VALENT U.S.A. CORPORATION
VALENT DUBLIN LABORATORY
DUBLIN, CALIFORNIA

THE DETERMINATION OF CLETHODIM RESIDUES IN CROPS, CHICKEN AND BEEF TISSUES, MILK, AND EGGS
METHOD: RM-26B-3

DATE: AUGUST 23, 1990
REVISION DATE: JANUARY 20, 1994

INTRODUCTION

This analytical method measures total residues of clethodim as two common moieties in plant and animal tissues by gas chromatography. The method cannot distinguish between clethodim and sethoxydim containing the 2-cyclohexen-1-one moiety.

Briefly, the method involves extraction with methanol and/or water, followed by cleanup with alkaline precipitation and acidic back extraction into dichloromethane, alkaline hydrogen peroxide oxidation converting sulfides and sulfoxides to sulfones and cleavage to dicarboxylic acids, derivatization to dimethyl esters, partitioning into dichloromethane and measurement of the pentanedioic acid dimethyl esters (DME sulfone and DME-OH sulfone) by gas chromatography using a flame photometric detector in the sulfur mode. Quantitation is made from a standard curve, and the total residue is expressed as clethodim equivalents.

This method modifies Chevron Chemical Company method RM-26B-2, dated August 23, 1990, to update measurement parameters, calculation procedures, and to modify the optional silica gel column cleanup procedure.

REAGENTS

Acetic Acid - Glacial, ACS reagent grade.

Acetone - Pesticide Quality

Barium Hydroxide - Prepare fresh each day. Add 10 g barium hydroxide octahydrate to 1 L deionized water. Heat with stirring until solid is dissolved. Gravity-filter solution through a 10-cm filter funnel lined with Whatman 2V filter paper while solution is still hot. Solution should be clear.

Calcium Hydroxide - Powder, reagent grade.

Catalase - Suspension from bovine liver (Boehringer Mannheim Biochemicals Cat. No. 106828). Analyze each lot of catalase for activity (65,000 U/mg for complete reduction of hydrogen peroxide) by manufacturer's procedure.

Celite 545 - Suitable for pesticide analysis.

Dichloromethane - Pesticide Quality

Hexane - Pesticide Quality

Hydrochloric Acid - Concentrated, ACS reagent grade.

Hydrogen Peroxide - Reagent grade, 30% stabilized solution. (Concentration of the hydrogen peroxide must be no less than 29% to ensure complete oxidation.)

Methyl Alcohol - Pesticide Quality

Potassium Metabisulfite or Pyrosulfite - ACS reagent grade, certified.

Silica Gel - Chromatographic grade, E. Merck, A. G. Darmstadt (Germany), 70-230 mesh, or equivalent.

Sodium Bicarbonate Solution - Saturated: Add 50 g sodium bicarbonate to 500 mL deionized water and stir vigorously for 15 minutes.

Sodium Chloride - Certified ACS grade.

Sodium Hydroxide Solution - 2 N: Dilute 10 mL 50% solution to 100 mL with deionized water or prepare from pellets.

Sodium Sulfate - Anhydrous, granular.

Water - Deionized

Clethodim [MW = 360] or clethodim sulfoxide [MW = 376](referred to as CS or CSO, respectively, reference standard used for fortification:
Prepare 50 or 10 μg/mL (or other concentration) acetone solution by diluting 1 mg/mL acetone stock solution. Check diluted solutions at regular intervals to verify stability.

5-Hydroxy clethodim sulfoxide [MW = 392] or 5-hydroxy clethodim sulfone [MW = 408](referred to as 5-OH CSO or 5-OH CSO_2, respectively) reference standard used for fortification:
Prepare 50 or 10 μg/mL (or other concentration) acetone solutions by diluting 1 mg/mL acetone stock solutions. Check diluted solutions at regular intervals to verify stability.

