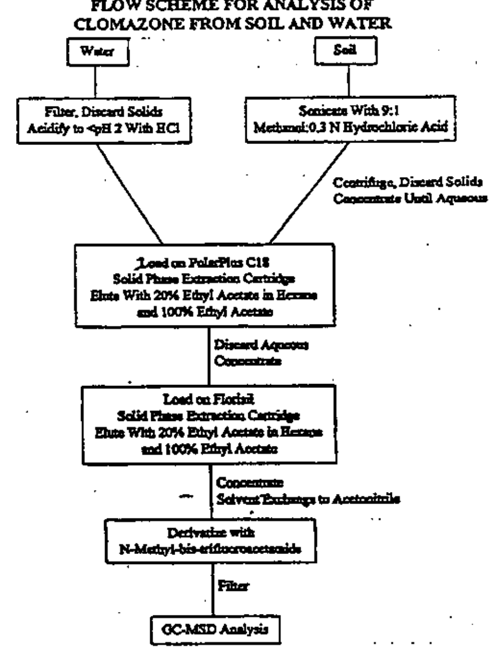
B. Method Flow Scheme

FIGURE 1

FLOW SCHEME FOR ANALYSIS OF CLOMAZONE FROM SOIL AND WATER



IV. MATERIALS

A. Analytical Standards

The chemical names, CAS numbers, structures, and purities of the analytical standards are listed in Section XI, Table 2. Individual stock solutions of approximately 1000 µg/mL of clomazone, FMC 55657, and FMC 65317 were prepared by dissolving 10 mg of the analytical standard in 10 mL of acetonitrile using a 10 mL volumetric flask. Mixed fortification solutions containing approximately 10 µg/mL of each standard were prepared by mixing 100 µL aliquots of each stock solution and diluting to 10 mL with acetonitrile. Dilutions of the 10 µg/mL fortification solutions were prepared in acetonitrile to produce individual calibration solutions ranging from 0.05 to 1.50 µg/mL. All standard solutions were stored at approximately -20°C when not in use. A summary of the analytical standard solutions prepared is shown in Section XI, Table 3.

B. Equipment

Balance, Model AE200, Mettler.
Balance, Model PM2000, Mettler.
Centrifuge, Centra-7, IEC.
Centrifuge Tubes, 50 mL, Glass.
Centrifuge Tubes, 50 mL, Polypropylene.
Flasks, Erhlenmeyer, Normal and Side-arm, 2 L
Filters, Gelman, A/E Glass Fiber
Funnels, Buchner.
Graduated Cylinders, various
Pasteur Pipets, disposable
Pipettors -100-1000 µL, EDP, Rainin (electronic air displacement).
250 µL, Microman, Gilson (manual positive displacement).
25 µL, Microman, Gilson (manual positive displacement).
Solid Phase Extraction Cartridges (C18), 2 g, 6 mL, PolarPlus™, Baker.
Solid Phase Extraction Cartridges (Florisil), 1 g, 6 mL, Baker Analyzed.
Solid Phase Extraction Reducing Adapter, Supelco.
Solid Phase Extraction Vacuum Manifold, Supelco.
Solid Phase Extraction Visidry Drying Attachment, Supelco.
Turbovap, Model II, Zymark
Ultrasonic Bath, Model 2200, Branson.
Vacuum Pump, Model 1400, Sargent Welch.
Vortexer, Genie, Vortexer II, VWR.
Volumetric Flasks, Class A (various sizes).

C. Reagents

Anhydrous Sodium Sulfate, Mallenkrodt
Deionized, distilled water (DDW), Milli-Q Plus water system, Millipore.
Hydrochloric Acid, Baker Analyzed, Reagent Grade, 37.4%.
Acetonitrile, High Purity, Burdick and Jackson.
Ethyl Acetate, Baker Analyzed.
Hexane, High Purity, Burdick and Jackson.
Methanol, High Purity, Burdick and Jackson.
N-Methyl-bis-trifluoroacetamide, Derivatization Grade, Aldrich.

D. Reagent Solutions

0.3 N Hydrochloric Acid Solution: Add 75 mL of concentrated hydrochloric acid to 2925 mL of deionized distilled water.

9:1 Methanol: 0.3 N Hydrochloric Acid: Dilute 200 mL 0.3 N hydrochloric acid to 2 L with methanol.

20% ethyl acetate in hexane: dilute 200 mL ethyl acetate to 1 L with hexane in a 1 L volumetric flask.

