a calculated LOD of 0.002 mg/kg.

8.3 Reported Limits of Quantitation and Detection

As shown in section 8.2, the calculated statistics support an LOQ for corn commodities of 0.008 mg/kg for ZA1296 and 0.003 mg/kg for MNBA. However, results should not be quantified for levels below which no recovery samples have been analyzed. Therefore, the reported LOQ for this method, as validated, is 0.01 mg/kg..

The calculated LOD for corn commodities was 0.004 mg/kg for ZA1296, and 0.002 mg/kg for MNBA. The statistical approach to the calculation of the LOD does not take into account the instrument-to-instrument variability of parameters such as signal-to-noise ratio. The value may also vary based on the background of each individual matrix. The calculated values reported here may vary with those obtained in other laboratories. Irrespective of any calculated LOD value is the requirement that the signal-to-noise ratio for the reported value be at least a factor of three. The calculated LOD for both analytes meet this requirement.

8.4 Extraction Efficiency

The extraction procedure reported in this report is consistent with that used in ZA1296 metabolism studies. Validation of this procedure is reported in Zeneca Report RR 96-007B (Reference 1).

8.5 Reagent Stability

The catalase reagent and the stannous chloride reagent must be prepared fresh daily. All other reagents can be prepared on a monthly basis. The catalase powder should be stored at a temperature of < 0° C. Once opened, the peroxide test strip container should be given an expiration date of one month.

8.6 Column Selection

8.6.1 HPLC Cleanup Column

The primary column for the HPLC cleanup (fraction collection) is either the Inertsil™ ODS-2, or the Phenomenex Prodigy™ ODS-2. These columns can be considered interchangeable. They are both produced with very high purity silica (99.999% pure) and very low metal content. Other ODS columns tend to produce a tailing peak for ZA1296. This compound may chelate with metal in the silica, producing the characteristic tailing. MNBA and AMBA do not tail significantly.

The Keystone Prism-RP™ column is a proprietary reverse phase packing and is used as an alternate HPLC cleanup column for confirmatory

analysis (see section 7.2). The tailing of ZA1296 observed with some reverse phase columns is evident with this column. New columns provide acceptable peakshape but tailing becomes more pronounced with use. This packing material is not as rugged as the Prodigy or Inertsil material, and as a result, care should be taken to flush the column with a acetonitrile : water (1:1) after each use.

8.6.2 Analytical Column

The majority of the method development work was performed using a Waters Spherisorb ODS-2 (250 mm x 3.0 mm i.d.) column for the analytical determination. Some work (sugar cane and corn forage) was performed using a 250 mm x 4.6 mm i.d., Spherisorb column. For this work the flow rate was increased to 1.5 mL/min and the injection volume was increased to 400 μ L. The results for corn grain were obtained using a column with a similar chemistry, the Metasil ODS-2 column. This column was 250 mm x 3.0 mm i.d. The use of this column illustrates the equivalent nature of the MetaSil packing material.

8.7 Gradient Elution Parameter

The HPLC parameters given in section 4.1.1 for the final analytical determination call for an isocratic elution of AMBA at a mobile phase composition of 16%, followed by a rapid gradient to wash the column of co-extractives and reaction by-products. These parameters were used to analyze the corn fodder samples (see Figures 6 and 7). The other commodities were analyzed using a solvent program starting at an initial %B of 20%. During analysis of the fodder samples, the 16% B program was found to provide a significant improvement in resolution, when compared to the 20% B program. Co-extractives that co-elute with AMBA using the 20% B program were well resolved when starting at 16%. *As a result, 16%B is the recommended starting point for the gradient.* These conditions, however may be modified to obtain optimum chromatography for a particular HPLC system, and specific samples.

8.8 MNBA Retention Adjustment

Column-to-column variation may provide slightly different selectivity during the UV calibration procedure (section 3.4.2). It is important to exclude any potential AMBA residues from the collected fractions. Therefore it may be necessary to modify the mobile phase composition to increase the resolution between MNBA and AMBA. This can be done by increasing the ionic strength of mobile phase A (section 2.2.16). Increasing the ammonium acetate concentration to 15-20 mM will

decrease the retention time of MNBA , with the retention time of AMBA remaining virtually unchanged.

8.9 Conversion Efficiency

As discussed in section 3.7 the optimum performance of the reactions converting the analytes to AMBA is a critical success factor for this method. The performance of the reactions converting ZA1296 to AMBA, and MBNA to AMBA are monitored by the calculation of conversion efficiencies, also shown in section 3.7. Comparing the response of the AMBA formed, resulting from the reduction of a known amount of MNBA, to the response of an AMBA calibration solution results in the % yield, or conversion efficiency of that reaction. Under the conditions given in section 3.6, conversion efficiencies of 90-95% can be expected for the reduction of MNBA to AMBA. The efficiency of the oxidation of ZA1296 to MNBA, and the subsequent reduction to AMBA, can be determined in a similar manner. This overall conversion is usually somewhat less than that of the MNBA-to-AMBA conversion since it requires two separate reactions, neither of which is 100 % complete. Under the conditions given in section 3.5, conversion efficiencies of 85-90% are normally obtained when converting ZA1296 to AMBA. Since residue levels are calculated based on the response of an AMBA reference standard, the conversion efficiency plays a direct role in residue determination, and recovery determination. *The conversion of the analytes to AMBA, at the rates given above, should be demonstrated prior to conducting residue determinations.*

8.10 Confirmation of Analyte Isolation

This method involves the conversion to, and quantitation of, the common moiety AMBA. As a result, the isolation of ZA1296 and MNBA from each other and from trace levels of AMBA that may be present in a sample is critical to the accuracy of the method. Upon initial use of the method it should be demonstrated that, in addition to adequate recovery of analyte fortified into control samples (method recovery), the presence of one analyte does not interfere (cause false positives) with the other. This can be accomplished by fortifying control extracts with ZA1296 and MNBA separately. The ZA1296 fraction (fraction B) of the sample fortified with MNBA can be analyzed for ZA1296, and shown to be free from interference. The MBNA fraction (fraction A) of the sample fortified with ZA1296, can be analyzed in a similar manner. Once isolation of the analytes and freedom from interferences is confirmed, method recovery can be demonstrated by the fortification of both analytes into a single unfortified control sample.

8.11 Methodology Logistics

8.11.1 Analysis Time

The method is normally performed over the course of two days. On day one, the extraction, evaporation, and reconstitution are completed. The HPLC fraction collection process is started and automated to run overnight. On day two, the conversion reactions and post-conversion cleanup are performed, and the analytical runs are started. Again, HPLC automation allows for the analytical determinations to run overnight on day two.

For a sample set of consisting of 6-8 samples the following time should be allowed. For day one, including extraction, evaporation and starting the overnight fraction collection run, 4-5 hours is required. For day two, including performing the conversion reactions and initializing the HPLC for the overnight analytical determination, 8 hours of laboratory time is required. If only one analyte to be determined, the amount of time required for day two may be reduced to 4-6 hours. *The estimated time requirements given above assume that certain tasks are conducted concurrently.* For example during the three evaporation steps, instrument runs can be set up, vials weighed, SPE column prepared, reagents prepared, etc.

8.11.2 Work Stoppages

Work on the method can be stopped at various points throughout the procedure for up to an hour. Stopping work at certain points should be avoided. These include:

1. After evaporation of HPLC cleanup fraction (section 3.3.5). After these fractions have been evaporated the conversion reactions should be carried out.
2. After addition of the sample aliquot to the C18 SPE column (section 3.6.2), the columns should be washed with the two, 1.5-mL wash volumes as soon as possible.