S-Methyl clethodim sulfoxide [MW = 362] or S-Methyl clethodim sulfone [MW = 378](referred to as S-Me CSO or S-Me CSO_2, respectively) reference standard used for fortification:
Prepare 50 or 10 μg/mL (or other concentration) acetone solutions by diluting 1 mg/mL acetone stock solutions. Check diluted solutions at regular intervals to verify stability.

DME sulfone [MW = 294](referred to as DME) reference standard, quantitation analyte: Prepare 1.0 mg/mL acetone stock solution.

DME-OH sulfone [MW = 310](referred to as DME-OH) reference standard, quantitation analyte: Prepare 1.0 mg/mL acetone stock solution.

S-methyl-DME sulfone [MW = 280](referred to as S-Me DME) reference standard, milk quantitation analyte: Prepare 1.0 mg/mL acetone stock solution.

GC standards: Using the 1.0 mg/mL stock solutions of DME, DME-OH and S-Me DME, prepare GC standards containing all three at 1.0, 2.5, 5.0, and 10.0 ug/mL acetone. For crops, the GC standard will contain only DME and DME-OH at 1.0, 2.5, 5.0, and 10.0 μg/mL in acetone because the S-Me chemical is not a plant metabolite.

EQUIPMENT

Büchner Funnels.

Filter Funnels.

Filter paper - Whatman No. 1, 2V or 4.

Glass wool - Pyrex (Dow Corning).

Heating Mantles - Suitable for 1 L round-bottomed flasks.

Hobart Food Chopper and meat grinder or equivalent.

Liquid Chromatography Columns - 400 x 25 mm i.d. with Teflon stopcocks.

Magnetic Stirrers.

Oxidation Glassware - See Figure 1.

pH Meter.

Potassium Iodide-starch Test Paper or Ether-peroxide Test Paper - Scientific Products, or equivalent.

Powerstats

Reflux Condensers - 500 mm jacket, with 24/40 ℱ inner and outer ground glass joints.

Rotary Evaporators fitted with water bath capable of being heated to 80°C.

Round-bottom Flasks - 500 mL and 1000 mL capacity with 24/40 ℱ ground glass joint.

Suction Flasks.

Ultrasonic bath.

Waring Blenders or equivalent.

Wiley Mill.

Gas Chromatograph, Hewlett-Packard model 5890 (or equivalent) equipped with a FPD in the sulfur mode, an autosampler and the following parameters (given only for reference since adjustments may be necessary to obtain optimum response):

A. Column: 0.53 mm I.D. x 10 m fused silica coated with 50% phenyl (HP-17 series 530 μ column), 2 μm film thickness

Flow Rates:

Carrier gas (Nitrogen)	-	21 mL/min
Make-up gas (Nitrogen)	-	19 mL/min
Air	-	100 mL/min
Hydrogen	-	70 mL/min

Injector Temperature:	250°C	
Detector Temperature:	250°C	
Column Oven Temperature:	Initial -	200°C, hold 3 min
	Rate -	10°C/min
	Final -	220°C, hold 2 min.

Retention Times - 4.8 min (DME) [See Figure 1]
5.4 min (DME-OH)
4.3 min (S-methyl-DME)

B. Column: 15 m x 0.53 mm I.D. methyl silicone (3 μm film thickness) capillary column (DB-1, J&W Scientific)

Injection Mode: Splitless (purge on 0.5 min)

Flow Rates:

Carrier gas (Nitrogen)	-	10 mL/min
Make-up gas (Nitrogen)	-	38 mL/min
Air	-	100 mL/min
Hydrogen	-	77 mL/min

Injector Temperature:	250°C	
Detector Temperature:	250°C	
Column Oven Temperature:	Initial -	180°C, hold 1 min
	Rate -	15°C/min
	Final -	230°C, hold 3 min.

Retention Times - 4.6 min (DME) [See Figure 2]
4.9 min (DME-OH)
4.1 min (S-methyl-DME)

Note: In all steps requiring reconstitution of residues in solvents after evaporation, use of sonication to assist in the reconstitution is strongly recommended.

ANALYTICAL PROCEDURE

SAMPLING

(Sample weights will vary according to crop type and desired method limit of detection. Evaporation water bath temperatures are approximations.)

