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STUDY TITLE

**Standard Operating Procedure [RAM 280/02]
Acetochlor: Method for the Determination of Residues Containing the
Common Moieties 2-Ethyl-6-Methylaniline (EMA) and 2-(1-Hydroxyethyl)-6-
Methylaniline (HEMA) in Crops**

DATA REQUIREMENT

N/A

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PROJECT/STUDY NUMBER

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10(d)(1)(A), (B), or (C).

COMPANY: Acetochlor Registration Partnership

COMPANY AGENT:

 12 Jan 01

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Registration Manager

Date:


GLP COMPLIANCE STATEMENT

Acetochlor Registration Partnership

This Standard Operating Procedure/ Analytical Methods is not subject to the requirements of Title 40 Code of Federal Regulations. Part 160, GOOD LABORATORY PRACTICE STANDARDS (FIFRA/FFDCA).

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Recipient: Regulatory FeedbackCopy No: 255**STANDARD OPERATING PROCEDURE****RAM 280/02**

ACETOCHLOR :
METHOD FOR THE DETERMINATION OF RESIDUES CONTAINING THE COMMON MOIETIES
2-ETHYL-6-METHYLANILINE (EMA) AND 2-(1-HYDROXYETHYL)-6-METHYLANILINE (HEMA)
IN CROPS

An External Standard Procedure using Gas Liquid Chromatography

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This SOP supersedes SOP RAM 280/01

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SCOPE

The analytical procedure described is suitable for the determination of the acetochlor common moieties 2-ethyl-6-methylaniline (EMA) (Figure 1) and 2-(1-hydroxyethyl)-6-methylaniline (HEMA) (Figure 2) in crops using external standardisation procedure. The limit of quantification has been set for both EMA and HEMA at 0.01 mg kg^{-1} , equivalent to 0.02 mg kg^{-1} acetochlor for each compound.

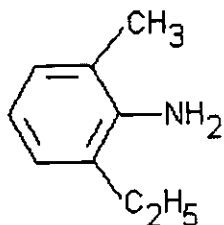


Figure 1 : 2-ethyl-6-methylaniline (EMA)

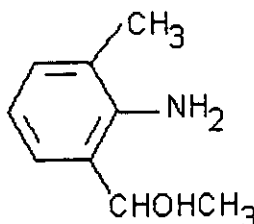


Figure 2 : 2-(1-hydroxyethyl)-6-methylaniline (HEMA)

SUMMARY

In summary, prepared crop samples are extracted by maceration with acetonitrile:water (80:20 v/v). Samples are then filtered under vacuum or centrifuged depending on crop matrix and aliquots equivalent to 0.5 g crop are taken and evaporated to dryness under a stream of dry air. Saturated potassium hydroxide solution and methanol are added to the samples and then the samples are placed in a heating block at $200\text{-}220^\circ\text{C}$ and refluxed for 30 minutes or if sample frothing occurs 60 minutes. The hydrolysate is diluted with water and saturated sodium chloride and partitioned with toluene. An aliquot of the toluene extract is derivatised with heptafluorobutyric acid anhydride (HFAA) to acylate the EMA and HEMA. Excess derivatising agent is removed by partition of the derivatised samples with sodium hydrogen carbonate solution and then the samples are analysed by gas liquid chromatography with mass selective detection. The results are quantified against the acylated EMA and HEMA standards prepared in the relevant crop matrix.

3

PROCEDURE

3.1

Sample Preparation

Samples should be prepared using an approved method of preparing samples for residue analysis, such as ZENECA Agrochemicals standard operating procedure 41/065/--.

3.2

Extraction and Filtration

3.2.1

Wet Crops eg Corn Forage

- a) Weigh representative amounts of prepared crop (10 g) into extraction vessels (250 ml size). At least one untreated control sample and two control samples fortified with known amounts (see example calculations in Appendix 7) of the acetochlor metabolites compound 24 (sulphonic acid) and compound 37 (Figures 3 and 4) should be analysed alongside the treated samples to enable verification of the method and recovery corrections to be made.

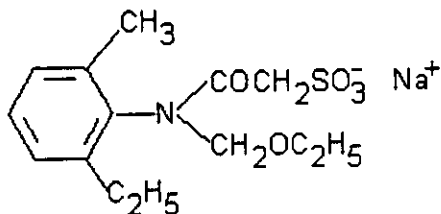


Figure 3 : Compound 24 (sulphonic acid)
sodium 2-sulfonato-*N*-ethoxymethyl-6'-ethylacet-*o*-toluidide

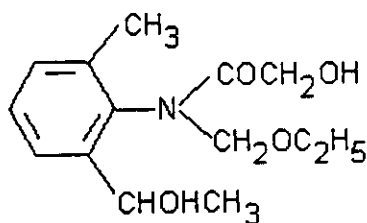


Figure 4 : Compound 37
2-hydroxy-*N*-ethoxymethyl-6'-(1-hydroxyethyl)acet-*o*-toluidide

- b) Add 80:20 (v/v) acetonitrile:water (50 ml) to the samples and macerate at high speed until the samples are well homogenised.
- c) Filter the samples under vacuum through two Whatman number 1 filter papers into round bottomed flasks (250 ml), rinse the extraction vessels and macerator head used for extraction with 80:20 acetonitrile:water (40 ml) and use to wash the filter cakes. Combine the filtrate washings with the main extracts and adjust the volume of the extracts to 100 ml with further extraction solvent. 5 ml aliquots of the extracts are now equivalent to 0.5 g crop.

3.2.2 Dry Crops e.g. Corn Grain

- a) Weigh representative amounts of prepared crop (10 g) into centrifuge bottles (250 ml size). At least one untreated control sample and two control samples fortified with known amounts (see example calculations in Appendix 7) of the acetochlor metabolites compound 24 (sulphonic acid) and compound 37 (figures 3 and 4) should be analysed alongside the treated samples to enable verification of the method and recovery corrections to be made.
- b) Add 80:20 (v/v) acetonitrile:water (50 ml \pm 1 ml) to the samples and macerate at high speed until the samples are well homogenised.
- c) Centrifuge the samples at 3000 rpm for five minutes and decant the supernatant solutions into round bottom flasks (250 ml). 2.5 ml aliquots of the extracts are now equivalent to 0.5 g crop.

3.2.3 Dry Bulky Samples eg Fodder and Straw

- a) Weigh representative amounts of prepared crop (10 g) into extraction vessels (250 ml size). At least one untreated control sample and two control samples fortified with known amounts (see example calculations in Appendix 7) of the acetochlor metabolites compound 24 (sulphonic acid) and compound 37 (figures 3 and 4) should be analysed alongside the treated samples to enable verification of the method and recovery corrections to be made.
- b) Add 80:20 (v/v) acetonitrile:water (100 ml \pm 1 ml) to the samples and macerate at high speed until the samples are well homogenised.
- c) Filter the samples under vacuum through two Whatman number 1 filter papers into round bottomed flasks (250 ml). Note : the filter papers should not be pre-wet or the filtrates washed with further solvent as aliquots will be taken directly from the filtered extracts. As soon as the samples have filtered the vacuum should be turned off otherwise sample concentration may occur through evaporation of the acetonitrile. 5 ml aliquots of the extracts are now equivalent to 0.5 g crop.

Base Hydrolysis of Sample Extracts

- a) Transfer aliquots of the crop extracts equivalent to 0.5 g into 30 ml boiling tubes and evaporate to dryness in a heating block at 40°C under a stream of dry air. Ensure that no aqueous solution remains. The samples should be removed from the air stream as soon as they are dry otherwise loss of volatile residues may occur. The evaporation step should take between 30-60 minutes.
- b) Add saturated potassium hydroxide solution (3 ml), methanol (0.5 ml) and a few anti-bumping granules to the boiling tubes and ultrasonicate thoroughly to ensure the complete uptake of material adhering to the sides of the tubes.
- c) Attach condensers to each boiling tube, place the tubes in a heating block at a temperature of 200-220°C and heat under reflux for 30 minutes. Ensure that the condensers have been washed with deionised water and acetone prior to use.

