

US EPA ARCHIVE DOCUMENT

REPORT TITLE

**ANALYTICAL METHOD FOR THE DETERMINATION OF MON 12000 IN  
RAW AGRICULTURAL COMMODITIES AND PROCESSED FRACTIONS**

Author: Jean Culkin  
Method Developed by: Jean Culkin and William Ridley  
Effective Date: December 1997  
Document Number: RES-109-97-4

Prepared by: Jean Culkin Date: 12-16-97  
Approved by: William Ridley Date: 12-16-97

## I. SCOPE

The analytical method described here is for the determination of MON 12000 parent compound in cotton, cotton processed fractions (i.e. refined oil, hulls and meal), rice, rice processed fractions (i.e. bran, polished rice and hulls), corn, sugarcane, milo and tree nut matrices. The basis of the determination is the conversion of MON 12000 residue to its rearrangement ester (a compound unique to MON 12000). The method has been validated over a range from 0.05 to 0.20 parts per million. The sections in this document list the equipment and chemicals used, describe the preparation of standard solutions and procedures for quantitating this analyte.

## II. PRINCIPLES

The procedure for determining residues consist of extraction, cleanup and gas chromatographic analysis. Crop samples are extracted with acetonitrile-water using a blender, then filtered and partitioned into dichloromethane. The samples are then evaporated to dryness; followed by a preliminary separation and cleanup, using Florisil Solid Phase Extraction (SPE) columns. The column fractions containing the MON 12000 are stirred with 0.5 M potassium carbonate for conversion to the Rearrangement Ester (RRE). Final sample cleanup is performed using Silica SPE columns. The extracts are reconcentrated and brought to a final volume. Quantitation is by gas chromatography (GC) with nitrogen specific (TSD) detector.

## III. MATERIALS AND METHODS

### A. EQUIPMENT

The following supplies are used in this analytical method. Specific brands are listed to aid the chemist in acquiring the items. In most cases equivalent equipment, obtained from other vendors, can be used.

Buchner funnels, 126 mm plate diameter, VWR No. 30310-164

Stir Plates: Fisher No. 11-496-60

Mechanical shaker: Fisher No. 14-261

Balance: Mettler PM 4800

Analytical balance: Mettler AE 240

Round bottom flask: Fisher No. 10-067- 2D (250 mL), 10-067-2F (500 mL)

Rotary evaporator: Fisher No. 09-548-100 (motor), Fisher No. HP-18325 (hot plate), Cole Parmer No. L-07274-00 (water baths), Kontes No. 926910-0500 (dewar vacuum trap), Fisher No. 01-096 (vacuum pump), Aldrich #Z16,405-4 (Splash Guard Trap)

Pasteur pipettes, 5.75 inch: Fisher No. 13-678-6A

Volumetric flask, 100 mL: Fisher No. 10-210C

Graduated cylinder, 1000 mL: 08-549-5J

Serological pipettes from 1 through 10 mL: VWR No. 53284-562, -584, -609, -620

Autosampler vials: Varian No. 66-000104-00

00050F0022

Gas Chromatograph: Varian 3700

Autosampler: Varian 8000 series

SPE Vacuum manifold: Supelco model 5-7030M

Supelco Teflon guide needles: 5-7047-M

Separatory Funnel, 500 mL: Fisher No. 10-437-2D

Whatman, Glass Microfibre Filters GF/C: Baxter Scientific No. F2832-11

Whatman, Glass Microfibre Filters 934-AH: Baxter Scientific No: F2835-11

Vacuum Filtration Adapter: Aldrich Z11-565-0

Funnels: Fisher No. 10-346-5B

Magnetic Stir Bars

Waring Blender: Fisher No. 14-509-10

Blender jars: Fisher No. 14509-11A

#### B. REAGENTS AND STANDARDS

The following reagents are used in this analytical method. Specific brands are listed to aid the chemist in obtaining these items. In most cases equivalent reagents, obtained from other vendors, can be used.

Acetonitrile: VWR No. BJ015-48-4

Deionized Water: VWR No. BJ365-4 or purified with system such as a Milli-Q

Dichloromethane: VWR No. BJ300-4

Ethyl Acetate: VWR No. BJ099-4

Florisil SPE, 5 g: Alltech No. 227950

Isooctane: VWR No. BJ362-4

Methanol: VWR No. BJ230-4

Potassium Carbonate: VWR No. JT3012-5

Silica SPE, 2 g: Alltech No. 209362

Sodium Chloride: VWR No. JT3628-1

25% water in acetonitrile (V/V): To prepare 4 L, mix 1000 mL water and 3000 mL acetonitrile

00060022

5% methanol in dichloromethane (V/V): To prepare 4 L, mix 200 mL methanol and 3800 mL dichloromethane

15% methanol in dichloromethane (V/V): To prepare 4 L, mix 600 mL methanol and 3400 mL dichloromethane

2% ethyl acetate in isooctane (V/V): To prepare 4 L, mix 80 mL ethyl acetate and 3920 mL isooctane

20% ethyl acetate in isooctane (V/V): To prepare 4 L, mix 800 mL ethyl acetate and 3200 mL isooctane

50% ethyl acetate in isooctane (V/V): To prepare 4 L, mix 2000 mL ethyl acetate and 2000 mL isooctane

0.5 M Potassium Carbonate (W/V): To prepare 1 L, dissolve 69.1 g  $K_2CO_3$  in 1L of water

MON 12000: 1H-Pyrazole-4-carboxylic acid, 3-chloro-5-[[[(4,6-dimethoxy-2-pyrimidinyl)amino] carbonyl]amino]sulfonyl]-1-methyl-, methyl ester of known analytical purity (if purity is less than 95%, purity corrections should be made). Molecular weight = 434.8.

MON 5781 Rearrangement Ester (RRE): 1 H-Pyrazole-4-carboxylic acid, 3-chloro-5-[(4,6-dimethoxy-2-pyrimidinyl)amino]-1-methyl, methyl ester of known analytical purity (if purity is less than 95%, purity correction should be made). Molecular weight = 327.7

## C. PREPARATION OF STANDARDS

### 1. GC Quantitation Standards

#### Rearrangement Ester (RRE)

The stock calibration standard is prepared by weighing  $0.0150 \pm g$  of RRE into a 100 mL volumetric flask. The standard is diluted to the volumetric mark with 2% ethyl acetate/isooctane. Mix well by repeated inversions and sonicate for complete dissolution. This stock solution contains 200  $\mu g/mL$  of RRE expressed as MON 12000 equivalents (150  $\mu g/mL$  RRE). A five mL aliquot of this solution is diluted to 100 mL with 2% ethyl acetate/isooctane in a second volumetric flask to provide a stock solution containing 10  $\mu g/mL$  in MON 12000 equivalents (7.5  $\mu g/mL$  RRE).