Oily crops (i.e., Cottonseed): Weigh 20 gm of cottonseed macerate into blender vessel. Proceed to EXTRACTION.

Dry crops (i.e., Soybean seed, forage, hay, meal, hulls): Weigh 10 gm of macerated or ground crop into blender vessel. Proceed to EXTRACTION.

Oils: Weigh 50 gm sample of well mixed sample into beaker. (For recovery purposes, fortify a control sample with an aliquot of a solution of clethodim and/or clethodim metabolites. See Note 2 at end of method.) Quantitatively transfer the oil from the beaker into a 250-mL separatory funnel. Rinse beaker with 50 mL hexane and transfer to separatory funnel. Mix well. Partition hexane and oil mixture with six 100-mL portions of acetonitrile. Drain acetonitrile layer (Note: acetonitrile is normally the lower layer but with some oil types, the phases are reversed; check layer with the addition of a drop of hexane) into a clean 1-liter round bottom flask. Evaporate the combined acetonitrile extracts to dryness on a vacuum rotary evaporator using a 50°C water bath. Add 100 mL methyl alcohol and 200 mL distilled water to residue. Mix well to ensure that any residue adhering to flask is dissolved. Use of an ultrasonic cleaner is recommended to assist in dissolving the residues. Proceed to PRECIPITATION CLEANUP.

Soapstock: Weigh 5 gm of a well mixed sample into blender vessel. Proceed to EXTRACTION.

Beef and Chicken tissues: Cut tissue into small pieces. Grind sample to fine consistency with meat grinder. Mix manually and weigh 25 grams (tissues) for extraction. (For recovery purposes, fortify a control sample with an aliquot of a solution of clethodim and/or clethodim metabolites. See Note 2 at end of method.) Add 300 mL methanol and blend for 5 min. Add 20 g Celite 545 and blend for 2 sec. Vacuum filter the extract into a filter flask through a Büchner funnel containing a 1-cm layer of Celite on Whatman #1 or #4 filter paper. Rinse pad with 2 x 50 mL methanol, transfer extract and washes to a 1-liter roundbottom flask and rotovap to dryness in 50 - 55° water bath. Add 100 mL methanol and 200 mL distilled water to dissolve residue. Proceed to PRECIPITATION CLEANUP.

Milk and eggs: Mix sample thoroughly to obtain homogeneous sample. Weigh 50 grams into container for extraction. (For recovery purposes, fortify a control sample with an aliquot of a solution of clethodim and/or clethodim metabolites. See Note 2 at end of method.) Add 500 mL methanol and blend for 5 min. Add 20 g of Celite and blend for 2 sec. Vacuum filter the extract into a 1 L filter flask through a Büchner funnel containing a 1-cm layer of Celite on Whatman #1 or #4 filter paper. Rinse pad with 2 x 50 mL methanol, transfer extract and washes to a 1-liter roundbottom flask and evaporate to dryness using a 50 - 55° water bath. Add 100 mL methanol and 200 mL distilled water to dissolve residue. Proceed to PRECIPITATION CLEANUP.

EXTRACTION

(For recovery purposes, fortify a control sample with an aliquot of a solution of clethodim and/or clethodim metabolites. See Note 2 at end of method.) Add 100 mL water and soak sample for 1 hour. Add 300 mL (100 mL for soapstock only) methanol and blend for 5 minutes. During the last 30 seconds of blending add 5 g Celite to the extract. Vacuum filter the extract into a filter flask through a Büchner funnel containing a 1-cm layer of Celite on a Whatman #1 or #4 filter paper. Rinse pad with 25 mL methanol twice. Transfer the extract to a 1-liter roundbottom flask and evaporate to approximately 180 mL volume using a rotary evaporator and a 30° water bath. Adjust volume to 200 mL with methanol in a 250 mL graduated cylinder, return to the 1-liter roundbottom flask and add 100 mL deionized water. (Extract composition should be approximately 2 parts water to one part methanol.) Proceed to PRECIPITATION CLEANUP.