8.11.3 Overnight HPLC Cleanup

It is convenient to automate the HPLC cleanup fraction collection process (section 3.3.4). Start the process on the afternoon/evening of day one of the procedure. Allowing the collected fractions to remain in the fraction collector until morning is acceptable. The method has also been successful when the collected fractions have remained in the fraction collector for up to 60 hours (over a weekend). However it is

recommended that this practice be avoided until sufficient experience with the method is obtained.

8.12 Minimization of Physical Losses

The nature of this method, including the use of small volume reactions, makes it susceptible to low recoveries due to physical loss of the extract at various stages of the procedure. These losses occur primarily due to the repeated opening and closing of the 4-mL reaction vials. During workup, small droplets of the vial contents can lodge on the PTFE liner of the vial cap and in the neck of the vial near the very top. These small droplets may then enter the thread area of the vial or cap, where they are lost. Also, the vial may be under some pressure after the catalase reaction. Any liquid residing near the vial cap may be forcibly expelled when the cap is removed, due to even a small amount of positive pressure. Additionally, when the Eppendorf pipet is used to transfer the sample to the SPE cleanup column (section 3.6.2) small droplets may be transferred from the neck of the vial to the outside of the pipet tip. The amount lost for each occurrence may very small (1-5 μ L). Considering the percentage of the total volume of sample, when repeated, these losses may result in an overall loss in the range of 2-5%. Considering the recoveries obtained and reported in Table 1, losses of this magnitude should not be overlooked.

For this reason, it is recommended that prior to opening these vials, they be centrifuged for a short time. 1-2 minutes at 1000-1500 rpm should suffice. This will force all liquid down away from the cap and neck and minimize the losses described above. Steps that should include this procedure are indicated in the method by inclusion of the statement, "*Centrifuge prior to opening vial*".

8.13 Safety Precautions

Personnel untrained in the routine safe handling of chemical and good laboratory practices should not attempt to use this procedure. Information of any specific chemical regarding physical properties, hazards, toxicity, and first-aid procedures can be found on the Material Safety Data Sheet accompanying the chemical, available from the supplier or from the Good Laboratory Practices Archive. In general, always wear safety glasses with side shields, work in a well ventilated area, avoid inhaling vapors and avoid contact of the chemicals with skin and clothing.

Specific hazards include the use of hydrogen peroxide. The 30% reagent is a strong oxidizer and should be kept away from organic material or other sources of combustion. Flammable solvents used in the method include methanol, acetonitrile, and ethyl acetate.

Conclusions

The method is specific for the analysis of ZA1296 and MNBA residues in corn grain, corn fodder, corn forage, and sugar cane. Only commercially available laboratory equipment and reagents are required. The analysis can be completed by one person in one 4-hour and one 8-hour period if an adequately homogenized sample is available. If available, untreated and fortified samples should be extracted and analyzed with each set of samples to demonstrate absence of interferences and adequate recovery. If residues of ZA1296 and MNBA at concentrations other than 0.01 and 0.10 ppm are required, suitably fortified samples must be analyzed to validate the method at that level.

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Figure 1.	Chromatograms of UV Calibration Standard and HPLC Cleanup of Corn Forage Crude Extract
Figure 2.	Sample Chromatograms: Corn Forage Fortified with 0.01 mg/kg ZA1296
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Figure 9.	Sample Chromatograms: Sugar Cane Fortified with 0.01 mg/kg MNBA

Table 1. Recovery of ZA1296 and MNBA From Corn Forage

Sample No.	Amount Added, Each Analyte (ppm)	Amount ZA1296 Found, % ¹	Amount MNBA Found, % ¹
N255-4 (replicate F-1)	0.01	71	72
N255-4 (replicate F-1)	0.01	78	77
N255-4 (replicate F-3)	0.01	82	77
N255-4 (replicate F-4)	0.01	76	77
N255-4 (replicate F-5)	0.10	80	85
N255-4 (replicate F-6)	0.10	86	85
N255-4 (replicate F-7)	0.10	77	92
N255-4 (replicate F-8)	0.10	74	83

Data Summary, ZA1296	Average			
	Recovery, %	CV, %	N	Range
Corn Forage, all 0.01 mg/kg fortifications	77	6	4	71-82
Corn Forage, all 0.10 mg/kg fortifications	79	7	4	74-86
Corn Forage, all fortifications	78	6	8	71-86

Data Summary, MNBA	Average			
	Recovery, %	CV, %	N	Range
Corn Forage, all 0.01 mg/kg fortifications	76	3	4	72-77
Corn Forage, all 0.10 mg/kg fortifications	86	4	4	83-92
Corn Forage, all fortifications	81	8	8	72-92

Note: Two control samples were analyzed with these samples. Interferences equivalent to 0.0003 mg/kg (3% LOQ) were encountered in the analysis ZA1296. One control sample analyzed for MNBA was lost, the other contained an amount equivalent to 0.0002 mg/kg (2% LOQ).

¹ Percent Recovery, corrected by subtraction of average amount found in controls analyzed concurrently with sample

Table 2. Recovery of ZA1296 and MNBA From Corn Grain

Sample No.	Amount Added, Each Analyte (ppm)	Amount ZA1296 Found, % ¹	Amount MNBA Found, % ¹
² AOAC (replicate F-1)	0.01	99	81
AOAC (replicate F-2)	0.01	74	84
AOAC (replicate F-3)	0.01	76	82
AOAC (replicate F-4)	0.01	92	82
AOAC (replicate F-5)	0.10	82	85
AOAC (replicate F-6)	0.10	79	85
AOAC (replicate F-7)	0.10	80	81
AOAC (replicate F-8)	0.10	82	83

Data Summary, ZA1296	Average		N	Range
	Recovery, %	CV, %		
Corn Grain, all 0.01 mg/kg fortifications	85	14	4	74-99
Corn Grain, all 0.10 mg/kg fortifications	81	2	4	79-82
Corn Grain, all fortifications	83	10	8	74-99

Data Summary, MNBA	Average		N	Range
	Recovery, %	CV, %		
Corn Grain, all 0.01 mg/kg fortifications	82	1	4	81-84
Corn Grain, all 0.10 mg/kg fortifications	83	2	4	81-85
Corn Grain, all fortifications	83	2	8	81-85

Note: Two control samples were extracted and analyzed concurrently with the above samples. Interferences equivalent to 0.0020 and 0.0022 mg/kg of ZA1296 were found. Similar amounts were found in reagent blanks and reaction blanks, indicating a potential reagent contamination. Interferences equivalent to 0.0014 and 0.0019 mg/kg MNBA were found in the same samples. Amounts found in MNBA reagent and reaction blanks were negligible, indicating an average level of crop-induced interference of 0.0016 mg/kg or 16% of LOQ.

¹ Percent Recovery, corrected by subtraction of average amount found in controls analyzed concurrently with sample

² Samples taken from a composite of field corn grain samples from study TEFL-96-MR-01 previously used for AOAC collaborative study for glyphosate method.

Table 3. Recovery of ZA1296 and MNBA From Corn Fodder

Sample No.	Amount Added, Each Analyte (ppm)	Amount ZA1296 Found, % ¹	Amount MNBA Found, % ¹
M601-007 (replicate F-1)	0.01	79	81
M601-007 (replicate F-2)	0.01	76	82
M601-007 (replicate F-3)	0.01	83	81
M601-007 (replicate F-4)	0.01	75	--- ²
M601-007 (replicate F-5)	0.01	72	--- ²
M601-007 (replicate F-6)	0.01	80	83
M601-007 (replicate F-7)	0.01	77	79
M601-007 (replicate F-8)	0.01	79	84
M601-007 (replicate F-9)	0.10	84	85
M601-007 (replicate F-10)	0.10	78	85
M601-007 (replicate F-11)	0.10	78	85
M601-007 (replicate F-12)	0.10	81	88
M601-007 (replicate F-13)	0.10	83	86

Data Summary, ZA1296	Average			
	Recovery, %	CV, %	N	Range
corn fodder, all 0.01 mg/kg fortifications	78	4	8	72-83
corn fodder, all 0.10 mg/kg fortifications	81	4	5	83-84
corn fodder, all fortifications	79	4	13	72-84

Data Summary, MNBA	Average			
	Recovery, %	CV, %	N	Range
corn fodder, all 0.01 mg/kg fortifications	82	2	6	79-84
corn fodder, all 0.10 mg/kg fortifications	86	2	5	85-88
corn fodder, all fortifications	84	3	11	79-88

Note: Six control samples were analyzed with these samples. Average interferences equivalent to 0.0017 mg/kg (17% LOQ) were encountered in the analysis ZA1296. MNBA controls contained an amount equivalent to 0.0003 mg/kg (3% LOQ).