NOTE : Certain matrices eg corn grain may froth up the tubes during the hydrolysis causing the hydrolysis reaction to proceed at a slower rate. In cases where frothing occurs the samples should be refluxed for 1 hour to ensure the reaction goes to completion.

- d) Remove the tubes from the heating block, whilst still connected to the condensers. Allow to cool and wash down the condensers with water (5 ml), followed by saturated sodium chloride solution (10 ml). Detach the tubes from the condensers, stopper them immediately and transfer to a cold water bath to cool down further and prevent losses of EMA and HEMA through volatility.

Partition

- a) Add toluene (2 ml) to the tubes, shake for 30 seconds and leave to separate. If the two layers do not separate, centrifuge the samples at 2500 RPM for 5 minutes.
- b) Once the layers have separated, transfer the upper organic layer using Pasteur pipettes into 10 ml graduated centrifuge tubes.
- c) Repeat the partition with further toluene (2 ml) and combine the two organic fractions in the centrifuge tubes. Adjust the combined fractions to a total volume of 4 ml to give a sample concentration of 0.125 g ml⁻¹.

Preparation of Derivatives for GC-MSD

- a) Transfer 1 ml aliquots of the toluene from 3.4 (c) to a 7 ml Pierce reacti-vial or similar vessel. Note : Care must be taken to ensure that no water droplets from the partition are present at this stage otherwise the derivatisation will be inhibited. Add 50 μ l of heptafluorobutyric acid anhydride (HFAA) to the vials, cap tightly, and place in a heating block at 50°C for 15 minutes, shaking the vials occasionally.
- b) Remove the samples and allow to cool. Add 4 ml of 0.5 M sodium bicarbonate solution to the vials, shake for 30 seconds and allow the two layers to separate. Transfer the upper organic layer to GC vials and centrifuge the vials at 3000 rpm for 5 minutes to remove any precipitate formed during derivatisation.

Note : For accurate quantification of the results by GC-MSD it is necessary to use matrix matched standards. The matrix standard should be prepared by transferring a 0.95 ml control aliquot from 3.4 (c) into a reacti-vial and adding 50 μ l of a 0.1 μ g ml⁻¹ mixed EMA and HEMA standard in toluene to give a 0.005 μ g ml⁻¹ matrix standard. This should be derivatised alongside the samples for each analytical run using the above procedure (3.5 (a)).

Other concentrations of standards can be prepared if required by varying the concentration and volume of the EMA and HEMA mixed standard added to the control aliquot. However the total volume of the solution should always add up to 1 ml and the volume of standard added should not exceed 100 μ l. Standards used for the GC-MSD analysis should always be in the concentration range of expected residues or recoveries.

3.6

Analysis by GC-MSD

The following instruments and conditions have been found to be suitable for this analysis in this laboratory. Other instruments can equally be used, however optimisation may be required to achieve the desired separation and sensitivity. The operating manuals for the instrument should always be consulted to ensure safe and optimum use. The following instrumentation has been found to be suitable using a Hewlett Packard HP5890A gas liquid chromatograph fitted with either a HP5970 or HP5971 mass selective detector.

3.6.1

Chromatographic conditions

Column	: Rtx200 with a retention gap
Dimensions	: 30 m × 0.25 mm id film thickness 0.25 µm
Retention gap	: 5 m × 0.25 mm id diphenyl methyl silicone deactivated fused silica
Injection port	: OPTIC programmable temperature inlet used in splitless mode with an open liner packed with a small amount of silanised glass wool
Carrier gas and head pressure	: Helium at 7.5 psi
Splitless time	: 0.8 minutes
Injection volume	: 2 µl
Injector temperature	: 275°C
Transfer line temperature	: 275°C
Temperature program	: 80°C hold for 1 minute then program at 10°C per minute to 190°C and immediately program at 40°C per minute to 300°C and hold for 5 minutes

3.6.2

MSD Conditions

Electron energy : 70eV

System calibration : Manual tune using ions 131, 264 and 314

Manual tune optimises on the ions m/e 131, 264, and 314. These ions are formed from the fragmentation of the calibration mixture. The calibration mixture is perfluorotributylamine (PFTBA).

On the HP5971 it is also possible to improve sensitivity by adjusting X-ray, repeller and electron multiplier voltages. The other tuning parameters can be adjusted but their effects are less noticeable. This adjustment should only be carried out if sensitivity is a problem.

Acquisition mode : Selected ion monitoring, low resolution for ions m/e 331 and 162 EMA heptafluorobutyl derivative and ions m/e 329 and 314 HEMA heptafluorobutyl derivative.

The ions of m/e 162 are used for the calculation of EMA residues and ions of m/e 314 are used for the calculation of HEMA residues.

Under these conditions the retention times for the heptafluorobutyl derivatives of HEMA and EMA using the Rtx200 column are approximately 8.3 and 10.8 minutes respectively.

3.7

Calculation of Results

EMA and HEMA residues may be calculated in mg kg⁻¹ for each sample extract using a mean standard response from each of the injections bracketing the sample as follows:

$$\text{Residue} = \frac{\text{PK Area (Sample)}}{\text{PK Area (Standard)}} \times \frac{\text{Standard Conc.}}{\text{Sample Conc.}}$$

PK Area (Sample) = Peak area of sample

PK Area (Standard) = Average peak area for bracketing standards

Standard Conc. = The concentration of standard reference material (µg ml⁻¹)

Sample Conc. = The concentration of the final sample solution (g ml⁻¹)

These sample residues should be further corrected using the average percentage recovery (ie calculate each recovery as above and express as a percentage of the fortification level. Use the average of all the recoveries for use in the calculation below).

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average Percentage Recovery}} \quad (\mu\text{g g}^{-1})$$

Results should be corrected to two significant figures.

However, It is important to note that when calculating recovery values a correction factor must be applied to the calculation of the EMA and HEMA recoveries.

The method converts recoveries fortified with compound 24 (sulphonic acid) and compound 37 to EMA and HEMA respectively and the recoveries are determined against an EMA and HEMA standard.

To calculate the EMA recovery values the fortification level of the compound 24 must be divided by 2.50 to give the fortification level expressed as EMA. This is because the molecular weight of compound 24 is 337 g and the molecular weight of EMA is 135 g and so a recovery fortified with 1 μg of compound 24 will contain 0.4 μg of EMA after hydrolysis i.e. 2.5 times less.

To calculate the HEMA recovery values the fortification level of the compound 37 must be divided by 1.77 to give the fortification level expressed as HEMA. This is because the molecular weight of compound 37 is 267 g and the molecular weight of HEMA is 151 g and so a recovery fortified with 1 μg of compound 37 will contain 0.56 μg of HEMA after hydrolysis i.e. 1.77 times less.

To express any residues of EMA and HEMA found in samples as acetochlor equivalents EMA residues must be multiplied by 2 and HEMA residues must be multiplied by 1.78 as the molecular weight of acetochlor is 270 g.

4 **LIMIT OF QUANTIFICATION**

The limit of quantification has been set at 0.01 mg kg⁻¹ for both HEMA and EMA in these laboratories which is 0.02 mg kg⁻¹ expressed as acetochlor equivalents. The chromatographic response for recoveries at this level should exceed the background signal noise factor by a factor of at least four to be considered an acceptable quantitative limit of quantification.

5 **CONTROL AND RECOVERY EXPERIMENTS**

Control and external recovery experiments should be completed as Section 3 for each set of samples analysed. Provided the recovery values are acceptable they may be used to correct any residues found. A minimum of one control and two external recovery experiments should be run alongside each set of samples analysed (that is untreated samples accurately fortified with known amounts of compound 24 and compound 37 prior to extraction).

Fortification levels should be based on the expected crop residue level. When no residues are expected the recoveries should be fortified at low levels, typically 0.1 mg kg⁻¹ with at least one recovery fortified at the limit of quantification.