RRE Working Standards (Expressed in MON 12000 equivalents)

1. Dilute 5.0 mL of the 10  $\mu g/mL$  stock solution to 100 mL with 2% ethyl acetate/isooctane in a volumetric flask to provide a 0.5  $\mu g/mL$  solution of RRE expressed as MON 12000 equivalents.
2. Dilute 3.5 mL of the 10  $\mu g/mL$  stock solution to 100 mL with 2% ethyl acetate/isooctane in a volumetric flask to provide a 0.35  $\mu g/mL$  solution of RRE expressed as MON 12000 equivalents.
3. Dilute 2.0 mL of the 10  $\mu g/mL$  stock solution to 100 mL with 2% ethyl acetate/isooctane in a volumetric flask to provide a 0.2  $\mu g/mL$  solution of RRE expressed as MON 12000 equivalents.

00070F0022

4. Dilute 1.5 mL of the 10  $\mu\text{g/mL}$  stock solution to 100 mL with 2% ethyl acetate/isooctane in a volumetric flask to provide a 0.15  $\mu\text{g/mL}$  solution of RRE expressed as MON 12000 equivalents.
5. Dilute 1.0 mL of the 10  $\mu\text{g/mL}$  stock solution to 100 mL with 2% ethyl acetate/ isooctane in a volumetric flask to provide a 0.1  $\mu\text{g/mL}$  solution of RRE expressed as MON 12000 equivalents.
6. Dilute 0.75 mL of the 10  $\mu\text{g/mL}$  stock solution to 100 mL with 2% ethyl acetate/ isooctane in a volumetric flask to provide a 0.075  $\mu\text{g/mL}$  solution of RRE expressed as MON 12000 equivalents.
7. Dilute 0.5 mL of the 10  $\mu\text{g/mL}$  stock solution to 100 mL with 2% ethyl acetate/isooctane in a volumetric flask to provide a 0.05  $\mu\text{g/mL}$  solution of RRE expressed as MON 12000 equivalents.

2. Fortification Standards

MON 12000 Stock

The stock fortification standard is prepared by weighing  $0.1000 \pm 0.0004$  g of MON 12000 into a 100 mL volumetric flask. The standard is diluted to the volumetric mark with acetonitrile. The final solution contains 1000 mg/mL of MON 12000. A ten mL aliquot of this solution is diluted to 100 mL with acetonitrile in a second volumetric flask to provide 100  $\mu\text{g/mL}$  MON 12000. A 10 mL aliquot of this 100  $\mu\text{g/mL}$  solution is then transferred to a third 100 mL volumetric flask and diluted to the volumetric mark with acetonitrile. The final solution contains 10  $\mu\text{g/mL}$  of MON 12000.

MON 12000 Working Solutions

1. Dilute 2.5 mL of the 100  $\mu\text{g/mL}$  stock solution to 100 mL with acetonitrile in a volumetric flask to provide a 2.5  $\mu\text{g/mL}$  solution of MON 12000.
2. Dilute 1.25 mL of the 100  $\mu\text{g/mL}$  stock solution to 100 mL with acetonitrile in a volumetric flask to provide a 1.25  $\mu\text{g/mL}$  solution of MON 12000.

All standards are stored in amber glass bottles. RRE solutions are stored in the refrigerator ( $0-8^{\circ}\text{C}$ ) and MON 12000 solutions are stored in the freezer ( $<0^{\circ}\text{C}$ ).

D. ANALYTICAL PROCEDURE

Crop samples are received and stored frozen until just prior to analysis. The crop matrix is thoroughly mixed with dry ice in a Hobart Mixer and subsampled for analysis.

1a. Sample Extraction [General]

1. Weigh crop samples ( $12.5 \pm 0.05$  g) into a one quart Waring blender jar.

00080F0022

2. Fortify samples by pipetting the fortification standard directly on the sample (e.g. Use a 1.0 mL aliquot of the 1.25 µg/mL MON 12000 solution for a 0.1 ppm fortification to a 12.5 gram sample).
3. Add 150 mL of 25% water/acetonitrile to the blender jar and blend at medium speed for 5 minutes.
4. Place two filters (one GFC and one 934-AH) in a buchner funnel and connect to a 500 mL flask using an adapter suitable for applying vacuum. Wet the filters with 25% water/ acetonitrile.
5. Filter the extract through the buchner funnel into the 500 mL flask.
6. Rinse the blender and filter cake with approximately 50 mL 25% water/ acetonitrile.

**Oily Matrices (e.g. tree nuts, corn or cotton):**

- a. Transfer the extract from step 1.6 to a 500 mL separatory funnel.
- b. Add 100 mL of hexane.
- c. Shake gently on a mechanical shaker for 5 minutes. Allow phases to separate.
- d. Discard the hexane (upper) layer. Retain the aqueous (lower) phase and continue with step 2 Partition.

1b. Sample Extraction [Cotton Seed Refined Oil, process fraction]

1. Weigh 12.5 ± 0.05 g of the refined oil directly into a 500 mL separatory funnel.
2. Fortify samples by pipetting the fortification standard directly on the sample (e.g. Use a 1.0 mL aliquot of the 1.25 µg/mL MON 12000 solution for a 0.1 ppm fortification to a 12.5 gram sample).
3. Add 150 mL 25% water/acetonitrile to the separatory funnel and shake for 5 minutes on a mechanical shaker.
4. Add 100 mL hexane to the separatory funnel and shake for an additional 5 minutes on a mechanical shaker.
5. Discard the hexane (upper) layer and continue with Step 2 Partition.

2. Partition

1. Transfer sample from Step 1.6 or Step 1.6d Extraction to a 500 mL separatory funnel.
2. Add 150 mL water and 130 mL dichloromethane and shake for about 30 seconds.

3. Add approximately five grams of sodium chloride and allow the phases to separate.
4. Drain the lower organic layer through approximately 20 g anhydrous sodium sulfate into a 500 mL round bottom flask.
5. Re-extract the aqueous layer with 50 mL dichloromethane.
6. Drain the organic layer through anhydrous sodium sulfate and into the flask containing the extract from Step 2.4.
7. Rinse the anhydrous sodium sulfate with approximately 10 mL dichloromethane.
8. Evaporate the combined organic solutions to dryness by rotary evaporation in a water bath (approximately 22°C).
9. Discard the aqueous layers.

3. Florisil SPE Column

1. Condition a five gram Florisil SPE column with 20 mL methanol followed by 20 mL dichloromethane. Do not allow the columns to go dry between additions.
2. Dissolve the residue from Step 2.9 in 2.5 mL 15% methanol/dichloromethane then add an additional 10 mL of dichloromethane.
3. Apply the sample to the column.
4. Rinse the flask with 30 mL dichloromethane and apply to the column.
5. Rinse the column with 40 mL 5% methanol/dichloromethane.
6. Discard all rinses to this point.
7. Place a container in the vacuum manifold, apply vacuum (typically 5-10 in/Hg) and elute the column with 100 mL 15% methanol/dichloromethane. Elute rapidly, but with drops still visible.