V. ANALYTICAL PROCEDURE

A. Initial Soil Extraction and Water Preparation.

For Soil:

1. Air dry the soil and homogenize using a suitable device.
2. Weigh five grams of soil and place it into 50-mL polypropylene centrifuge tubes. Fortify the required control samples with various volumes of the fortification solution using pipettors. Allow the tubes to remain uncovered for approximately 20 minutes to evaporate the fortification solvent. Add 40 mL 9:1 methanol:0.3 N HCl to each sample. Vortex the tubes until the soil is suspended and then sonicate for 20 minutes. Centrifuge for five minutes at approximately 3000 rpm and then decant the supernatants into 250-mL 1-mL endpoint Turbovap tubes. Repeat the extraction three additional times (collecting the supernatant for each extraction in the same Turbovap tube).

3. Bring each extract up to a total volume of 200 mL by adding ca. 40 mL of 0.3 N HCl. Concentrate each sample to 50 mL on a Turbovap. Bring each sample up to a final volume of 100 mL with 0.3 N HCl.

For Water:

1. Measure 250 mL aliquots of water using a 500-mL graduated cylinder. Fortify the required control water aliquots with various volumes of the fortification solution using pipettors. Filter each water aliquot through Gelman glass fiber filters using a Buchner funnel to remove any soil sediment. Add 6 mL of concentrated HCl to adjust the water aliquot to ~0.3 N. Check the pH of each aliquot with pH paper and adjust to less than 2 with concentrated HCl, if necessary.

B. Solid Phase Extraction

1. Assemble the SPE vacuum manifold with 2 gram, 6 mL PolarPlus™ C18 cartridges. Condition each cartridge with 24 mL methanol, followed by 24 mL 0.3 N HCl, discarding the waste. Do not allow the cartridges to dry before loading samples.

2 Using a Pasteur pipet, transfer portions of each sample to its corresponding cartridge. Connect each cartridge to a sample using a SPE reducing adapter and a length of Teflon® tubing. Raise the sample slightly above the manifold to create a siphon effect.

3. Apply the samples to the columns at a flow rate of approximately 5 mL/minute using vacuum. The vacuum should be set at <10" Hg. Once all of the sample has been applied, rinse each Turbovap tube with 10 mL 0.3 N HCl and apply the rinsate to the cartridges. Rinse each cartridge with 10 mL deionized distilled water to remove some of the acid. All of the eluate may be discarded.

4. When the solution has completely passed through the SPE cartridge, remove the tubing and add the nitrogen drying attachment. Dry the columns under a strong nitrogen flow for at least 30 minutes at ambient temperature. Remove any remaining droplets adhering to the sides of the cartridge with a cotton swab or clean tissue. It is crucial that the water is removed or problems will occur during subsequent steps.

5. Remove the drying attachment. Rinse the columns with 12 mL hexane, discarding the rinse.

6. Elute each column under vacuum at a flow rate of approximately 1 mL/minute into a 50 mL graduated centrifuge tube with 24 mL 20% ethyl acetate in hexane followed by 24 mL 100% ethyl acetate.

7. Concentrate the samples to 0.5-1 mL on a Turbovap; do not allow the sample volume to drop below 0.5 mL. Remove and discard any aqueous layer. Bring the sample volume up to 10 mL with hexane and mix. Reconcentrate to 2-3 mL. Bring the final volume up to 5 mL with hexane.

8. Assemble the SPE vacuum manifold with 1 gram florisil SPE columns. Add anhydrous sodium sulfate to a height of approximately 0.5 cm to each cartridge. Condition each cartridge with 12 mL of 20% ethyl acetate in hexane and then with 12 mL of hexane, discarding the waste.

9. Load the hexane samples from step 2-7 to the columns at a flow rate of 1-2 mL/minute with vacuum. Rinse each Turbovap tube with 2 mL hexane and pass the rinsate through the column.

10. Elute the columns under vacuum at a flow rate of approximately 1 mL/minute with 24 mL of 20% ethyl acetate in hexane followed by 24 mL 100% ethyl acetate.

11. Concentrate the sample to 0.5 - 1 mL using a Turbovap and tubes with 1 mL tips; do not allow the sample volume to drop below 0.5 mL. Add 5 mL of acetonitrile to each tube. Reconcentrate to 0.5 - 1 mL. Bring the final volume up to 1.0 mL with acetonitrile.

C. Derivatization

1. Add 100 µL MBTFA (N-methyl-bis-trifluoroacetamide) to each Turbovap tube. Using a disposable pipette, transfer each solution from B-11 to a 2-mL screw-capped glass vial.

2. To derivatize calibration solutions, aliquot 1 mL of each calibration solution into an empty 2 mL screw-cap glass vial. Add 100 µL MBTFA to each calibration solution. (Note: Calculate the concentration of the calibration solutions based on a 1.1 mL volume)

3. Incubate the vials for 30 minutes in a 70°C oven (a GC oven works fine for this). Allow the vials to cool to room temperature before opening.