PRECIPITATION CLEANUP

Add 1 gm (2 gm for soapstock and oil samples) of calcium hydroxide per 10 gram sample to extract in the 1-liter roundbottom flask. Mix well and let stand for 30 minutes. Vacuum filter through a Büchner funnel containing Whatman #1 filter paper with a 1-cm layer of Celite into a 1-liter filter flask. Rinse pad twice with 25 mL of 2:1 water:methyl alcohol. Combine filtrates. Proceed to PARTITION, then follow remaining steps sequentially as listed.

PARTITION

Acidify solution with 5 mL concentrated hydrochloric acid. Add sufficient sodium chloride to saturate (ca 100 gm) the solutions. Mix vigorously for 15 - 30 minutes using a magnetic stirrer. Transfer sample to a 1-liter separatory funnel. Rinse the filter flask with 100 mL dichloromethane and decant rinse into separatory funnel. Shake separatory funnel for one minute and let layers separate. Drain organic (lower) layer into a 1-liter roundbottom flask. Partition the aqueous layer with three additional 100 mL portions of dichloromethane, collecting and combining each organic layer into the round-bottom flask. Discard the aqueous layer. Evaporate off the dichloromethane using a rotary evaporator and a 50°C water bath. Some water may remain in the flask.

OXIDATION

(See Figure 1 for oxidation set-up)

Add 100 mL 1% aqueous barium hydroxide solution (freshly prepared and filtered before use) and a magnetic stirring bar to the roundbottom flask from partitioning step. Place flask in heating mantle, which is on top of a magnetic stirrer. Attach flask to reflux condenser and begin stirring sample. Turn on power to powerstat controlling heating mantle. Powerstat is set at mid-point range for medium heating. Once the sample begins to reflux, immediately begin the slow addition of 10 mL 30% hydrogen peroxide through Bantam-Ware separatory funnel. Let mixture reflux for 10 minutes. Add another 10 mL 30% hydrogen peroxide to mixture via the attached separatory funnel and reflux for an additional 15 minutes. Allow mixture to cool to room temperature before removing reflux condenser. An ice bath may be used for cooling the sample.

EXCESS HYDROGEN PEROXIDE REMOVAL

Loosen residue on flask using an ultrasonic bath. Add 1 mL concentrated HCl to flask and mix. Use 2 N sodium hydroxide and 2 N hydrochloric acid to adjust the solution to approximately pH 7. Add 50 μL catalase suspension. Caution: possible oxygen evolution. After oxygen has evolved, add potassium pyrosulfite crystals until pH of 4.0-4.5 is obtained. Test with potassium iodide-starch indicator paper to determine if oxidant has been completely destroyed (Blue color indicates remaining oxidant). If oxidant is still present, repeat adjustment to pH 7, catalase addition and pH 4-4.5 adjustment.

Add 5 mL glacial acetic acid. Evaporate sample to dryness on a 70° C water bath. Proceed to methylation step.

METHYLATION

Dissolve residue in solution by adding 90 mL anhydrous methanol and mixing well; loosen any residue with aid of ultrasonic bath. Add 10 mL of concentrated hydrochloric acid. Place flask in heating mantle, attach flask to reflux condenser, begin stirring sample and apply heat to heating mantles. Reflux for 30 minutes.

Cool mixture to room temperature before removing reflux condenser. Carefully add 150 mL saturated sodium bicarbonate solution to contents of round-bottom flask. (Sample will bubble from evolving carbon dioxide gas during addition of sodium bicarbonate.) Check pH with pH paper to ensure that solution is neutral or weakly alkaline. If pH is not $\geq$ 7, add saturated bicarbonate solution until pH is $\geq$ 7. Transfer contents of flask to 500 or 1000 mL separatory funnel.

Partition with two 100-mL portions of dichloromethane, filtering the lower dichloromethane layer through sodium sulfate (pre-washed with dichloromethane prior to use) in a 10-cm funnel plugged with glass wool into a 500-mL roundbottom flask. Rinse filter pad with another 50 mL dichloromethane, combining rinse with filtrate in flask. Evaporate to dryness using a rotary evaporator and a 50°C water bath.