Recovery data is generally considered acceptable when the mean values are between 70% and 110% with a coefficient of variation of $\leq 20\%$.

6 **METHOD VALIDATION**

A method validation (95JH225) study has been carried out on the procedure described in Section 3. This is reported in RJ2075B, See Appendix 6 for a summary of the method validation data.

7 **REFERENCE**

1. SOP No: 41/065/-- Approved Methods of Preparing Crop Samples for Residue Analysis.

Filename : RAM28002.doc
Location : E:\GROUP\WP\SOPRAM
Reference : NJR/JD
Date : 14 January 1998

Appendix 1

Apparatus

Apparatus Used During Analysis of Crop Samples

- a) Quickfit round-bottomed flasks with ground glass joints (250 ml size).
- b) An ultrasonic bath, available from Sonikor Instrument Company, Copiagne, NY, USA.
- c) Hewlett Packard HP5890a gas liquid chromatograph fitted with either a HP5970 or HP5971 mass selective detector and HP7673 automatic liquid sampler.
- d) Fused silica capillary GC column, Rtx200 25 m x 0.25 mm id, 0.25 μ m film thickness available from Restek Corporation, 110 Benner Circle, Bellefonte, PA, 1682308812 USA. Part number 15023.
- e) Pyrex splitless injection liner for OPTIC injector available from Ai Cambridge, London Road, Pampisford, Cambridge, CB2 4EF, UK. Tel (01223) 834420. Part number 126100.
- f) Deactivated glass wool available from Restek Corporation. Part number 20789.
- g) Universal Press-Tight™ connectors available from Restek Corporation. Part number 20400.
- h) Fused silica phenyl-methyl silicone deactivated guard column 5 m x 0.25 mm id available from Restek Corporation. Part number 10043.
- i) Quickfit test tubes with light wall and 19/26 socket.
- j) Glass or polypropylene centrifuge bottles 250 ml size.
- k) Stoppered graduated glass centrifuge tubes, 10 ml capacity with 0.1 ml graduations.
- l) 7.4 ml glass screw capped vials with solid phenolic closures and Teflon liners. Available from Supelco UK LTD., Fancy Road, Poole, Dorset BH17 7NH, UK. Part number 2-7150.
- m) Heating block with compressed air sample concentrator e.g. Techne™ Dri-block DB3D with FSC400D sample concentrator available from Fisons Scientific Equipment, Bishops Meadow Road, Loughborough, Leicestershire LE11 0RG, UK.
- n) Extraction vessel for wet crops e.g. storage jar (250 ml) size.
- o) OPTIC temperature programmable inlet for gas chromatography available from Ai Cambridge, London Road, Pampisford, Cambridge, CB2 4EF, UK. Tel (01223) 834420.

- p) Apparatus suitable for the base hydrolysis of the sample extracts, consisting of an aluminium block 800 mm × 100 mm × 50 mm with ten 26 mm diameter holes drilled to a depth of 30 mm into the block. The holes are filled with approximately 5 ml of Dow Corning 710 silicone fluid to aid heat transfer and the block is placed upon three Bibby B290 ceramic hot plates (290 mm × 290 mm size) placed next to each other. Glass fibre insulating material covered with tin foil is placed around the sides of the block on top of the ceramic hot plates to insulate the aluminium block.

The aluminium block and glass fibre insulation are not commercially available and need to be specially made.

The hot plates are available from Orme Scientific Equipment, Stakehill Industrial Park, Middleton, Manchester M24 2RH, UK. Part number H15-106.

The silicone fluid is available from BDH Laboratory Supplies, Poole BH15 1TD, UK. Tel (01202) 669700.

- q) Quickfit Leibig condensers C1/22/SC 400 mm length, 19/26 cone size fitted with PTFE sleeves (part number S28-275) to prevent seizing. Available from Orme Scientific Equipment, Stakehill Industrial Park, Middleton, Manchester M24 2RH, UK.
- r) Macerator for extraction of samples e.g. Janke and Kunkel Ultra Turrax T25, available from Orme Scientific Equipment, Stakehill Industrial Park, Middleton, Manchester M24 2RH, UK.
- s) Laboratory centrifuge e.g. Wifug 31500 available from Eltex of Sweden Ltd. Lane Close Mills, Bartle Lane, Great Horton, Bradford BD74QQ, UK.

Appendix 2

Reagents

All solvents/reagents must be of high purity i.e. HPLC grade solvents. If a source of solvent/reagent has not been previously evaluated, then individual solvents/reagents should be examined for possible interfering impurities prior to analysis.

- a) Acetonitrile, HPLC grade.
- b) Methanol, HPLC grade.
- c) Toluene, glass distilled grade.
- d) Ultra pure water.
- e) Analytical grade potassium hydroxide pellets.
- f) Analytical grade sodium chloride.
- g) Analytical grade sodium hydrogen carbonate.
- h) Heptafluorobutyric acid anhydride (HFAA), derivatisation grade.
- i) Analytical standard of sodium 2-sulfonato-*N*-ethoxymethyl-6'-ethylacet-*o*-toluidide of known purity.
- j) Analytical standard of 2-hydroxy-*N*-ethoxymethyl-6'-(1-hydroxyethyl)acet-*o*-toluidide of known purity.
- k) Analytical standard of 2-ethyl-6-methylaniline of known purity.
- l) Analytical standard of 2-(1-hydroxyethyl)-6-methylaniline of known purity.
- m) Anti-bumping granules.

Appendix 3

Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in doubt, consult the appropriate safety manual (e.g. ZENECA Laboratory Safety Manual) containing recommendations and procedures for handling chemicals, and a monograph such as 'Hazards in the Chemical Laboratory' edited by S G Luxon, The Chemical Society, London.

a) **METHANOL**

Highly flammable
Harmful by inhalation and in contact with skin
Do not breathe vapour
Avoid contact with eyes and skin
(OES long term exposure limit 200 ppm)

b) **ACETONITRILE**

Highly flammable
Serious risk of poisoning by inhalation or swallowing
Gives off poisonous vapour
Do not breathe vapour
(OES long term exposure limit 70 ppm)

c) **TOLUENE**

Highly flammable
Harmful vapour
Avoid breathing vapour
Avoid contact with eyes and skin
Wear rubber gloves and eye protection
(OES long term exposure limit 50 ppm)

d) **POTASSIUM HYDROXIDE**

Causes severe burns
Irritating to eyes and skin
Prevent contact with eyes and skin
(OES short term 2 mg m^{-3})

e) **HEPTAFLUOROBUTYRIC ACID ANHYDRIDE**

Very toxic by inhalation
Causes severe burns
Danger of very serious irreversible effects
Do not breathe vapour
Avoid contact with eyes and skin

- f) **2-ETHYL-6-METHYLANILINE**
Toxic by inhalation or contact with skin
Highly flammable
Avoid breathing vapour
Avoid contact with skin and eyes
- g) **2-HYDROXY-N-ETHOXYMETHYL-6'-(1-HYDROXYETHYL)ACET-O-TOLUIDIDE**
Avoid contact with eyes and skin
Avoid breathing vapour
- h) **2-(1-HYDROXYETHYL)-6-METHYLANILINE**
Toxic by inhalation or contact with skin
Avoid breathing vapour
Avoid contact with skin and eyes
- i) **SODIUM 2-SULFONATO-N-ETHOXYMETYL-6'-ETHYLACET-O-TOLUIDIDE**
Avoid contact with eyes and skin