¹ Percent Recovery, corrected by subtraction of average amount found in controls analyzed concurrently.

² Samples lost during processing

Table 4. Recovery of ZA1296 and MNBA From Sugar Cane

Sample No.	Amount Added, Each Analyte (ppm)	Amount ZA1296 Found, % ¹	Amount MNBA Found, % ¹
N228-301 (replicate F-1)	0.01	78	82
N228-301 (replicate F-2)	0.01	77	81
N228-301 (replicate F-3)	0.01	70	69
N228-301 (replicate F-4)	0.01	74	83
N228-301 (replicate F-5)	0.10	72	83
N228-301 (replicate F-6)	0.10	72	86
N228-301 (replicate F-7)	0.10	76	86
N228-301 (replicate F-8)	0.10	73	78

Data Summary, ZA1296	Average Recovery, %	CV, %	N	Range
sugar cane, all 0.01 mg/kg fortifications	75	5	4	70-78
sugar cane, all 0.10 mg/kg fortifications	73	2	4	72-76
sugar cane, all fortifications	74	4	8	70-78

Data Summary, MNBA	Average Recovery, %	CV, %	N	Range
sugar cane, all 0.01 mg/kg fortifications	79	8	4	69-83
sugar cane, all 0.10 mg/kg fortifications	82	4	4	78-86
sugar cane, all fortifications	81	7	8	69-86

Note: Two control samples were analyzed with these samples. Interferences equivalent to 0.0003 mg/kg (3% LOQ) were encountered in the analysis ZA1296. MNBA controls contained an amount equivalent to 0.0003 mg/kg (3% LOQ).

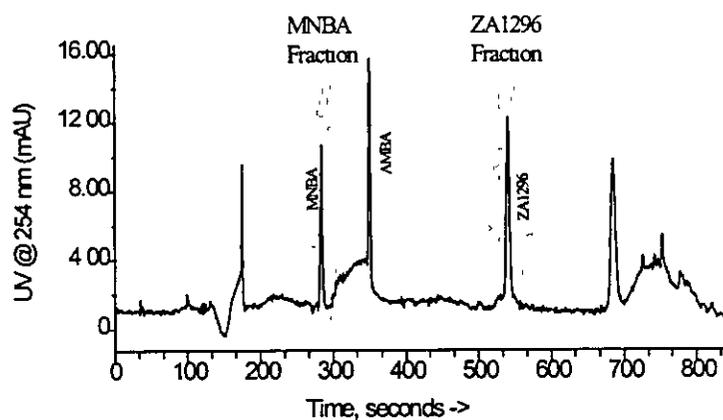
¹ Percent Recovery, corrected by subtraction of average amount found in controls analyzed concurrently with sample.

Table 5. Recovery Summary, All Commodities

<u>Data Summary, ZA1296</u>	<u>Average Recovery, %</u>	<u>CV, %</u>	<u>N</u>	<u>Range</u>
All 0.01 mg/kg fortifications	79	7	20	70-99
All 0.10 mg/kg fortifications	79	4	17	72-86
All fortifications	79	7	37	70-99

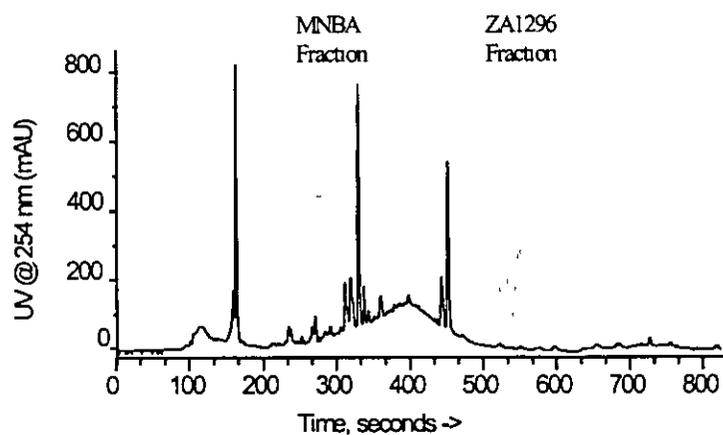
<u>Data Summary, MNBA</u>	<u>Average Recovery, %</u>	<u>CV, %</u>	<u>N</u>	<u>Range</u>
All 0.01 mg/kg fortifications	80	4	18	69-84
All 0.10 mg/kg fortifications	85	3	17	78-92
All fortifications	82	5	35	69-92

Figure 1 Chromatogram of UV Calibration Standard and HPLC Cleanup of Corn Forage Crude Extract



UV Calibration Standard

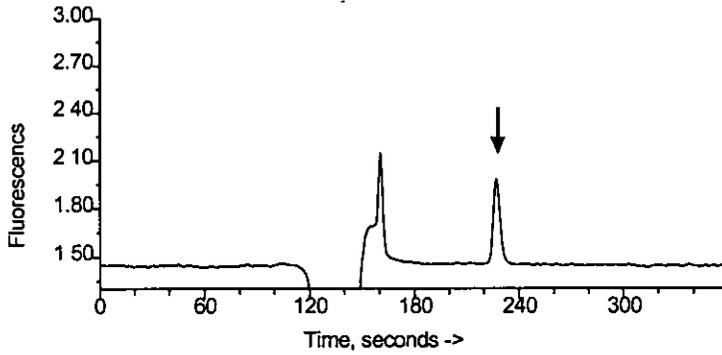
Determination of fraction collection windows (0.20 ug/mL each analyte)



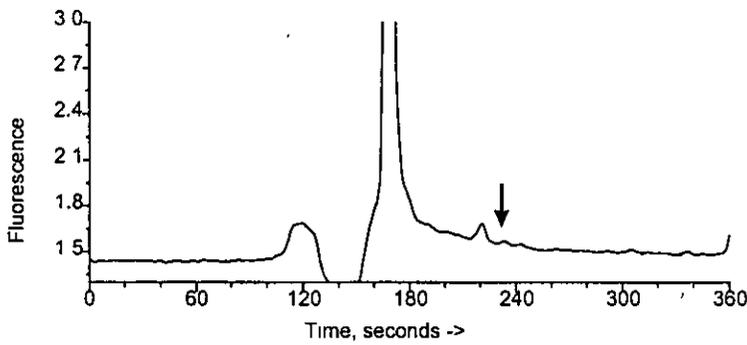
Crude Corn Forage Extract

Shown are fraction collection windows for each analyte. (extract concentration 0.125 g/mL)

Figure 2. Sample Chromatograms: Corn Forage Fortified with 0.01 mg/kg ZA1296

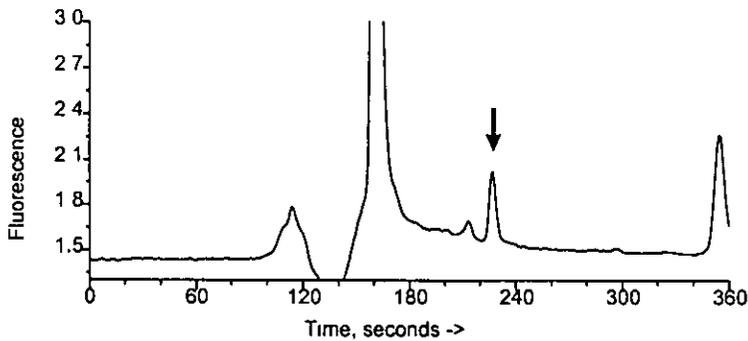


AMBA calibration standard,
0.12 ng/mL (0.189 ng/mL
ZA1296 equivalents)