Transfer sample to a 50-mL roundbottom flask with three 5-mL portions of acetone and evaporate to dryness using a rotary evaporator and a 50°C water bath. Redissolve residue in 1.0 mL or 2.0 mL acetone, depending on the desired limit of detection. Proceed to MEASUREMENT. If, upon initial GC analysis, the sample causes major sensitivity loss such that the limit of detection cannot be reached, the sample may be evaporated to dryness using a rotary evaporator and a 50°C water bath and redissolved in hexane (this procedure may be necessary for fat samples). If GC analysis shows matrix peaks which interfere with measurement, an aliquot of the sample extract should be evaporated and the SILICA GEL COLUMN CLEANUP step should be utilized after the METHYLATION step.

MEASUREMENT

NOTE: Frequent replacement of the silanized glass wool and injector liner is highly recommended to assure optimum sensitivity.

Transfer the solutions to be measured to autosampler vials (use of vial inserts are recommended to conserve sample for possible further cleanup or dilution) for use on the automatic liquid sampler. Load the sample tray in a specified order, such as the following: conditioning shot, conditioning shot, standard, standard, fortified sample, control sample, standard, sample, sample, standard,..... Set the syringe to deliver a specified aliquot up to 3 µL. The standard vials contain reference standards containing 10.0, 5.0, 2.5 or 1.0 µg/mL concentrations each of DME and DME-OH. The standards are interspersed throughout the run. Dilute sample with acetone if peaks are not within the range of the standard concentrations used for the standard curve.

SILICA GEL COLUMN CLEANUP (Optional - See Note 1)

(This cleanup step is provided in the event additional cleanup is necessary.)

NOTE: The activity of silica gel is found to vary from batch to batch and must be checked by conducting recoveries of DME and DME-OH sulfone standards through this cleanup step.

Prepare a silica gel column as follows: Place a glass wool plug at the bottom of a 400 x 25 mm i.d. glass chromatographic column equipped with a Teflon stopcock. Add 50 mL 15% (v/v) acetone in hexane followed by 10 g silica gel. Tap the column gently to release air bubbles from the silica gel. Rinse the sides of the column with the mixed solvent and allow the solvent to drain to the top of column packing.

Remove an aliquot of the sample extract and evaporate to dryness. Using a total of 20 mL 15% (v/v) acetone in hexane, quantitatively transfer the sample from the flask (methylation step) onto the column. Allow the solvent to drain to the top of the column. Rinse the flask with an additional 100 mL 15% acetone in hexane. Transfer the rinsate to the column and allow to drain to the top of the column. Elute the DME and DME-OH with 200 mL methanol-acetone-hexane (5+10+85, v/v/v) collecting the eluate in a round bottom flask. (Note: The methanol-acetone-hexane mixture must be clear. If not, remix.) Evaporate the eluate to near dryness using a rotary evaporator.

Transfer the sample to a 50-mL round bottom flask with three 5-mL portions of acetone and evaporate to dryness using a rotary evaporator and a ≤50°C water bath. Redissolve the residue in the same volume of acetone aliquotted for cleanup. Proceed to MEASUREMENT.

CONCENTRATION DETERMINATION

Generate a standard curve correlating the concentration of the standards with their respective measured average peak units.

A computerized data regression program in LOTUS 123 Version 2.0 or 2.2 is used to predict the concentration (y) from the peak units (x) by the equation $y=ax^b$, where a is the intercept or constant and b is the slope or x coefficient, and using natural logarithms for y and x.