4. Conversion to RRE

1. Transfer the solution from Step 3.7 to a 250 mL round bottom flask, add 100 mL 0.5 M potassium carbonate and a magnetic stirring bar.
2. Loosely stopper the flask and stir briskly overnight.
3. Transfer the mixture to a 500 mL separatory funnel.
4. Rinse the flask twice with 20 mL dichloromethane and add the rinses to the separatory funnel.

5. Drain the lower organic layer through approximately 20 g anhydrous sodium sulfate into a 250 mL round bottom flask.
6. Re-extract the aqueous layer with 40 mL dichloromethane and allow the layers to separate.
7. Drain the organic layer through anhydrous sodium sulfate and into the flask containing the extract from Step 4.5.
8. Rinse the sodium sulfate with approximately 10 mL dichloromethane.
9. Evaporate the sample to dryness by rotary evaporation in a water bath (approximately 22°C).

5. Silica SPE Column

1. Dissolve the residue in two mL ethyl acetate.
2. Condition a two gram silica SPE column with 20 mL ethyl acetate followed by 20 mL 50% ethyl acetate/ isooctane. Do not allow the column to go dry between additions.
3. Place a container in the vacuum manifold, apply vacuum (typical vacuum 5-10 in/Hg) and collect all eluent.
4. Add two mL isooctane to the sample flask and apply mixture to the column.
5. Rinse the flask with 5 mL 50/50 ethyl acetate/isooctane and add to the column.
6. Elute with 40 mL 20% ethyl acetate/isooctane. Elute rapidly, but with drops still visible.
7. Evaporate the sample to dryness by rotary evaporation in a water bath (approximately 22°C).
8. Dissolve the residue sample in five mL 50% ethyl acetate/isooctane.
9. Appropriate dilutions of samples and fortifications should be made to ensure their response falls within the range of the quantitation standards.

IV. INSTRUMENT PARAMETERS

The isolated residue is quantified by gas chromatography with a nitrogen specific detector (TSD). The following operating parameters are used in this step. Similar, but different, equipment or column conditions may be used, but may require modification of these parameters.

00110022

A. GLC/TSD Operating Conditions

Column: Rtx-5 0.53  $\mu\text{m}$ , 3  $\mu$  film, 60 m  
Program: 195 °C (2 min), 10 °C/min to 280 °C (hold 10 minutes)  
Injector Temp.: 250 °C                      Detector Temp.: 300 °C  
Air flow: 165 mL/min                      Nitrogen flow: 22 mL/min  
3  $\mu\text{L}$  injection  
Hydrogen flow: 4.00-5.00 mL/min  
See Figures 1a - 1g for sample chromatograms.

B. Calibration

The GC system is calibrated by using external analytical standards run with each analytical set. These standards are placed periodically among the samples (generally two samples between standards). The calibration curves are generated by plotting the peak height of the detector response against the concentration of each calibration standard. Linear least squares estimates of the data points are then used to define the calibration curve. See figure 2a

V. INTERFERENCES

Detailed interference studies have not been performed. No interference owing to solvents or labware has been observed.

VI. CONFIRMATORY METHOD

A full confirmatory method is not currently available, however the RRE can be analyzed by GC/MS in the positive ion electron ionization mode using 30m x 0.25 mm ID (0.25  $\mu\text{m}$  film) DBMS column and monitoring  $M/Z = 327$ . Alternatively, samples can be quantified using a SPB-1 column (0.75 mm I.D. x 60 m, 1  $\mu$  film).

VII. TIME REQUIRED FOR ANALYSIS

A set of 12 samples requires approximately 2 days from initial extraction to setting the samples up for GC analysis.

VIII. MODIFICATIONS OR POTENTIAL PROBLEMS

Control and fortified samples should be run in the same analytical set as treated samples.

During rotary evaporation steps heat may be applied to maintain room temperature (approx. 22°C).

The liquid/liquid extraction Steps 2 and 4 result in emulsions. A six mL aliquot of saturated aqueous sodium chloride may be sufficient to break up the emulsions and substituted for the solid sodium chloride. Additional sodium chloride, either solid or saturated aqueous solution, may be needed after the reextraction. However, too much sodium chloride may cause the phases to invert. This does not effect the recovery, if the correct phase is saved.

Stirring times for section 4 has been found to give acceptable results from 2 up to 64 hours.

Variations in lots or manufacturers of Florisil and silica used in the clean up steps may result in a shift of the elution pattern. Adjustments should be made to compensate for such shifts.

Before fortifications, shake the standard solution bottle and pipette an aliquot into a small capped vial. Return the standard bottle to the freezer. When the aliquot is warmed to room temperature take the fortification from this solution. Dispose of any remaining solution in the vial.

In Steps 2 and 4, a powder funnel with either filter paper or a glass wool plug is used to hold the anhydrous sodium sulfate.

## IX. METHOD OF CALCULATION

This section contains the necessary steps for calculating the residue levels of MON 12000.

### A. CALIBRATION CURVE

The concentration of the RRE expressed as MON 12000 equivalents is determined based upon the peak height of the analyte. The concentration is determined by interpolation of an external standard linear calibration curve. See Figure 2 for an example of a calibration curve.

$$\mu\text{g/mL found} = (\text{PKHT})m + b$$

Where,

PKHT = the height of the analyte peak

m = the slope of the linear least squares fit of the calibration standards.

b = the y-intercept of the linear least squares fit of the calibration standards.

### B. ANALYTE IN SAMPLE

The amount of analyte in the sample is determined by multiplying the  $\mu\text{g/mL}$  amount found by the adjusted volume of the injected sample. This gives the total  $\mu\text{g}$  amount found in the injected sample that represents the total  $\mu\text{g}$  amount in the sample carried through the chromatography. Division of this number by the sample weight results in the  $\mu\text{g/g}$  or ppm value.

$$\text{ppm found} = (\mu\text{g/mL found} \times \text{adjusted volume}) / \text{sample weight}$$

Check samples are spiked with a known amount of the analyte before extraction. After the ppm is found in the spiked sample, that amount (after subtracting any amount found in the unfortified check sample) is divided by the amount fortified in ppm. The result is the Percent Recovery of that individual fortified sample.

$$\% \text{ Recovery} = (100 \times \text{ppm found}) / \text{ppm added}$$

## X. RESULTS/DISCUSSION

### A. Accuracy and Precision

A total of 161 samples, fortified with MON 12000, were analyzed using this method. The total number of nut matrix samples run using this method was 37 samples, rice matrices totaled 68 samples and the cotton matrices totaled 56 samples. All were fortified with MON 12000 at levels ranging from 0.05 ppm (lower limit of method validation) to 0.4

ppm. Average recoveries for MON 12000 were 91%, 89% and 88% for tree nuts, cotton and rice, respectively. The following table summarizes the validation results for each matrix.