Untreated Control

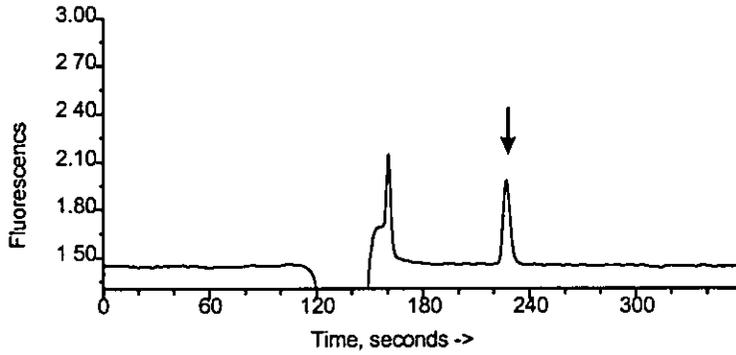
Replicate C-1, fraction B
0.02 g matrix/mL extract



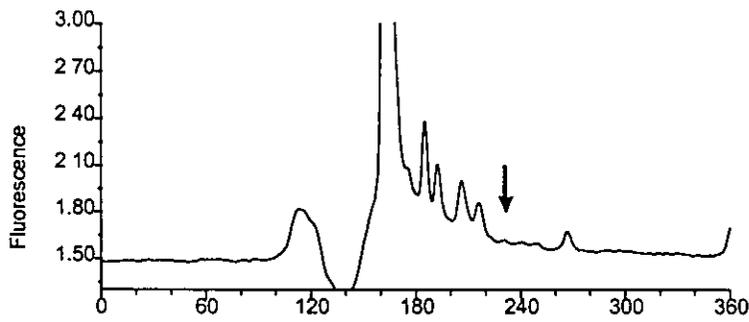
Untreated Control, fortified
with ZA1296 at 0.01 mg/kg.
78% Recovery

Replicate F-2, fraction B
0.02 g matrix/mL extract

Figure 3. Sample Chromatograms: Corn Forage Fortified with 0.01 mg/kg MNBA

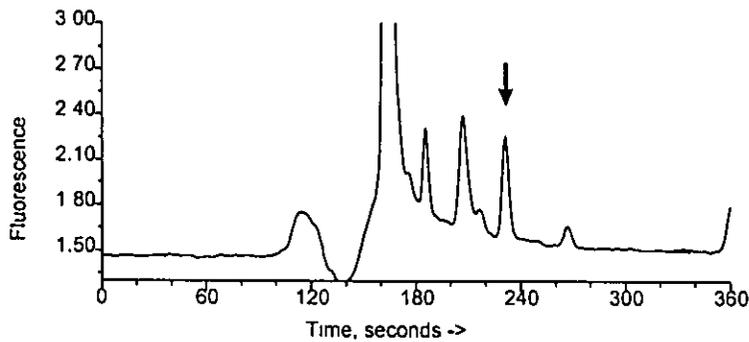


AMBA calibration standard, 0.12 ng/mL (0.137 ng/mL MNBA equivalents)



Untreated Control

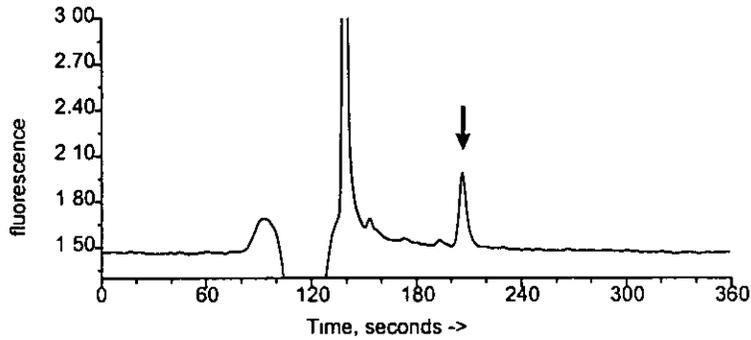
Replicate C-1, fraction A
0.02 g matrix/mL extract



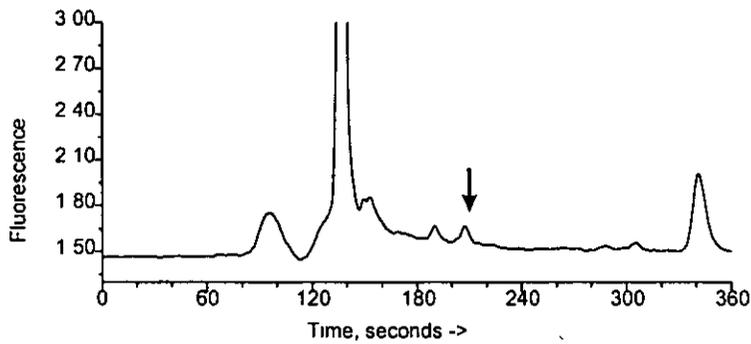
Untreated Control, fortified with MNBA at 0.01 mg/kg
77% Recovery

Replicate F-2, fraction A
0.02 g matrix/mL extract

Figure 4. Sample Chromatograms: Corn Grain Fortified with 0.01 mg/kg ZA1296

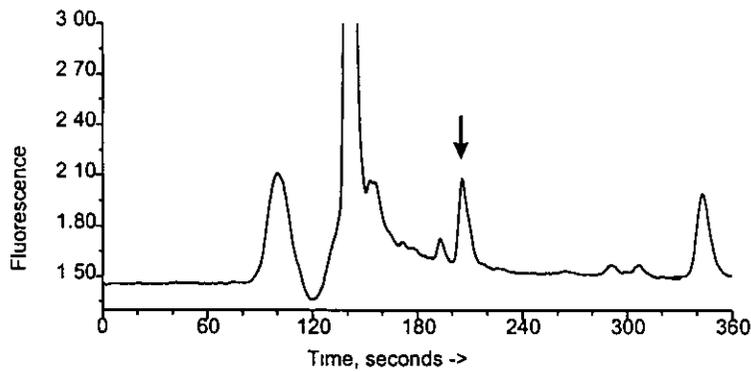


AMBA calibration
standard, 0.12 ng/mL
(0.189 ng/mL ZA1296
equivalents)



Untreated Control

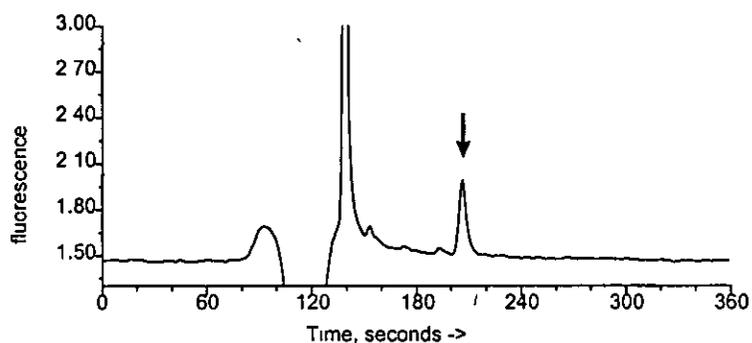
Replicate C-1, fraction B
0.02 g matrix/mL extract