Appendix 4
Preparation of Analytical Standards

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

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

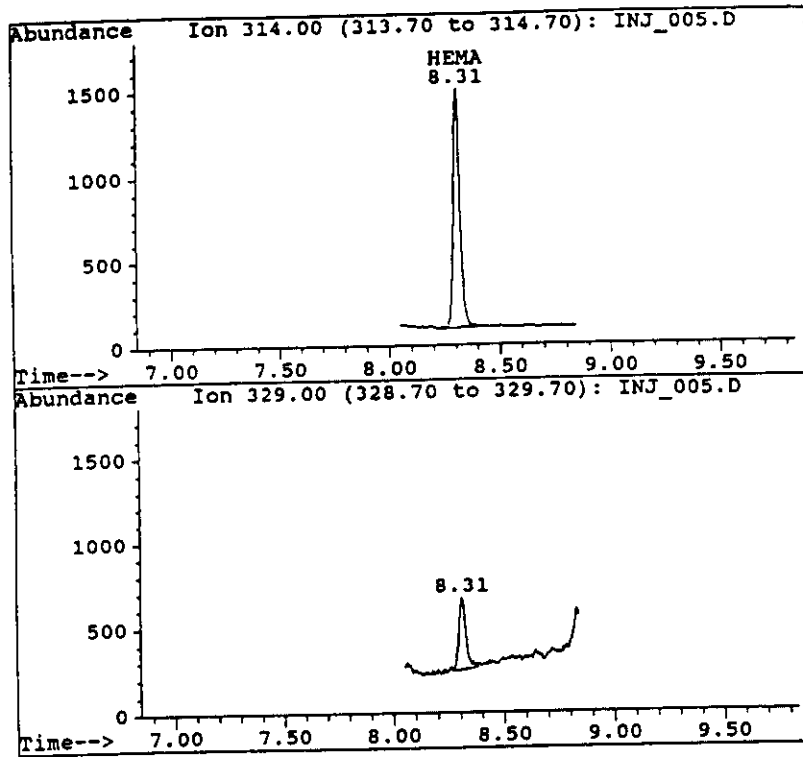
Weigh out accurately, using a five figure balance, sufficient EMA and HEMA analytical standards to allow dilution in toluene to give $1000 \mu\text{g ml}^{-1}$ stock solutions in volumetric flasks. Then prepare a $100 \mu\text{g ml}^{-1}$ mixed standard by pipetting 10 ml of each stock solution into a volumetric flask (100 ml) and diluting to 100 ml with toluene. Make serial dilutions of this mixed standard solution to give $10 \mu\text{g ml}^{-1}$, $1.0 \mu\text{g ml}^{-1}$ and $0.1 \mu\text{g ml}^{-1}$ standard solutions in toluene. These solutions should be used for derivatisation and GC-MSD analysis.

Weigh out accurately, using a five figure balance, sufficient sodium 2-sulfonato-*N*-ethoxymethyl-6'-ethylacet-*o*-toluidide (compound 24) and 2-hydroxy-*N*-ethoxymethyl-6'-(1-hydroxyethyl)acet-*o*-toluidide (compound 37) analytical standards to allow dilution in 90:10 acetonitrile:water to give $1000 \mu\text{g ml}^{-1}$ stock solutions in volumetric flasks. Make serial dilutions of these standards to give $100 \mu\text{g ml}^{-1}$, $10 \mu\text{g ml}^{-1}$ and $1.0 \mu\text{g ml}^{-1}$ standard solutions in 90:10 acetonitrile:water. These solutions should be used for fortification of samples prior to extraction.

When not in use, always store the standard solutions in a refrigerator at $<8^{\circ}\text{C}$ to prevent decomposition and/or concentration of the solution. Analytical standards should be replaced with freshly prepared standards after four months of use.

Appendix 5
Typical Chromatograms

Figure 1: 0.005 $\mu\text{g ml}^{-1}$ HEMA Standard (HFAA derivative) in the Presence of Soyabean Seed Control Matrix

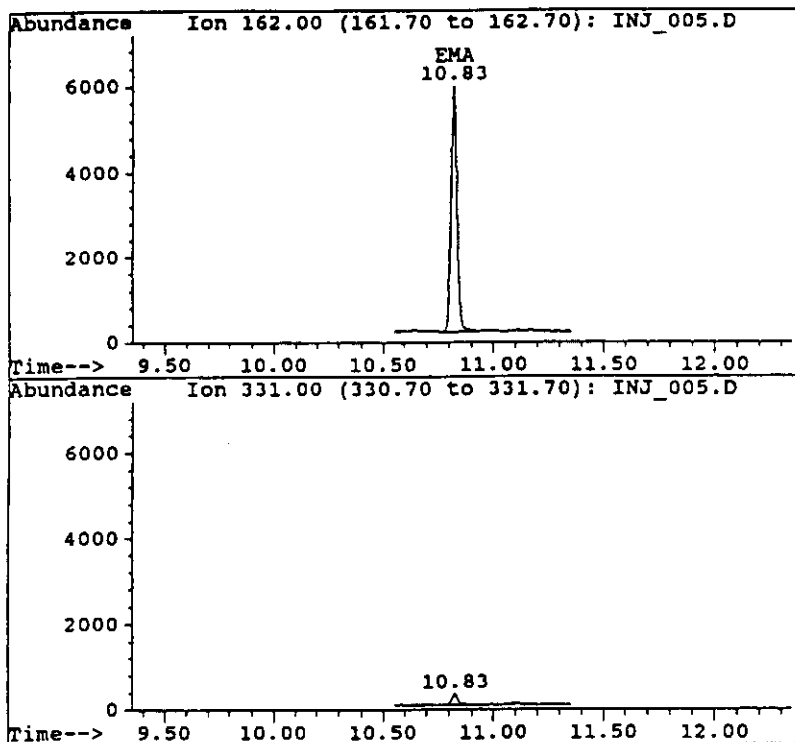


File C:\HPCHEM\1\DATA\NCA8416A\INJ_005.D
 Study No: 95JH225
 Sequence Number : G807.S
 Operator: EA
 Date Acquired: 8 Dec 95 7:55 pm
 Method File: HEMA Compound Name: HEMA
 Sample Name: 0.005 UG/ML DER.DIL STD J0509/27B
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	314	8.31	29047
Qual 1	329	8.31	8819

Figure 2 : 0.005 $\mu\text{g ml}^{-1}$ EMA Standard (HFAA derivative) in the Presence of Soyabean Seed Control Matrix

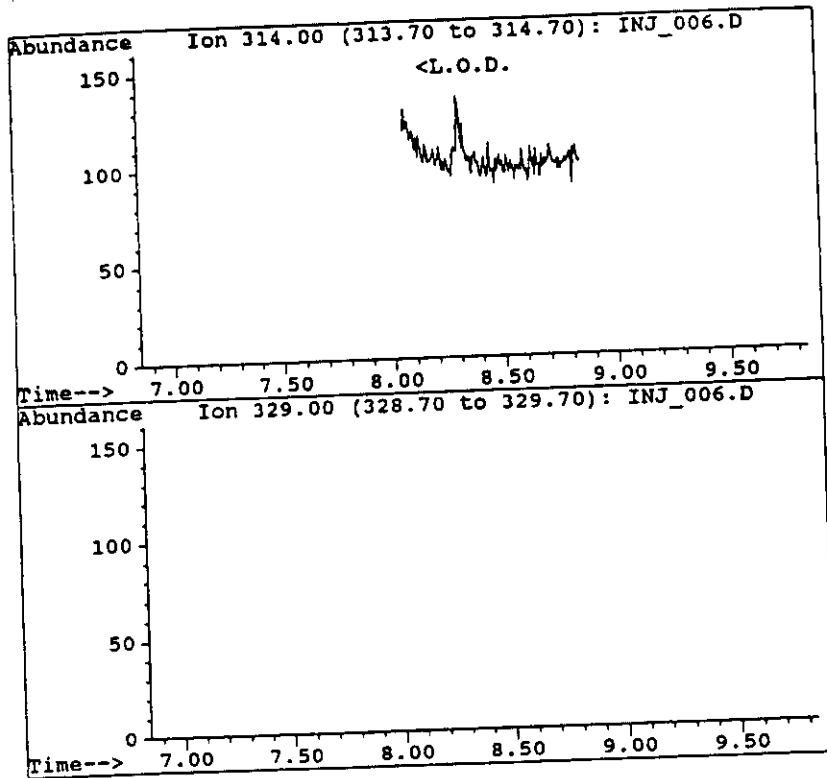


File C:\HPCHEM\1\DATA\NCA8416A\INJ_005.D
 Study No: 95JH225
 Sequence Number : G807.S
 Operator: EA
 Date Acquired: 8 Dec 95 7:55 pm
 Method File: HEMA Compound Name: EMA
 Sample Name: 0.005 UG/ML DER.DIL STD J0509/27B
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	162	10.83	111906
Qual 1	331	10.83	5292