<u>MATRIX</u>	<u>AVERAGE RECOVERY%</u>	<u>MINIMUM RECOVERY%</u>	<u>MAXIMUM RECOVERY%</u>	<u>STANDARD DEVIATION</u>
Pecans	92.3	76.1	111.6	12.6
Pistachios	91.5	87.2	101.2	5.7
Almond Nutmeat	92.0	65.0	118.8	12.6
Almond Hulls	87.5	72.4	120.3	13.5
Rice Grain	83.4	63.3	104.0	11.2
Rice Straw	92.0	68.1	114.2	11.3
Cotton Undelinted Seed	87.5	68.9	99.1	6.8
Cotton Gin Trash	91.1	69.0	113.7	11.6

Note: The validation data summarized in the above table will be archived with raw data for protocol 96-42-R-1, 96-42-R-2 and 96-42-R-3. The table includes data from MACS jobs: SOY5000BAYB, SOY5000BAXX, SOY5000BAZB, SOY5000BAZL and RIC5000CAEN.

B. Ruggedness Testing

Formal ruggedness testing has not been done for this method.

C. Limits of Quantitation (LOQ) and Limits of Detection (LOD)

The limits of quantitation and detection have not been statistically determined for this method. However, the lower limit of method validation (the lowest level of fortification demonstrated to have acceptable recovery and precision) is 0.05 ppm for MON 12000.

D. Limitations

This method is designed specifically for analysis of residue levels expected in crop matrices treated with MON 12000. The method has been validated for cotton, rice and tree nut matrices at residue levels from 0.05 to 0.40 ppm. Analysis of other matrices or residue levels above or below these limits would require appropriate adjustments and validation.

XI. CONCLUSIONS

The analytical method described is applicable to the determination of MON 12000 in crop matrices and processed fractions. No known interferences have been observed.