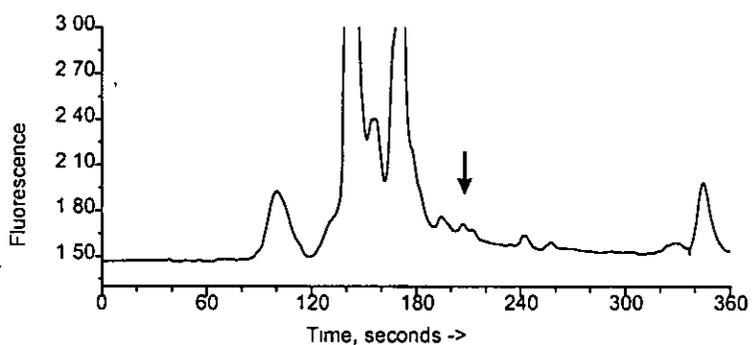
Untreated Control, fortified
with ZA1296 at 0.01 mg/kg
99% Recovery

Replicate F-1, fraction B
0.02 g matrix/mL extract

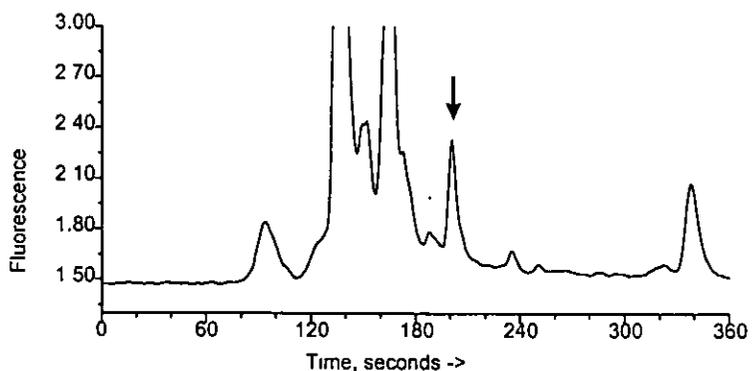
Figure 5. Sample Chromatograms: Corn Grain Fortified with 0.01 mg/kg MNBA



AMBA calibration
standard, 0.12 ng/mL
(0.137 ng/mL MNBA
equivalents)

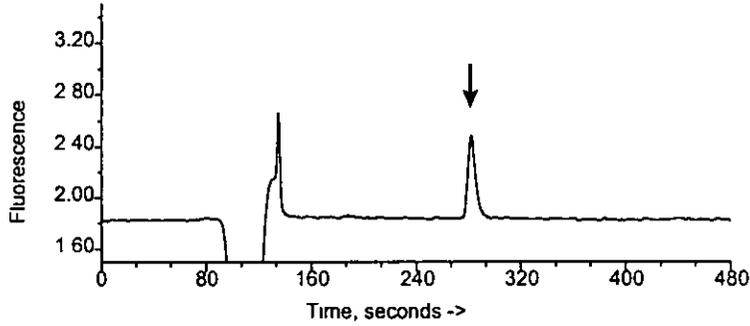


Untreated Control
Replicate C-1, fraction A
0.02 g matrix/mL extract

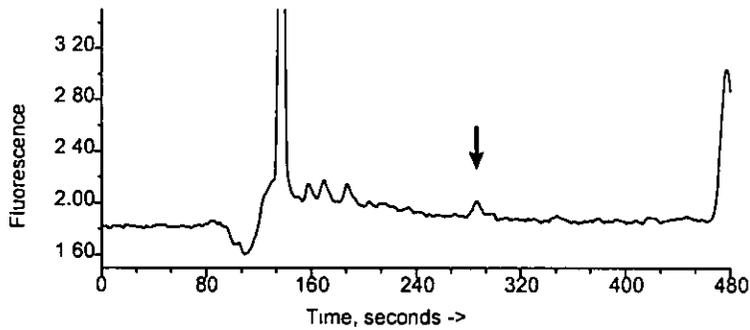


Untreated Control, fortified
with MNBA at 0.01 mg/kg
81% Recovery
Replicate F-1, fraction A
0.02 g matrix/mL extract

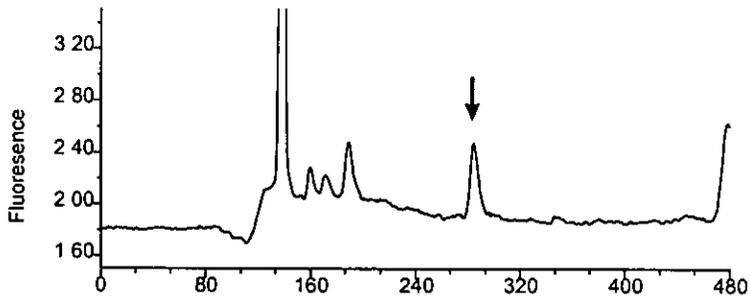
Figure 6. Sample Chromatograms: Corn Fodder Fortified with 0.01 mg/kg ZA1296



AMBA calibration
standard, 0.12 ng/mL
(0.189 ng/mL ZA1296
equivalents)



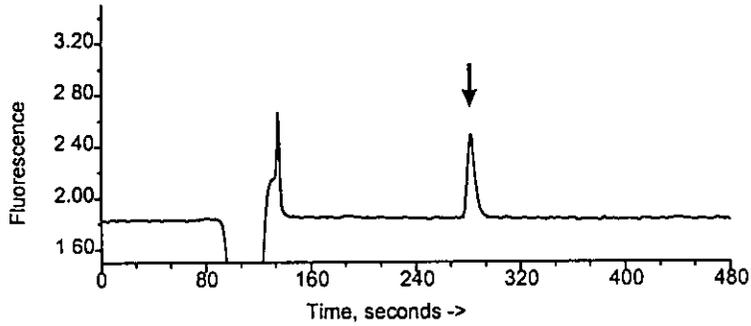
Untreated Control
Replicate C-3, fraction B
0.02 g matrix/mL extract



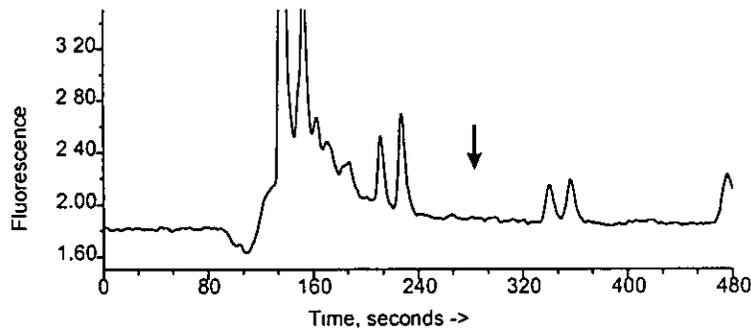
Untreated Control, fortified
with ZA1296 at 0.01 mg/kg
79% Recovery

Replicate F-1, fraction B
0.02 g matrix/mL extract

Figure 7. Sample Chromatograms: Corn Fodder Fortified with 0.01 mg/kg MNBA

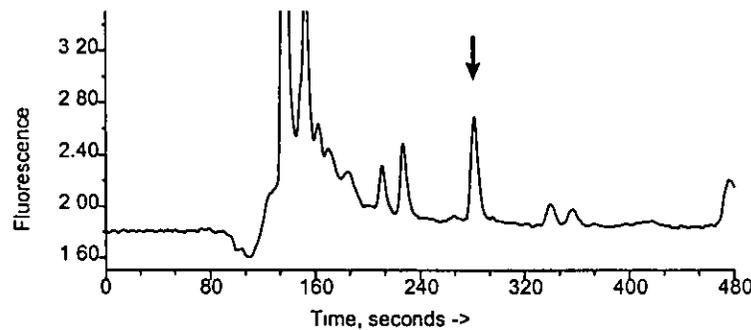


AMBA calibration
standard, 0.12 ng/mL
(0.137 ng/mL MNBA
equivalents)