Figure 3 : Control Soyabean Seed Sample, 0.125 g ml⁻¹

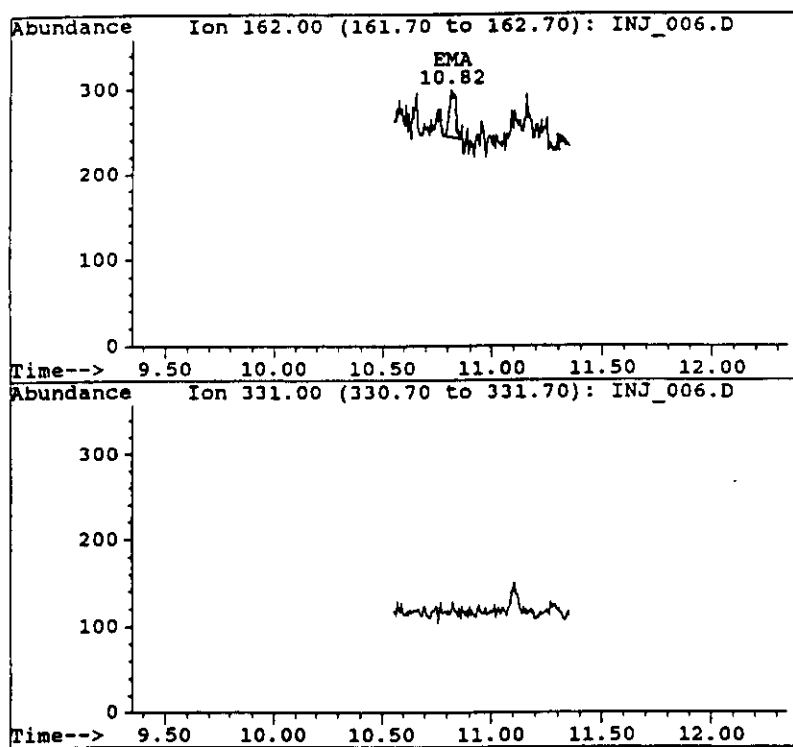


File C:\HPCHEM\1\DATA\NCA8416A\INJ_006.D
 Study No: 95JH225
 Sequence Number : G807.S
 Operator: EA
 Date Acquired: 8 Dec 95 8:20 pm
 Method File: HEMA Compound Name: HEMA
 Sample Name: 1317/1 95 CON
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
No HEMA detected at		8.34 mins	

Figure 4: Control Soyabean Seed Sample, 0.125 g ml⁻¹

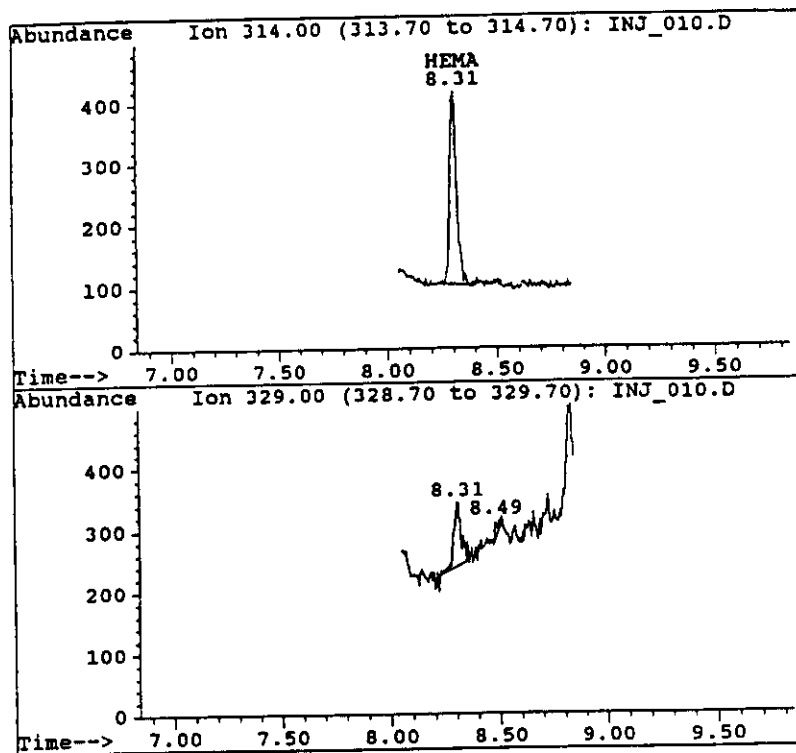


File C:\HPCHEM\1\DATA\NCA8416A\INJ_006.D
 Study No: 95JH225
 Sequence Number : G807.S
 Operator: EA
 Date Acquired: 8 Dec 95 8:20 pm
 Method File: HEMA Compound Name: EMA
 Sample Name: 1317/1 95 CON
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	162	10.82	1076
Qual 1	331	0.00	0

Figure 5 : Recovery Soyabean Seed Sample Fortified at 0.01 mg kg⁻¹
 HEMA Equivalent, 0.125 g ml⁻¹. HEMA Recovery 93%

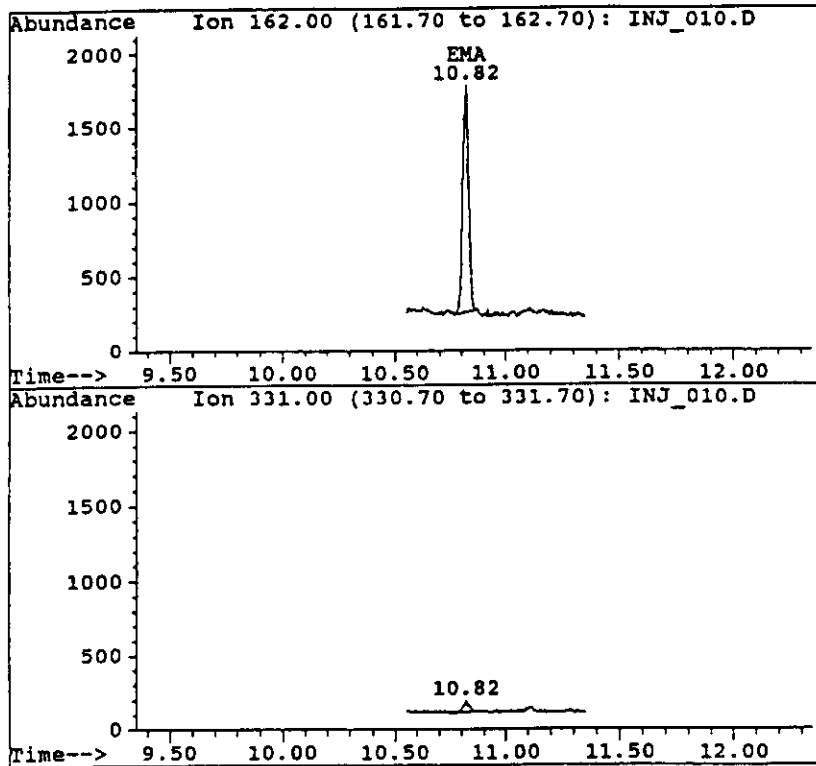


File C:\HPCHEM\1\DATA\NCA8416A\INJ_010.D
 Study No: 95JH225
 Sequence Number : G807.S
 Operator: EA
 Date Acquired: 8 Dec 95 9:59 pm
 Method File: HEMA Compound Name: HEMA
 Sample Name: R3 1317/4 95 0.01 MG/KG
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	314	8.31	6805
Qual 1	329	8.31	2032