PPM CALCULATION

After the concentration of each extract is determined, the results for the samples are calculated in clethodim equivalents by the following formula:

$$ppm = \frac{\text{conc. DME} \times \text{vol.} \times 1.22}{\text{wt. of sample}} \quad \text{or} \quad \frac{\text{conc. DME-OH} \times \text{vol.} \times 1.16}{\text{wt. of sample}}$$

$$\text{or} \quad \frac{\text{conc. S-methyl-DME} \times \text{vol.} \times 1.29}{\text{wt. of sample}}$$

where vol. = total volume, including dilution factors, if any,
conc. = µg/mL calculated from calibration curve.
1.22 = factor to convert DME to clethodim units.
1.16 = factor to convert DME-OH to clethodim units.
1.29 = factor to convert S-methyl-DME to clethodim units.

PERCENT RECOVERY CALCULATION

For clethodim fortified samples, the percent recovery is calculated by the following formula:

$$\% \text{ Recovery} = \frac{\text{ppm (cleth) found in Fortified - ppm (cleth) in Control}}{\text{ppm clethodim fortified}} \times 100$$

If clethodim metabolites are used for fortification purposes, the factor used to determine recoveries is calculated by dividing the molecular weight of the metabolite by the molecular weight of the corresponding analyte. For example, 1.32 is the factor to convert DME-OH to 5-OH clethodim sulfone and 1.28 is the factor to convert DME to clethodim sulfoxide. To calculate the recovery of 5-OH clethodim sulfone, the calculation would be:

$$\% \text{ Recovery} = \frac{\text{ppm DME-OH fd in fortified - ppm DME-OH in control}}{\text{ppm 5-OH clethodim sulfone fortified}} \times \frac{408}{360} \times 100$$

LIMIT OF DETECTION

The limit of detection (LOD) is determined by the following calculation:

$$\text{LOD (in ppm)} = \frac{1\ \mu g/mL \times \text{final volume of untreated sample}}{\text{sample weight}}$$

Note 1. The Silica Gel Column Cleanup is optional and is normally not required for routine analysis.

Note 2. In this method, the oxidation reaction converts sulfides and sulfoxides to sulfones and cleaves the cyclohexene-1-one ring to 3-alkyl and 3-alkyl-3-hydroxy substituted pentanedioic acids. Thus, imine and oxazole clethodim metabolites, along with clethodim and clethodim sulfoxide, are converted to DME while 5-OH clethodim sulfone and corresponding 5-hydroxy metabolites are converted to DME-OH and the S-methyl metabolites are converted to S-methyl DME.

Although clethodim or clethodim sulfoxide, 5-hydroxy clethodim sulfone and S-methyl clethodim sulfoxide are described in the procedure as fortifying standards when conducting method recovery efficiencies, this method measures all the aforementioned clethodim and clethodim metabolites as the three common DME-sulfone moieties.