Untreated Control

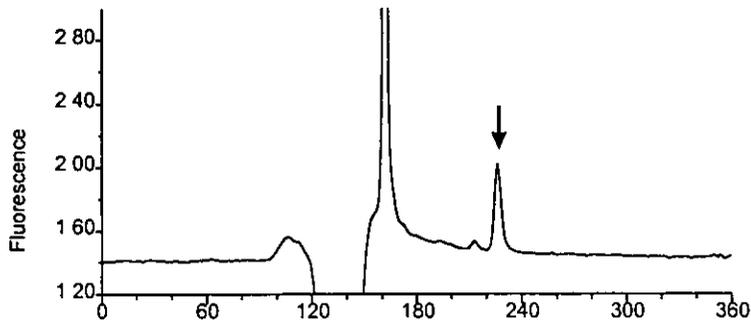
Replicate C-3, fraction A
0.02 g matrix/mL extract



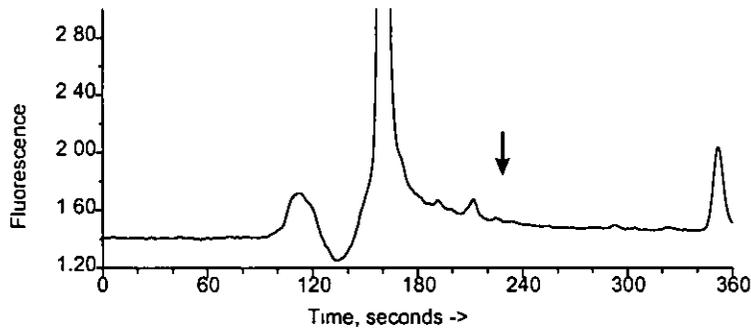
Untreated Control, fortified
with MNBA at 0.01 mg/kg
81% Recovery

Replicate F-1, fraction A
0.02 g matrix/mL extract

Figure 8. Sample Chromatograms: Sugar Cane Fortified with 0.01 mg/kg ZA1296

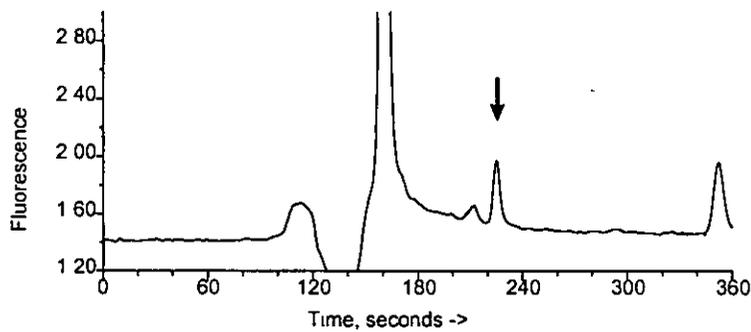


AMBA calibration
standard, 0.12 ng/mL
(0.189 ng/mL ZA1296
equivalents)



Untreated Control

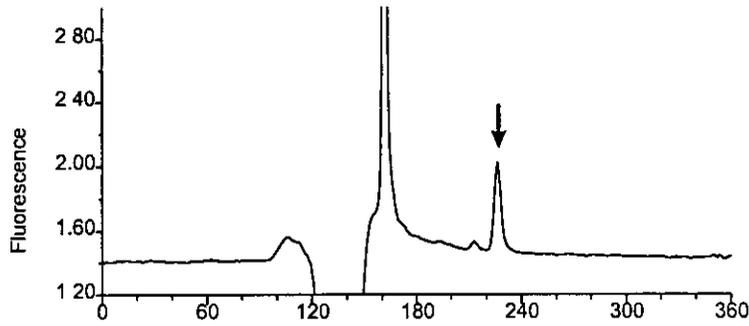
Replicate C-1, fraction B
0.02 g matrix/mL extract



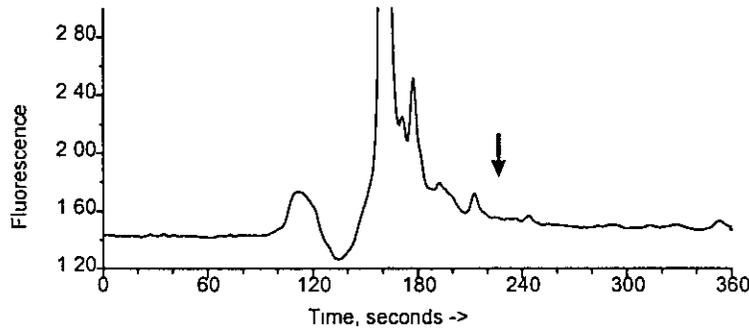
Untreated Control, fortified
with ZA1296 at 0.01 mg/kg
77% Recovery

Replicate F-2, fraction B
0.02 g matrix/mL extract

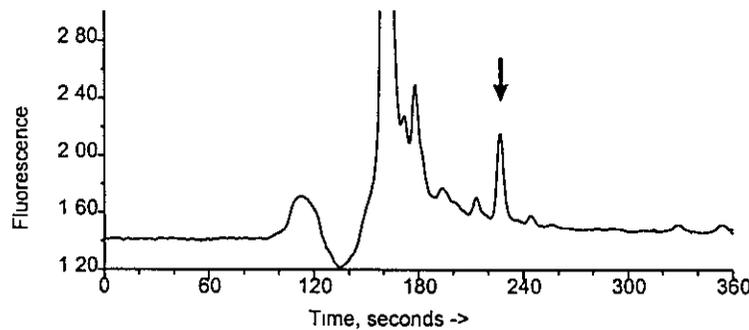
Figure 9. Sample Chromatograms: Sugar Cane Fortified with 0.01 mg/kg MNBA



AMBA calibration
standard, 0.12 ng/mL
(0.137 ng/mL MNBA
equivalents)



Untreated Control
Replicate C-1, fraction A
0.02 g matrix/mL extract



Untreated Control, fortified
with MNBA at 0.01 mg/kg
81% Recovery
Replicate F-2, fraction A
0.02 g matrix/mL extract

11

References

1. Tarr, J. B. (1996) [Phenyl- $u\text{-}^{14}\text{C}$] ZA1296; Nature of the Residue in Corn. Zeneca Inc., RR 96-007B
2. Raw data referenced in WRC notebooks No. 16294 and 16295

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Appendices

Appendix A: HPLC Cleanup: Example of Fraction Collection Window Determination

Appendix B: Sample Calculations

Appendix C: Modifications to Original Crop Method: TMR0643B

Appendix D: Flowchart of Analytical Procedure

Appendix A

HPLC Cleanup:

Example of Fraction Collection Window Determination

Determination of Fraction Collector Delay

DAD-to-FLD delay

Retention time of AMBA by uv (DAD) = 6.42 min.

Retention time of AMBA by fluorescence (FLD) = 6.47 min.

DAD-to-FLD delay = 0.05 min.

FLD-to-FC delay

Length of 0.02" i.d. tubing
connecting FLD to FC = 40 cm

Volume of tubing = $L \times \pi r^2$
= 40 cm x (0.01 in. x 2.54cm/in.)² x 3.14
= 0.081 cc (or mL)

FLD-to-FC delay = $\frac{0.081 \text{ mL}}{1.5 \text{ mL/min.}}$ = 0.05 min.

Total Fraction Collector Delay

DAD-to-FLD delay + FLD-to-FC delay = 0.05 + 0.05 min. = 0.10 min.