Figure 6 : Recovery Soyabean Seed Sample Fortified at 0.01 mg kg⁻¹
 EMA Equivalent, 0.125 g ml⁻¹. EMA Recovery 99%

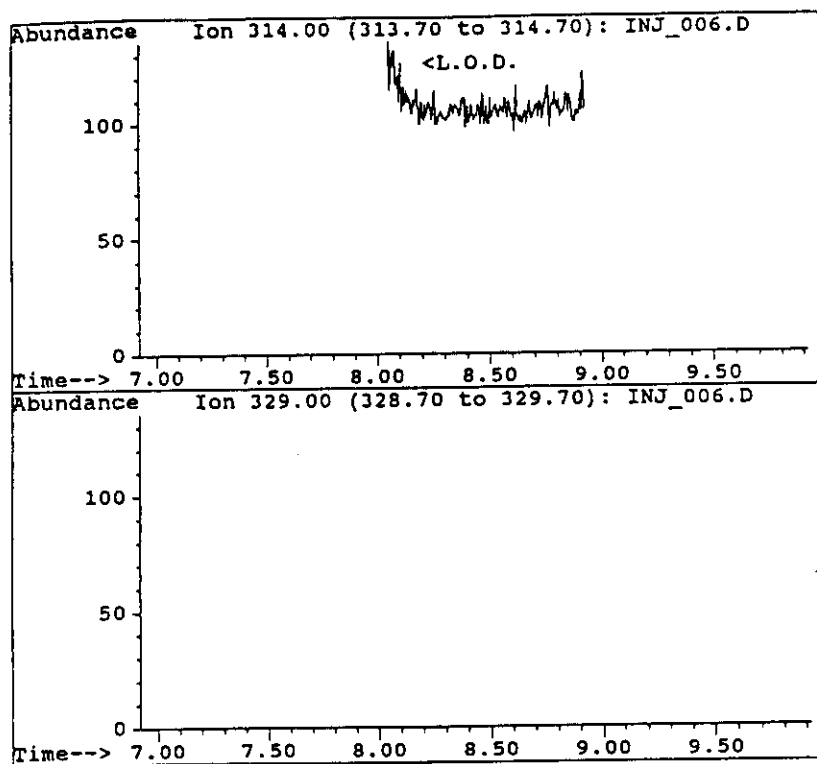


File C:\HPCHEM\1\DATA\NC8416A\INJ_010.D
 Study No: 95JH225
 Sequence Number : G807.S
 Operator: EA
 Date Acquired: 8 Dec 95 9:59 pm
 Method File: HEMA Compound Name: EMA
 Sample Name: R3 1317/4 95 0.01 MG/KG
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	162	10.82	27724
Qual 1	331	10.82	1561

Figure 7: Control Maize Forage Sample, 0.125 g ml⁻¹

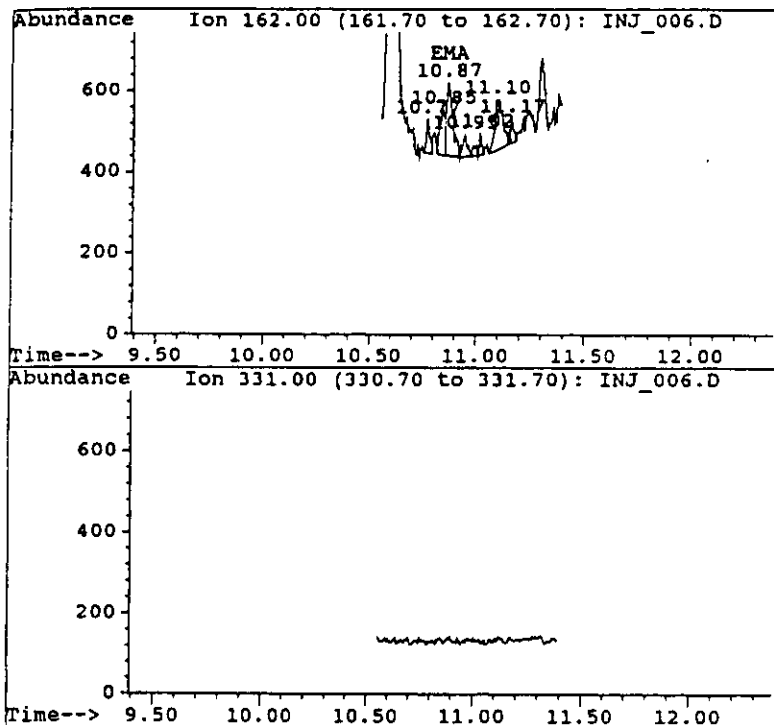


File C:\HPCHEM\1\DATA\NCA8370A\INJ_006.D
 Study No: 95JH225
 Sequence Number : G802.S
 Operator: E.AVER
 Date Acquired: 30 Nov 95 7:18 pm
 Method File: HEMA Compound Name: HEMA
 Sample Name: 1288/1 95 CON
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
No HEMA detected at		8.42 mins	

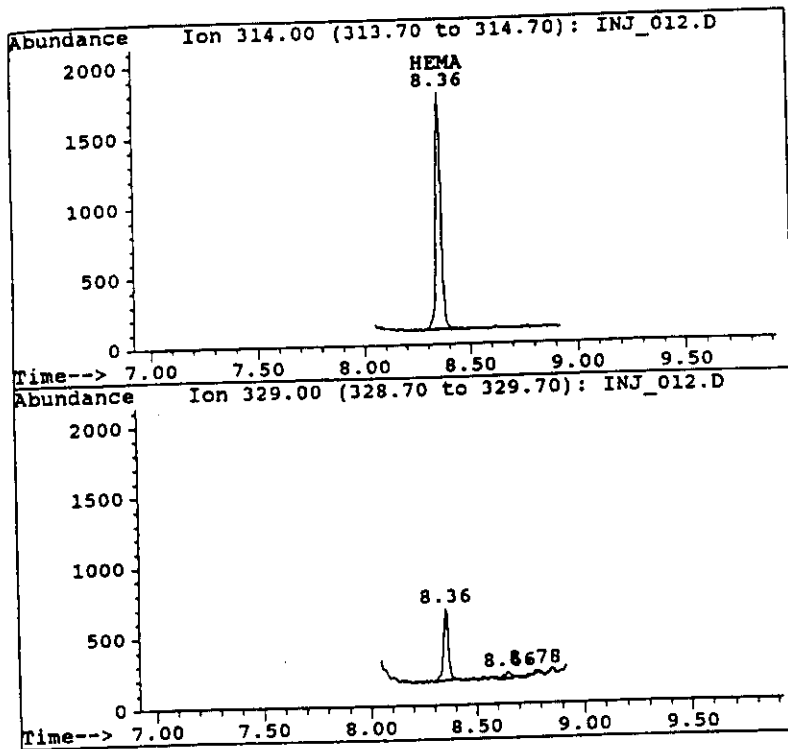
Figure 8 : Control Maize Forage Sample, 0.125 g ml⁻¹



File C:\HPCHEM\1\DATA\NCA8370A\INJ_006.D
 Study No: 95JH225
 Sequence Number : G802.S
 Operator: E.AVER
 Date Acquired: 30 Nov 95 7:18 pm
 Method File: HEMA Compound Name: EMA
 Sample Name: 1288/1 95 CON
 Type : SAMPLE Level : 1
 Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	162	10.87	3689
Qual 1	331	0.00	0