J. C. LAI 2-18-94

Reviewed by: 2-18-94

Figure 1

Oxidation Glassware

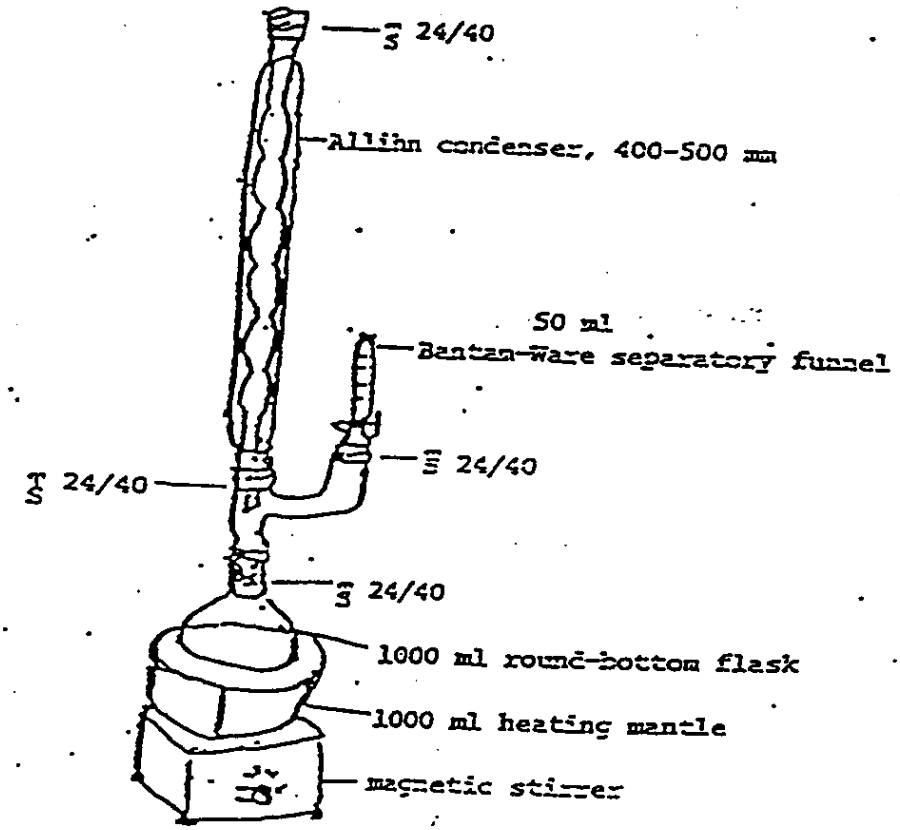
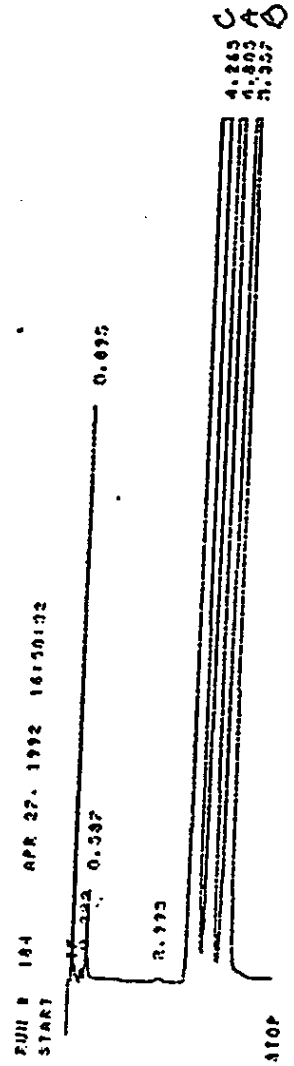
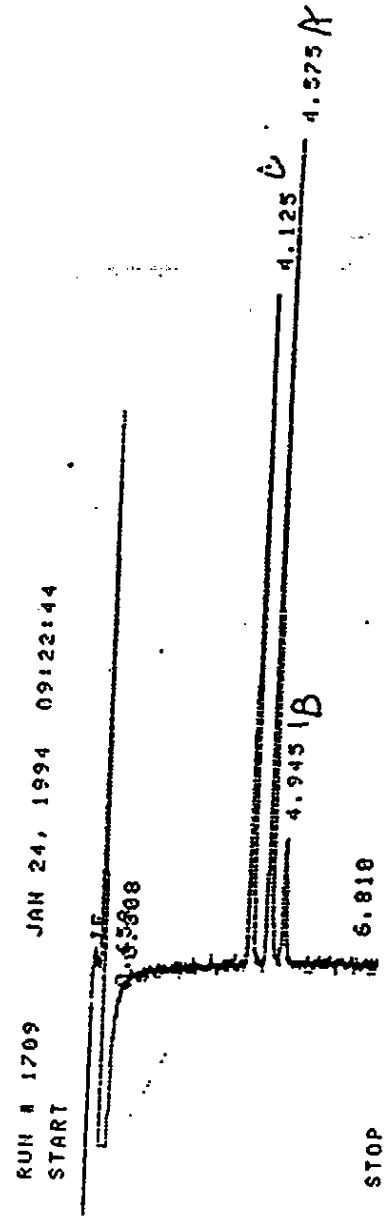


Figure 2
Reference Standards



20 ng DME = A
20 ng DME-OH = B
ca 20 ng S-Methyl-DME = C

Figure 3
Reference Standards



5 ng DME = A
5 ng DME-OH = B
ca 5 ng S-Methyl-DME = C

PROCEDURE SCHEME