Determination of Fraction Collection Windows

Analyte	Uv Rt (min.)	FC Delay (min.)	FC Elution Time (min.)	Nominal Window (min.)	Fraction Collection Window (min.)
MNBA	4.82	0.10	4.92	±0.4 min.	4.52 - 5.32
AMBA	5.83	0.10	5.93		
ZA1296	9.02	0.10	9.12	±0.6 min.	8.42 - 9.62

Appendix B

Sample Calculations

Sample Preparation, Determination of Final Crop:Solvent Ratio (CS)

Section 3.1 Extract 10 g of crop (containing 0.01 mg/kg of ZA1296), with 117 mL of extraction solution

$$\frac{10 \text{ g}}{125 \text{ mL}} = 0.08 \text{ g/mL}$$

Crop = 80% water (10 g x 0.80) + 117 = 125 mL total extraction volume

Section 3.2 Transfer 8 mL aliquot of extract to 8 mL vial. Evaporate to 2.5 - 3 mL, add 2 mL 1% formic acid in water. Bring to 8 mL volume with water

$$0.08 \text{ g/mL} \times \frac{8 \text{ mL}}{8 \text{ mL}} = 0.08 \text{ g/mL}$$

Section 3.3 Perform HPLC cleanup on a 250 µL aliquot

$$0.08 \text{ g/mL} \times 0.250 \text{ mL} = 0.02 \text{ g}$$

Section 3.4 Perform conversion reactions, add entire extract to SPE column, elute, evaporate, dissolve in 1.0 mL acetate buffer

$$\frac{0.02 \text{ g}}{1.0 \text{ mL}} = 0.02 \text{ g/mL}$$

Final Crop Solvent ratio = CS = 0.02 g/mL

Analyte Concentration in Sample

Assuming: Fortification Level = 0.01 mg/kg
 Crop concentration in extract, CS = 0.02 g/mL
 Area Response of converted AMBA in Fortified Sample (fraction B), R_Z = 2.23 area units
 Area Response of converted AMBA in Control (fraction B), R_Z = 0.19 area units

5.11 and 5.12 AMBA Response Factor

Standard Conc (C, ng/mL)	Area Response	Average Area Response (R)	Response Factor (RF)	Average Response Factor (RF _{avg})
0.12	2.41 2.23	2.32	0.05172	0.05340
1.0	18.31 18.45	18.38	0.05441	
10.0	185.2 184.6	184.9	0.05408	

Appendix B

Sample Calculations (cont.)

5.1.3 Crop in Extract

$$CS = \frac{W(\text{sample}) \times V(\text{injected})}{V(\text{extract}) \times V(\text{final})} = \frac{10 \text{ g}}{125 \text{ mL}} \times \frac{0.250 \text{ mL}}{1.0 \text{ mL}}$$

5.1.4 Concentration of Converted AMBA in Final Extract, A_z or M

	A_z	=		$R_z \times RF_{AVG}$	
Fortified Sample	A_z	=	2.23	x	0.05340
		=	0.11908		ng/mL
Control	A_z	=	0.19	x	0.05340
		=	0.01015		ng/mL

5.1.45 Concentration of Analyte (ZA1296) in Original Sample

$$\text{Analyte Conc (mg/kg)} = A_z \times \frac{MW \text{ ZA1296}}{MW \text{ AMBA}} \times \frac{1}{CS} \times \frac{1}{1000}$$

$$\begin{aligned} \text{Conc ZA296 (mg/kg) in Fortified Sample} &= 0.11908 \times \frac{339}{215} \times \frac{1}{0.02} \times \frac{1}{1000} \\ &= 0.00939 \text{ mg/kg} \end{aligned}$$

$$\begin{aligned} \text{Conc ZA296 (mg/kg) in Control} &= 0.01015 \times \frac{339}{215} \times \frac{1}{0.02} \times \frac{1}{1000} \\ &= 0.0008 \text{ mg/kg} \end{aligned}$$

Percent Recovery Calculation

$$\text{Percent Recovery from Fortified Sample} = \frac{(\text{Conc ZA1296 in Fortified Sample}) - (\text{Conc. ZA1296 in Control})}{\text{Fortification Rate}} \times 100$$

$$\text{Percent Recovery from Fortified Sample} = \frac{0.00939 - 0.0008}{0.01} \times 100 = 85.9$$

Appendix C

Modifications from Original ZA1296 Crop Method: TMR0643B

The original method for determination of ZA1296 and MNBA in crops was issued as TMR0643B. The following is a brief description of the differences between TMR0643B and the method contained in this report.

Primary Modifications

Crude Extract Cleanup

The ethyl acetate partition and silica gel SPE cleanup have been eliminated. These steps were replaced by an evaporation and acidic reconstitution step, effectively eliminating acetonitrile from the crude extract prior to the HPLC cleanup. The benefits are less sample work up, resulting in time saving, eliminating sources of error, and sources of analyte loss.

HPLC Cleanup

For work conducted prior to issuing TMR0643B, it was shown that the ethyl acetate partitioning and silica gel SPE cleanup removed up to 75% of co-extractives (determined by HPLC-UV). Removing these steps result in an extract that could overload of the HPLC column during this cleanup step. As a result, the injection volume was reduced to 250 μ L and the column diameter was increased from 3.0 mm to 4.6 mm.

Oxidation Reaction (ZA1296 \rightarrow MNBA)

The oxidation conditions were changed in an effort to increase the efficiency of this reaction. The original conditions were 6% hydrogen peroxide for 35 min at 70° C. The new conditions were 30% peroxide for 20 min at 85° C. These changes produced a 4-5% increase in the conversion rate. The estimated conversion rate for this reaction the ZA1296 to AMBA reaction is now 85-90%.

As a result of this change, minor modifications were required to compensate for the increased peroxide. The post-oxidation sample was diluted with water and frozen prior to adding the catalase. This slowed the reaction eliminating the peroxide, which due to the increase in peroxide level, would have become violent. As a result of the dilution with water prior to adding the catalase, the concentration of the stannous chloride/HCl reagent was increased to 90 mg per mL in 3 N HCl (from 60 mg per mL in 2 N HCl).

Final Determination of Converted AMBA

The final HPLC determination of the AMBA conversion product was conducted with a different HPLC column. The original method used the same column used for the HPLC cleanup (Inertsil or Prodigy). This method uses a Waters Spherisorb (or Metasil) column. The different column chemistry provides a cleaner profile. A isocratic elution system (with a rapid gradient to wash column of coextractives) was developed that reduced the run time for this analysis from 17 min to 13 min.

Quantitation using AMBA Calibration Standards

The use of procedural standards (ZA1296 and MNBA standards converted to AMBA) was eliminated in this procedure. A portion of losses now incurred are due to the fact that the conversion rates are less than 100%. Previous recovery data reported for TMR0643 showed recovery of ZA1296 to be 81 % with a CV of 17. Recovery of MNBA was reported at 88% with a CV of 12. Data later collected from routine use of the method in a number of studies found 85% recovery (CV = 18) for ZA1296 and 95% recovery (CV = 14) for MNBA.

Data reported here (Table 5) shows slightly lower recovery yet better reproducibility, as evidenced in a lowering of the CV values by more than 50%. Recovery of ZA1296 was 79% (CV = 7), while recovery of MNBA was 82% (CV=5).