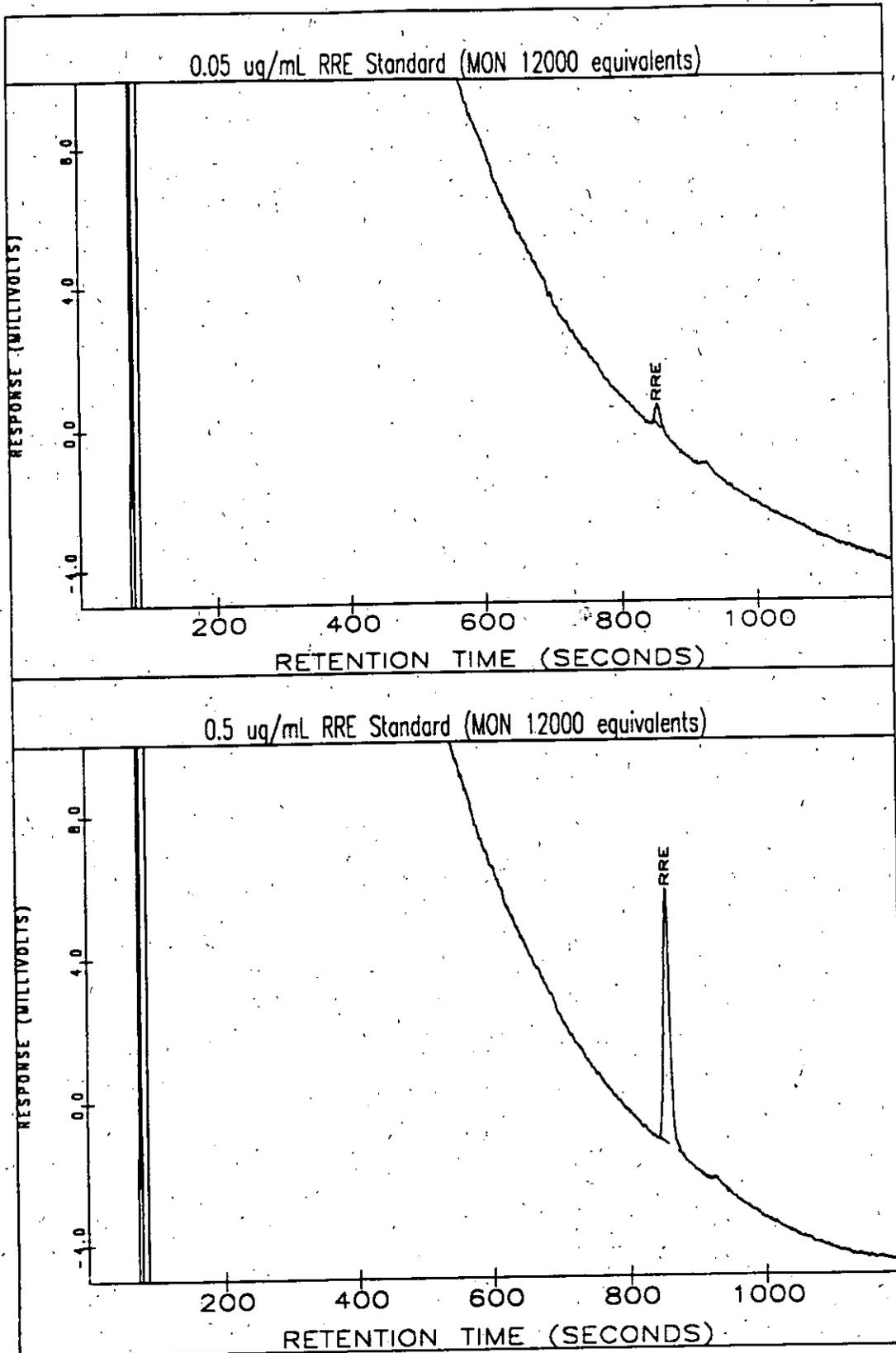


Figure 1a: Sample Chromatograms for Calibration Standards

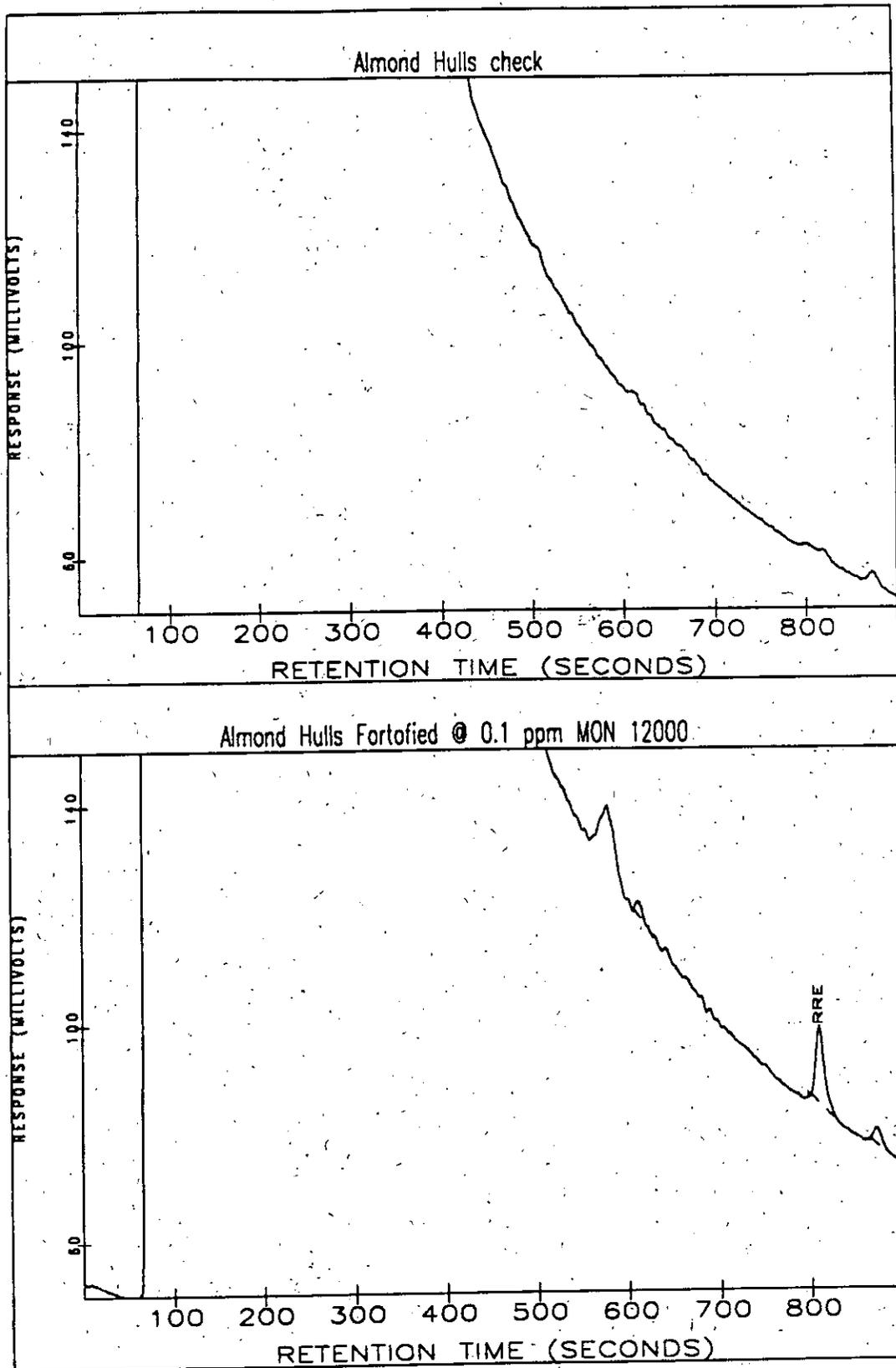


Figure 1b: Sample Chromatograms for Almond Hulls

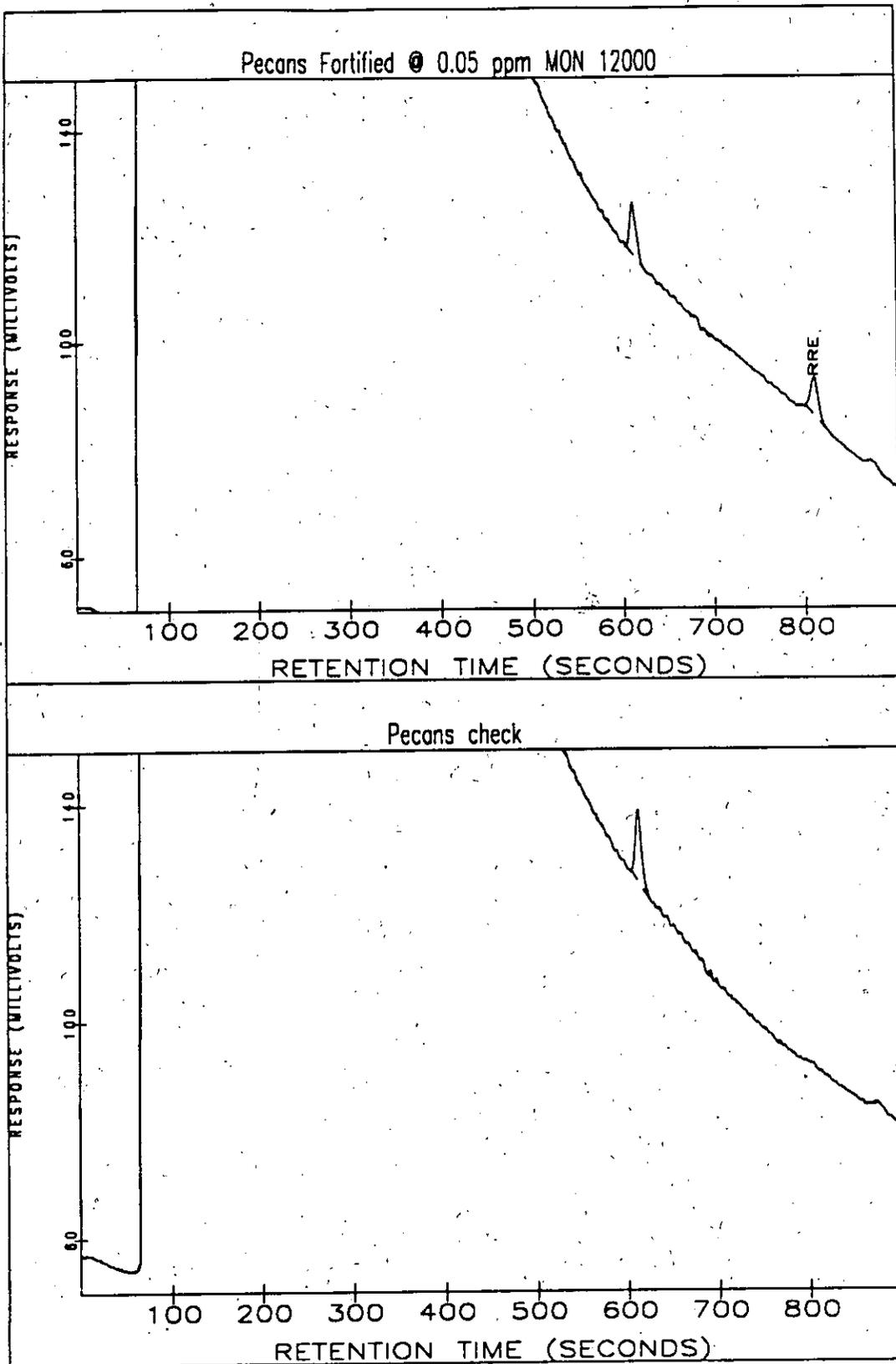


Figure 1c: Sample Chromatograms for Pecan Nutmeat

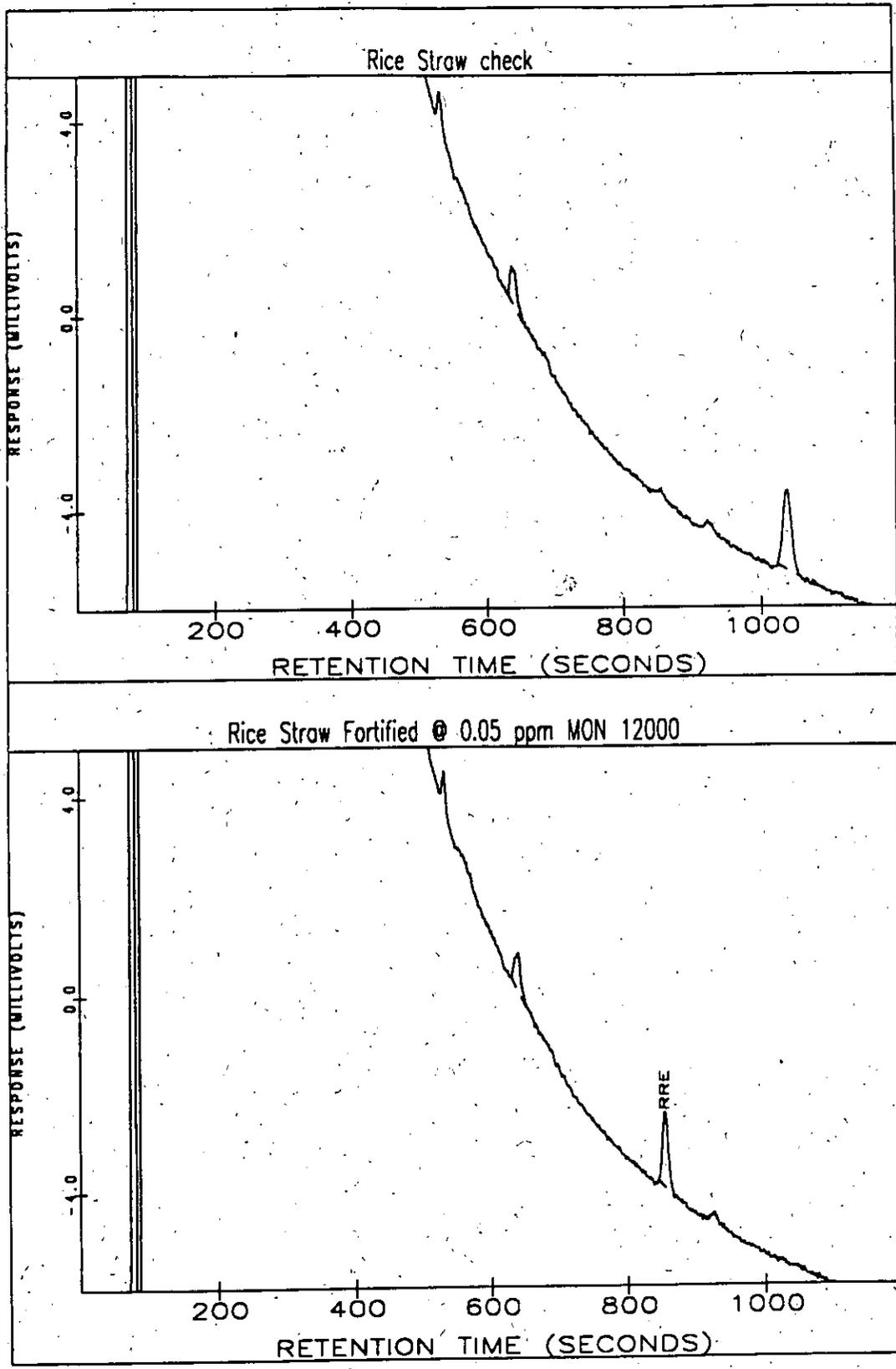


Figure 1d: Sample Chromatograms for Rice Straw

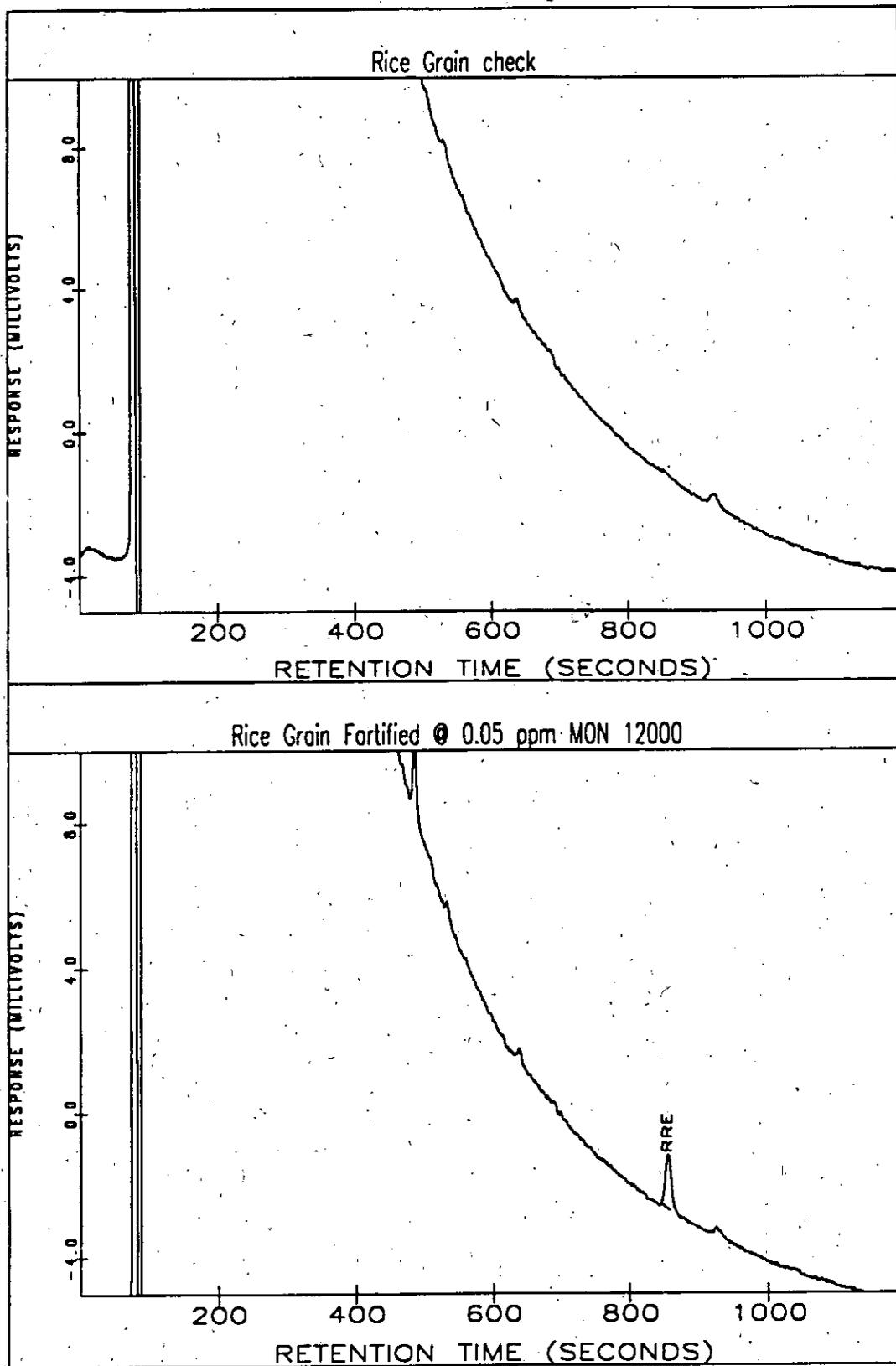


Figure 1e: Sample Chromatograms for Rice Grain

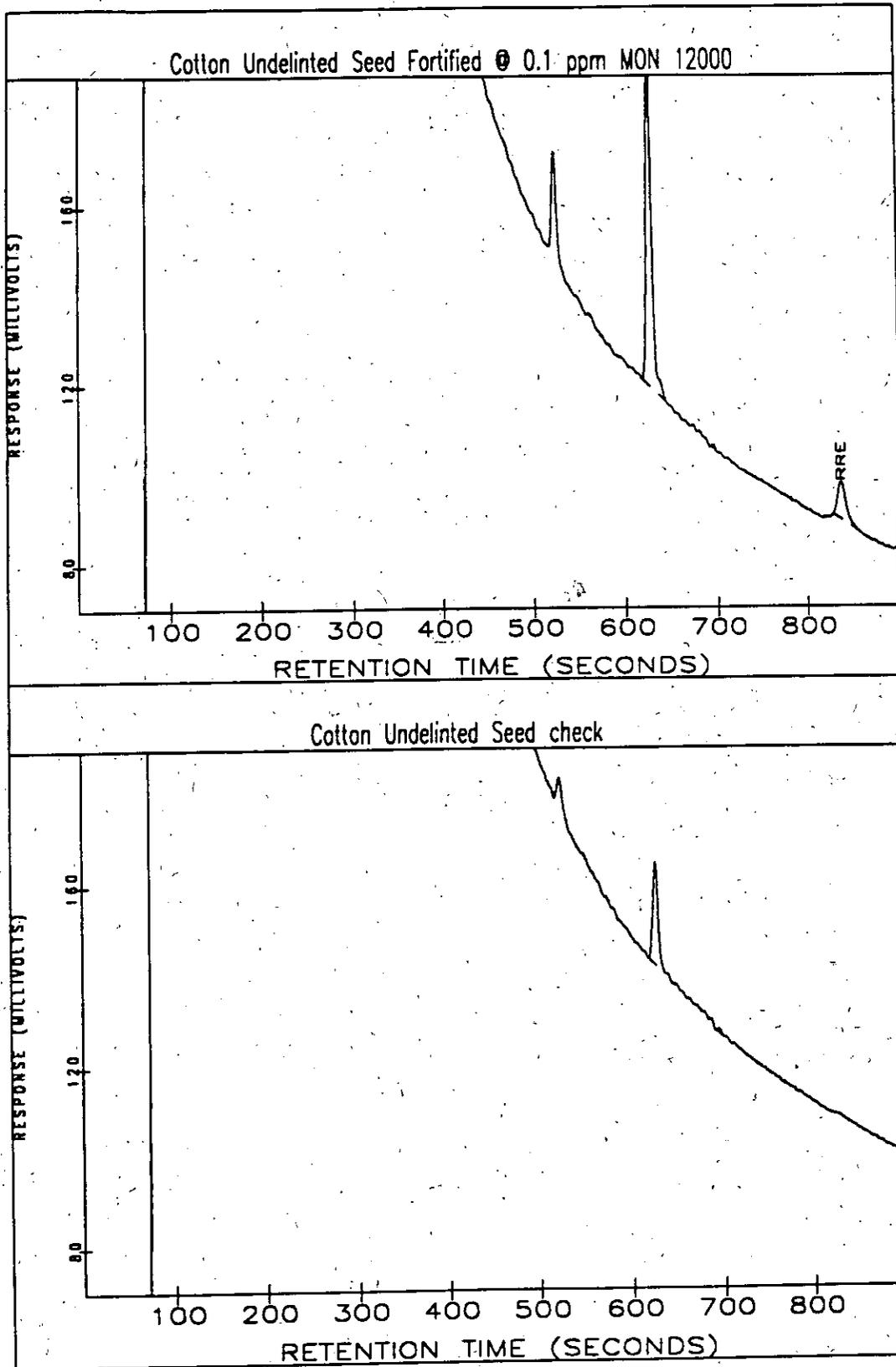


Figure 1f: Sample Chromatograms for Cotton Undelinted Seed

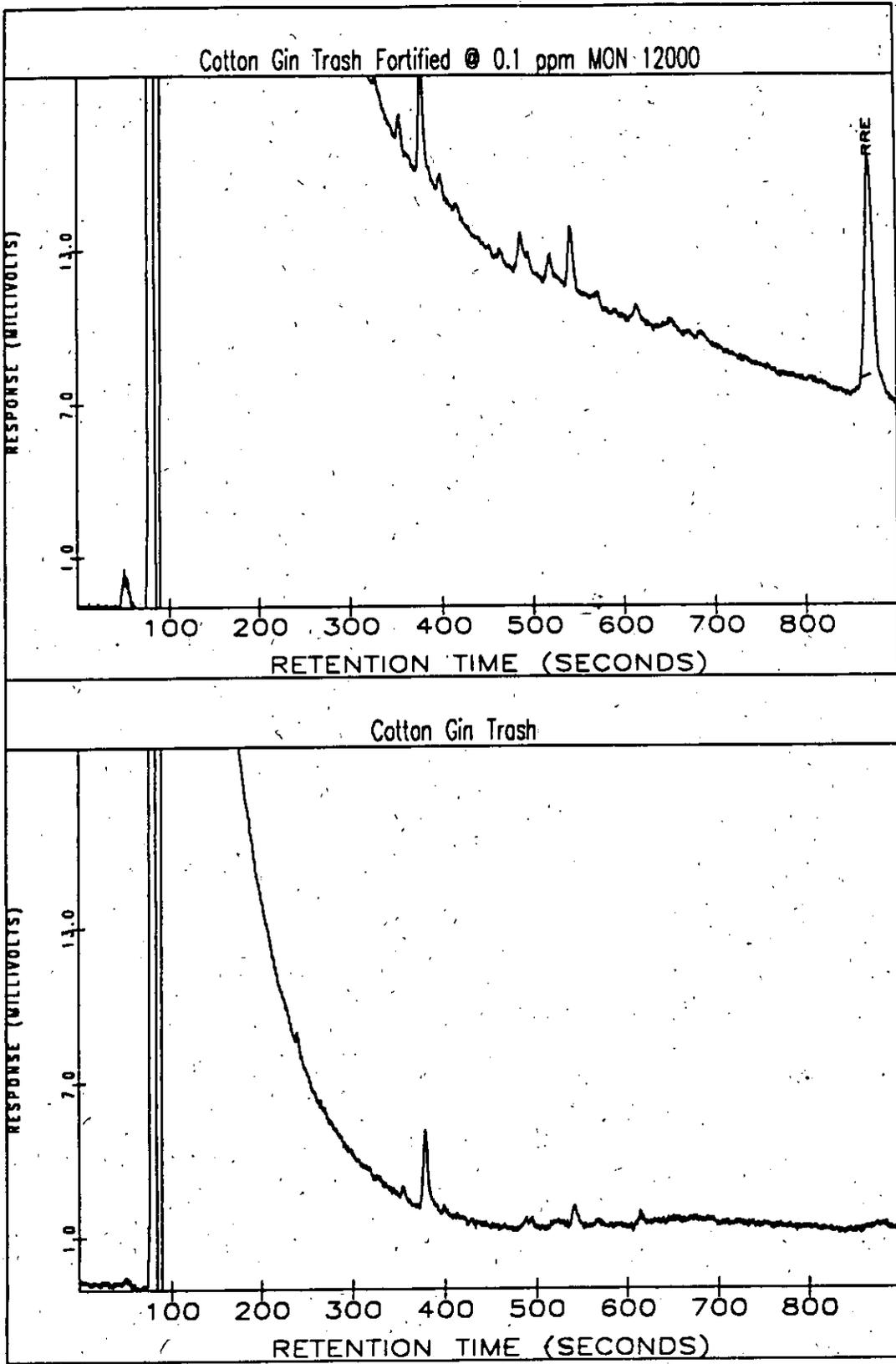