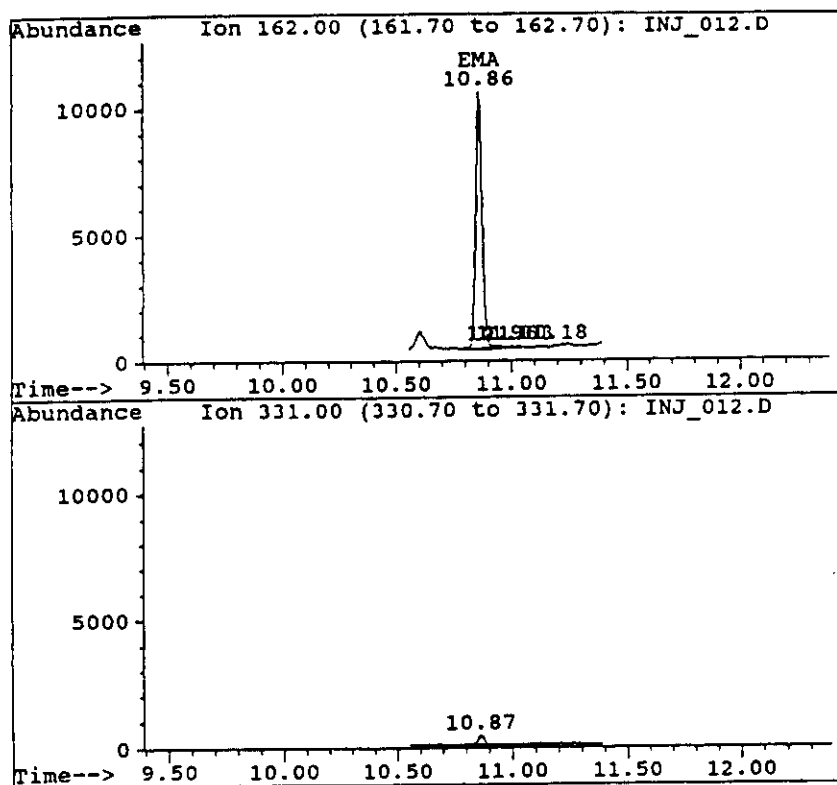
Figure 9 : Recovery Maize Forage Sample Fortified at 0.05 mg kg⁻¹
 HEMA Equivalent, 0.125 g ml⁻¹. HEMA Recovery 89%



File C:\HPCHEM\1\DATA\NCA8370A\INJ_012.D
 Study No: 95JH225
 Sequence Number : G802.S
 Operator: E.AVER
 Date Acquired: 1 Dec 95 9:16 am
 Method File: HEMA Compound Name: HEMA
 Sample Name: 1288/4 95 R3 0.05MG KG
 Type : SAMPLE Level : 1
 Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	314	8.36	29404
Qual 1	329	8.36	8489

Figure 10 : Recovery Maize Forage Sample Fortified at 0.05 mg kg⁻¹
EMA Equivalent, 0.125 g ml⁻¹. EMA Recovery 100%



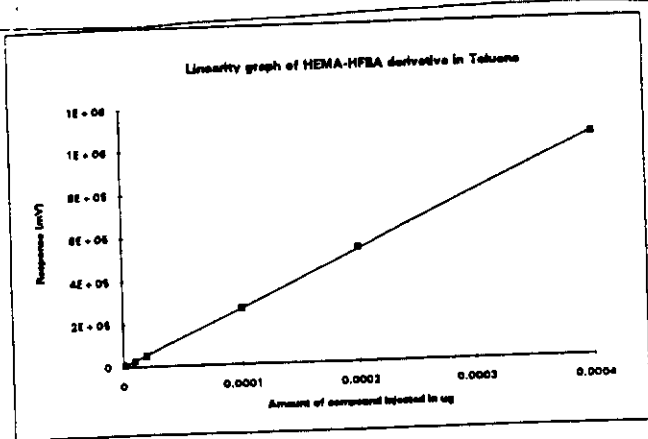
File C:\HPCHEM\1\DATA\NCA8370A\INJ_012.D
 Study No: 95JH225
 Sequence Number : G802.S
 Operator: E.AVER
 Date Acquired: 1 Dec 95 9:16 am
 Method File: HEMA Compound Name: EMA
 Sample Name: 1288/4 95 R3 0.05MG KG
 Type : SAMPLE Level : 1

Data Generated By Sequence

Type	Ion	Ret.Time	Area
Target	162	10.86	196576
Qual 1	331	10.87	6652

Appendix 6
Method Validation Data

Figure 11 : Linearity Graphs for HEMA and EMA Standards by GC-MSD



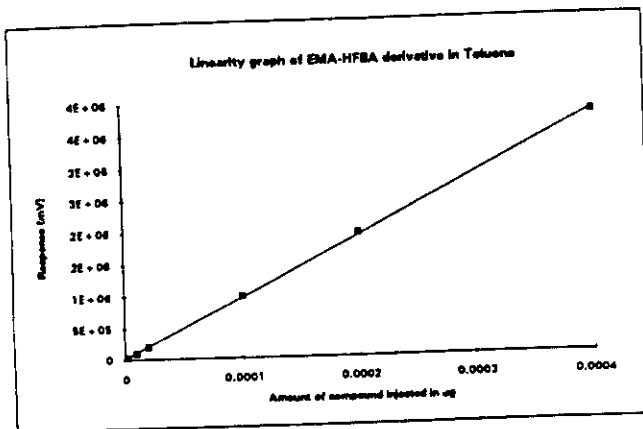
Response inj. 1 (mV)	Response inj. 2 (mV)	Response inj. 3 (mV)	Amount Of Compound Injected (ug)	Average Response (mV)
4760	4864	4810	0.000002	4796
22889	23084	23878	0.00001	23219
47785	47058	47889	0.00002	47609
283249	285148	284134	0.0001	286149
529711	531883	528118	0.0002	529627
1045859	1020851	1038817	0.0004	1038708

$$y = mx + b$$

$$m = 2.61E+06$$

$$b = -885$$

R² = 0.9996881



Response inj. 1 (mV)	Response inj. 2 (mV)	Response inj. 3 (mV)	Amount Of Compound Injected (ug)	Average Response (mV)
18808	18247	18234	0.000002	18694
93423	90617	95910	0.00001	93183
182890	189381	188627	0.00002	186299
989583	981119	993317	0.0001	981006
1928778	1858827	1844846	0.0002	1843317
3718973	3780225	3789828	0.0004	3788008

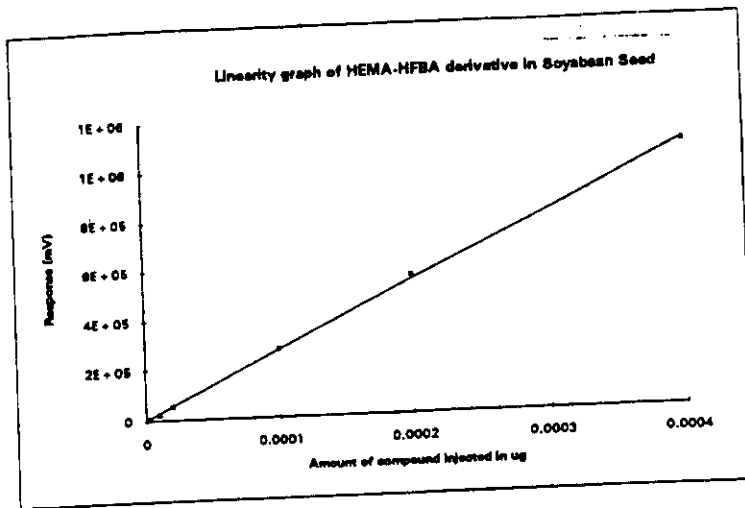
$$y = mx + b$$

$$m = 9.43E+09$$

$$b = 12820$$

R² = 0.9996501

Figure 12 : Linearity Graphs for HEMA and EMA Standards in the Presence of Soyabean Seed Control Matrix by GC-MSD