Secondary Modifications

Final SPE Cleanup

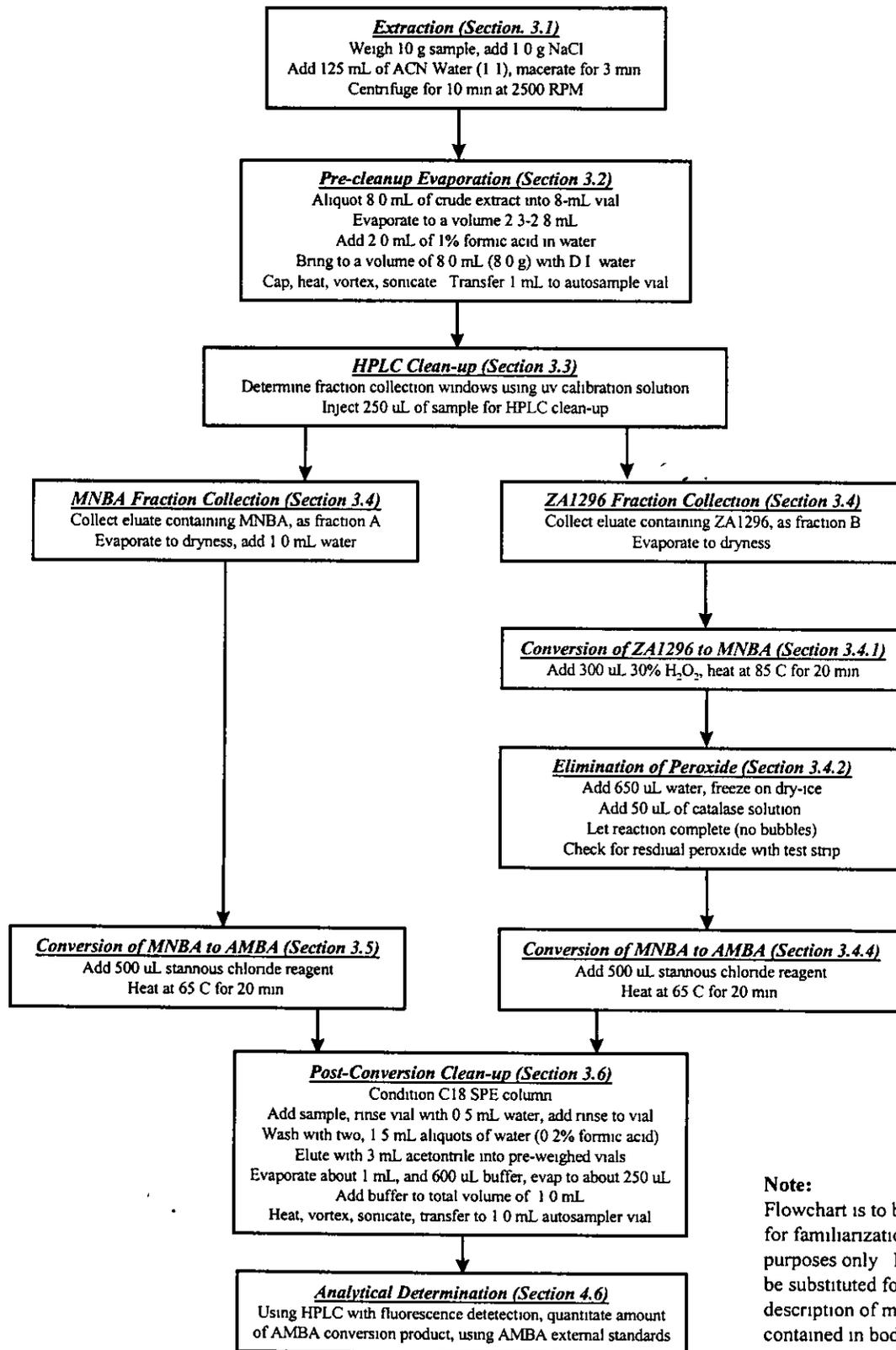
Minor modification were made in the final C18 SPE cleanup. These included the use of vacuum to facilitate elution at a flow rate of 0.5 - 1.0 mL/min. This was implemented as time saving measure.

Reduction of Physical Losses

The small volumes used in this method for the conversion reactions cause the method to be susceptible to physical loss of sample. Repeated capping and uncapping of small vials can cause sample to be lost through the transfer of liquid from the cap liner to the threads of the cap or vial. Another source of loss is sample being transferred from the neck of the vial to the outside of the Eppendorf pipet tips. Capped vials, after the completion of the catalase reaction, can be under positive pressure due to the formation of oxygen. Uncapping these vials when small amounts of liquid reside near the top of the vial and cap can cause expulsion of small amounts of liquid. As a result, the method recommends the centrifuging of these vials at various points in the method, prior to removal of the caps.

While the actual volume of loss in each of these examples is very low (perhaps 5 uL for each occurrence), these small volumes may result in appreciable recovery loss. With the actual recoveries averaging 79% for ZA1296, the elimination of losses in the range of 1-3 percent could be significant.

Appendix D: Flowchart of Analytical Procedure



Note:
Flowchart is to be used
for familiarization
purposes only. Not to
be substituted for
description of method
contained in body of
report.

EPA Addendum

PP#8F04954, MRID# 449017-19

Mesotrione & MNBA in/on Field Corn Grain, Forage and Fodder

- 1) Due to the variation of moisture content of the various sample matrices and the time required to determine the moisture content of each matrix, the ACB suggests the extraction procedure of the method be changed as follows:

A 10 gram sample is extracted with 50 ml of acetonitrile:water (1:1, v:v) in a tissuemizer/ blender for 3 minutes. The extract is filtered through a 9.0 cm GF/C filter in a buchner funnel attached by a vacuum adaptor directly into a 100 ml glass-stoppered graduated cylinder. The filter cake is rinsed several times until the total volume in the cylinder is ca 90 ml then the volume is made to 100 ml total volume with extraction solution.

- 2.) In step 2.2.16 for preparing the HPLC Mobile Phase A, (9 mM ammonium acetate and 0.25% formic Acid) and step 2.2.17 for preparing the HPLC Mobile Phase B, (95:5 acetonitrile : water), there is no caution or warning against filtering /degassing the mobile phases. The ACB has found that degassing mobile phase results in a loss of formic acid which leads to poor chromatography; i.e., poor peak shape and resolution. The ACB concludes that method users should not degas the mobile phases after the formic acid is added.
- 3.) The ACB found it necessary to modify the mobile phase for the fraction collection step to obtain satisfactory separation (i.e., avoiding excessive peak separation) of the two analyte peaks of interest. The ACB used 20% acetonitrile in a solution of 9 mM ammonium acetate and 0.25% formic acid(buffer solution) in water as bottle A; and 45% acetonitrile in buffer solution without acid as bottle B. The acid was found to be necessary to obtain adequate peak separation. The ACB used a 5 ul injection of 10 ug/ml of both mesotrione and MNBA to select the proper fraction collection window.
- 4.) The ACB found it necessary to modify the gradient for the analytical instrument due to an interference found in standard, control, and sample chromatograms. The gradients used by ACB and the petitioner are as follows:

Petitioner		ACB	
<u>Time</u>	<u>%B</u>	<u>Time</u>	<u>%B</u>
0.0	16	0.0	10
5.0	16	1.0	20
7.0	80	3.0	30
9.0	80	6.0	40
10.0	16	9.0	50
12.0	16	10.0	10

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- 5.) The ACB used a 0.2 micron syringe filter to filter the sample extract solution prior to fraction collection instead of centrifuging as suggested by the petitioner. The ACB has found no significant loss when injecting a known standard concentration before and after filtering the solution.

The ACB agrees that method users should incorporate the following recommendations made by the ILV laboratory (En-Cas Analytical Laboratories):

- 1.) Background peaks near the retention time of analytes were traced to contamination from sample contact sources, such as pipette tips and vials. Pre-rinsing all equipment which contacted the samples with acetone or methanol eliminated the interferences.
- 2.) It is recommended that the calculation and preparation of the SnCl_2 solution (Section 2.2.14) be clarified so the analyst knows whether to include the water in the formula. (Per Sponsor do not include water.)
- 3.) It is recommended that guidance be provided regarding the stability of standards and various sample aliquots (Section 2.3).
- 4.) It is recommended that guidance be provided on the accuracy needed for taring and weighing sample containers (Sections 3.2 and 3.6).
- 5.) To achieve adequate separation of background from the AMBA (in the HPLC/FL analyses), the ratio of solvent A (9mM NH_4OAc + 0.025% formic acid in water) to solvent B (95:5 acetonitrile:water) was changed from 84:16 to 88:12 (A:B). This yielded a retention time of ca 7.2 minutes. The flush at the end of each injection was changed from 20:80 to 10:90 (A:B).