Figure 1g: Sample Chromatograms for Cotton Gin Trash

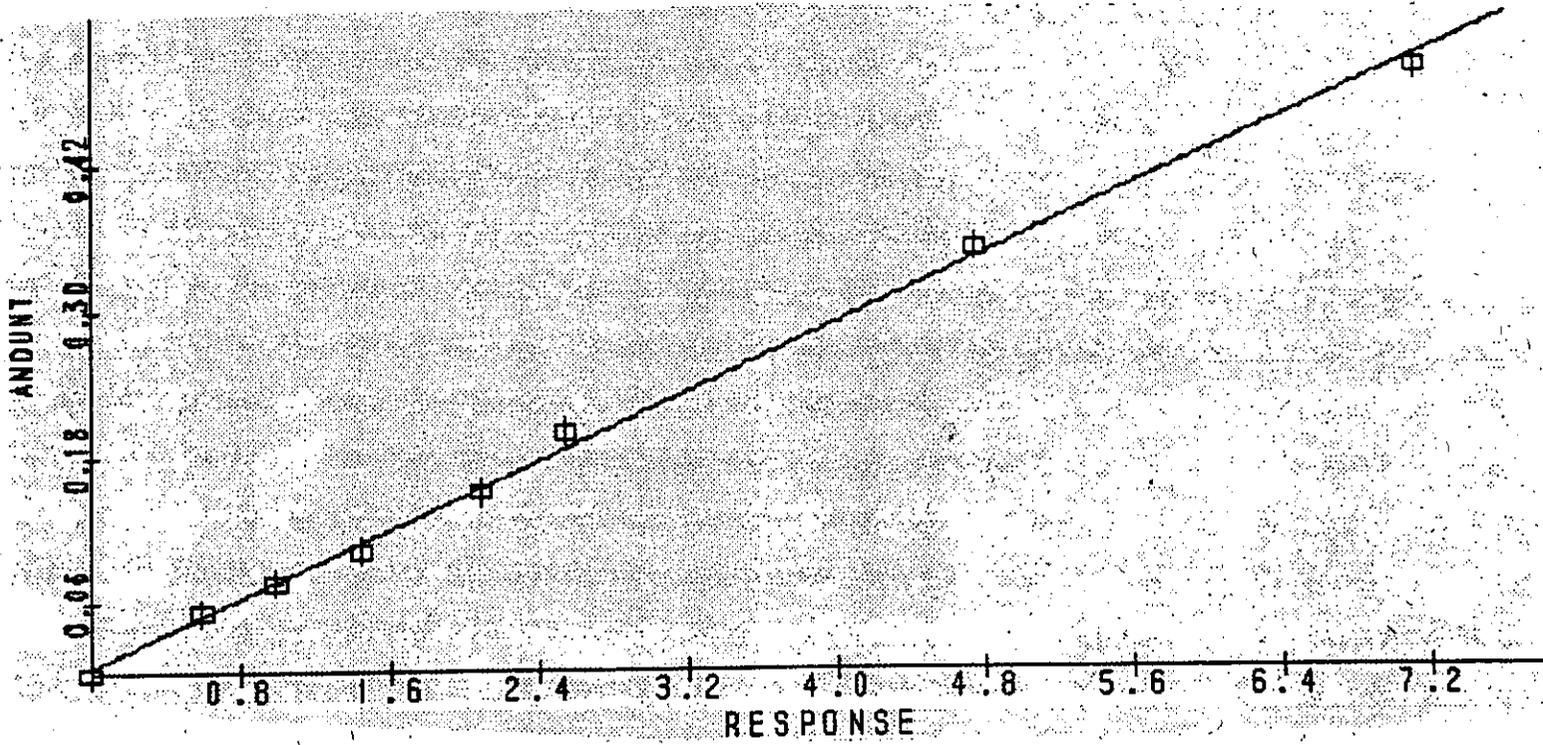


Figure 2a: Rearrangement Ester Calibration Curve

EPA ADDENDUM

"Analytical Method for the Determination of MON 12000 in Raw Agricultural Commodities and Processed Fractions",  
by Jean Culkin.

- 1) ACB used a Tekmar Tissumizer Mark II instead of a blender for all commodities except the almond nutmeat, without difficulties and good recoveries. The almond nutmeat though was ground too fine and would not filter when a Tissumizer was used. A blender gave good results for this commodity.