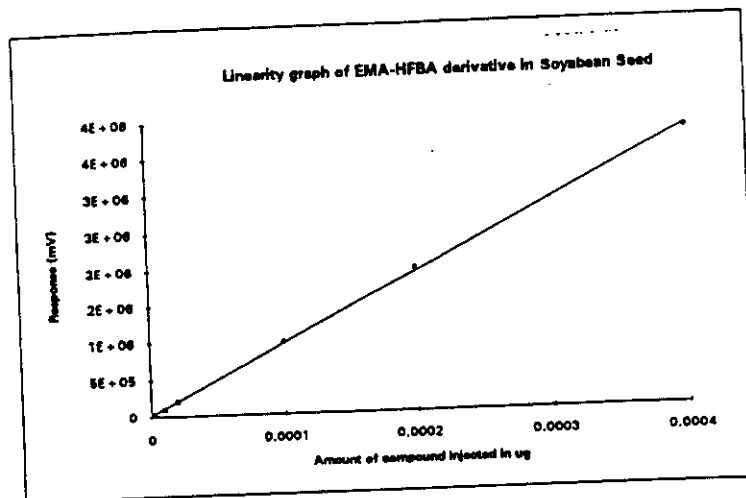
Response Inj. 1 (mV)	Response Inj. 2 (mV)	Response Inj. 3 (mV)	Amount Of Compound Injected (ug)	Average Response (mV)
4871	4613	4103	0.000002	4428
23474	23834	24151	0.00001	23820
50893	51028	51518	0.00002	51078
274432	270611	274433	0.0001	273158
544823	555348	565128	0.0002	555089
1073828	1084880	1088856	0.0004	1081148

$$y = ax + b$$

$$a = 2.88E + 08$$

$$b = 1314$$

R² = 0.9995648



Response Inj. 1 (mV)	Response Inj. 2 (mV)	Response Inj. 3 (mV)	Amount Of Compound Injected (ug)	Average Response (mV)
18877	17845	18850	0.000002	18457
94706	97245	98810	0.00001	96187
182781	185089	188421	0.00002	184780
857271	881862	878906	0.0001	872614
1834128	1844280	1878806	0.0002	1852408
3746898	3784188	3788485	0.0004	376788

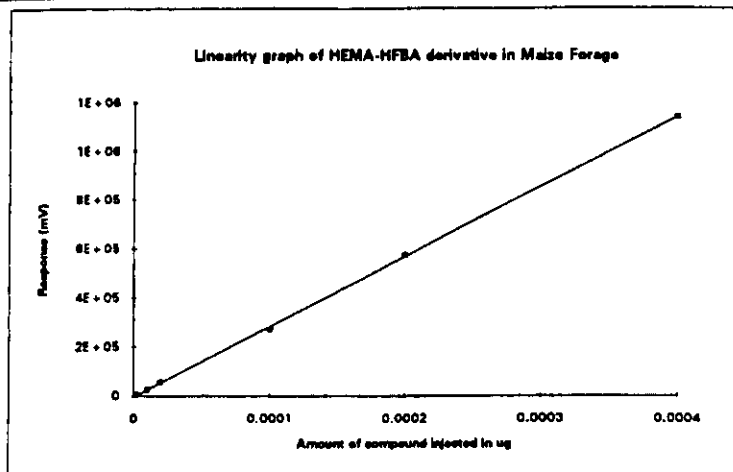
$$y = ax + b$$

$$a = 8.45E + 08$$

$$b = 14106$$

R² = 0.9995584

Figure 13 : Linearity Graphs for HEMA and EMA Standards in the Presence of Maize Forage Control Matrix by GC-MSD



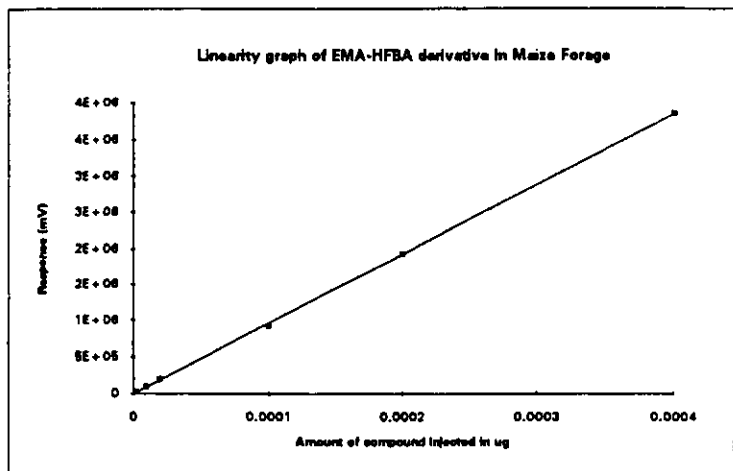
Response inj. 1 (mV)	Response inj. 2 (mV)	Response inj. 3 (mV)	Amount Of Compound Injected (ug)	Average Response (mV)
4737	4688	4787	0.000002	4703
25101	24111	25723	0.00001	24978
54938	54808	52984	0.00002	54178
263252	272010	268345	0.0001	268202
571847	572388	573350	0.0002	572554
1154410	1127785	1124580	0.0004	1138825

$$y = ax + b$$

$$a = 2.86E+08$$

$$b = -4858$$

R² = 0.9997818



Response inj. 1 (mV)	Response inj. 2 (mV)	Response inj. 3 (mV)	Amount Of Compound Injected (ug)	Average Response (mV)
20055	18734	18842	0.000002	18910
88485	84007	89806	0.00001	87427
183610	189883	203083	0.00002	198785
805413	806898	828271	0.0001	813484
1835818	1828382	1821012	0.0002	1828737
3878431	3882045	3840122	0.0004	3858533

$$y = ax + b$$

$$a = 8.95E+08$$

$$b = -7781$$

R² = 0.998783

Table 1 : Recovery Data Obtained During the Validation Analysis

Crop	** Fortification Level (mg kg ⁻¹)	Recovery (%)	
		HEMA	EMA
Sugar Beet Root	0.01	64, 79, 56, 74	50, 84, 92, 108
	0.05	83, 84	98, 78
	0.1	87, 84	100, 88
	0.2	96, 102	103, 95
Maize Sweetcorn Kernel on the Cob	0.01	78, 76, 72, 89	91, 91, 79, 101
	0.05	72, 86	85, 87
	0.1	97, 85	94, 88
	0.2	98, 94	91, 91
Maize Forage	0.01	*72, *69, *56, *79	*101, *97, *92, *109
	0.05	89, 74	100, 93
	0.1	89, 76	99, 96
	0.2	90, 98, 91, 86, 84 77, 95, 73, 97, 92	92, 95, 87, 88, 87 80, 97, 75, 99, 90
Soyabean Seed	0.01	102, 104, 93, 95	96, 101, 99, 112
	0.05	85, 89	106, 85
	0.1	103, 116	93, 101
	0.2	112, 116	92, 81
	Mean Recovery (%)	87	92
	Standard Deviation (%)	14	10
	Coefficient of Variation (%)	16	11
	Number of Analyses	48	48

* Mean of duplicate injections.

** Fortification level based on EMA and/or HEMA common moieties

Appendix 7
Example Fortification Calculations

For example a 10 g sample is required to be fortified at 0.02 mg kg⁻¹ with EMA and HEMA equivalent.

EMA Fortification Calculation :-

$$\frac{\text{Molecular Weight Compound 24}}{\text{Molecular Weight EMA}} = \frac{337}{135} = 2.5$$

Required amount of compound 24 to be added to the sample = 0.02 x 2.5 x 10 g = 0.5 µg

The sample should therefore be fortified with 0.5 ml of a 1 µg ml⁻¹ compound 24 standard.

HEMA Fortification Calculation :-

$$\frac{\text{Molecular Weight Compound 37}}{\text{Molecular Weight HEMA}} = \frac{267}{151} = 1.77$$

Required amount of compound 37 to be added to the sample = 0.02 x 1.77 x 10 g = 0.354 µg

The sample should therefore be fortified with 0.354 ml of a 1 µg ml⁻¹ compound 37 standard.