- 2) ACB used a Lab-line Environ-shaker, model # 3528-5cc, at 200 rpm for the shaker step for oily matrices using 500 ml boiling flasks, and also for the stirring step for conversion to RRE, with very satisfactory results.
- 3) ACB used a Hewlett Packard 5890 gas chromatograph with a Nitrogen Phosphorus Detector (GC/NPD) in place of GC/TSD with operating conditions as follows.  
Column: J&W DB-5, 30 m x 0.53 mm x 1.5 um  
Oven Program: 195°C(2 min.), 10°C/min to 280°C(hold 10 min.)  
Injector temp: 250°C  
Detector temp: 300°C  
Air flow: 60 ml/min, constant flow  
Hydrogen flow: 3.0 ml/min.  
Carrier flow: 6.0 ml/min, constant flow  
Injection: 3 ul, splitless
- 4) Method users should be aware of the following minor changes to the method that need to be made.
  - A) Method section III.C.1- the method calls for weighing 0.0150 g, ACB calculates this number to be 0.0151 g to make a 200 ug/ml solution as described.
  - B) Method section III.C.2- There is a typo in the third line, which should be corrected to read "The final solution contains 1000 ug/ml of Mon 12000."

- C) Method section III. D.3.2- There is a typo in the method which should be corrected to read "Dissolve the residue from Step 2.8 ....."
- D) Method section III.D.4 - There should be a note to discard aqueous layer after step 4.9.