4. Filter each derivatized solution through an Anopore® 0.2 µm, 10 mm syringe filter into GC vials for analysis.

D. Dilution

1. If after analysis any of the sample concentrations are outside the range of the calibration curve (or if a fortified sample is calculated to have an extract concentration outside the calibration range), dilute with ACN until the concentration falls within the range.

E. Analysis

1. Instrumentation:
   Gas Chromatograph, Hewlett-Packard, Model 5890 Series II.
   Mass Selective Detector, Hewlett-Packard, Model 5971A.
   Personal Computer, Hewlett-Packard ChemStation (DOS series)
   Software operating on a Hewlett-Packard Vectra Personal Computer Model QS/120(386/25)
   Column, capillary gas chromatography, DB-5, 15 m x 0.25 mm i.d., 0.25 µm film thickness, J&W, Supelco.

2. Analyze the extracts using the instrument operating conditions listed in Section XIII, Appendix A. Typically the autosampler is loaded with vials containing a solvent blank (acetonitrile), a complete set of calibration standards, and the sample solutions. Program the data system to inject calibration standards at the beginning of the analysis sequence and interspersed throughout the run between sample solutions. Another complete set of calibration solutions can also be analyzed at the end of the analysis sequence if desired.

F. Calculations

Calculate the equations for the least squares linear regression curves from the peak area response versus known concentrations of the calibration standards. The actual concentration of each analyte in the samples is determined from the linear regression curve.

The concentration of each analyte in a given sample is calculated as follows:

$$\text{Analyte concentration (ppb)} = \left(\frac{A-b}{m}\right) \times \frac{V_f \times D}{W} \times 1000$$

where:

- A = sample peak area for the analyte
- m = slope of the calibration curve for the analyte
- b = intercept of the calibration curve for analyte
- V_f = final extract volume (1.1 mL)
- D = dilution factor (if applicable)
- W = sample weight (soil) or volume (water)

Analyte concentration in water sample fortified with clomazone at 6.25 ppb.

$$4.88\ ppb = \frac{(17931 - 176)}{20003} \times \frac{(1.1 \times 2)}{400} \times 1000$$

$$Method\ Recovery\ \% = \frac{Concentration\ obtained}{Nominal\ Concentration} \times 1000$$

$$78.1\% = \frac{4.88\ ppb}{6.25\ ppb} \times 1000$$

G. Time Required for Analysis

One person can extract and prepare twelve water samples for analysis in one 8-hour work day and twelve soil samples in two 8-hour work days.

H. Modification or Potential Problems

1. Loading the 2-gram SPE cartridges can be difficult due to the cartridge's smaller solvent reservoirs. It is important that the C18 bed not go dry. The flow rate can be controlled both with vacuum pressure and siphon rate (raising or lowering the sample above the cartridge).

2. After loading the C18 SPE cartridge, it must be thoroughly dried to remove all traces of water before elution. No water can be present when the hexane eluate from the C18 cartridge is loaded onto the florisil SPE cartridge or the analytes will not be adequately retained on the cartridge. If the concentrated C18 eluant contains two layers or phases, this indicates that the

cartridges were not sufficiently dried before elution. This solution can be successfully dried by pipetting off most of the water and then adding anhydrous sodium sulphate.

3. Lower recoveries may result during the nitrogen concentration steps if the extracts are allowed to evaporate to less than 0.5 mL.

4. Other gas chromatographic columns also provide acceptable chromatographic performance. If the column performance deteriorates rapidly, adding a 1 m pre-column with a polar stationary phase (stabilwax) should provide more reproducible chromatography.

5. The purpose of step A-3 is to remove the MeOH before SPE. Sample preparation time might be reduced if the acidic methanol extracts are concentrated as they are produced instead of waiting for all four extracts to be combined. If this procedure is used, the 40 mL of 0.3 N HCl should be added to the final extract volume before MeOH is evaporated.

VI. METHOD VALIDATION

A. Experiment Design

Control samples of soil and water were fortified by adding known amounts of clomazone, FMC 55657, and FMC 65317 to each matrix. The analytical method was practiced at approximately 50, 250, and 500 ppb in soil and at 0.63, 3.13, and 6.25 ppb in water. A fortification solution containing clomazone, FMC 55657, and FMC 65317 was prepared and the solution was added using calibrated positive displacement pipettors onto an accurately weighed or measured aliquot of matrix. For each matrix an analysis set consisted of one reagent blank, one control sample, and three laboratory fortified control samples for each fortification level.

B. Test System

The test systems were comprised of soil (0-6" depth) and water from the control plot of trial number 03 located in Proctor, Arkansas. The trial site was representative of key rice growing regions in the southern United States. Soil cores from the control plot were shipped via Federal Express from Mid-South Ag Research, Inc. and received on May 20, 1995. The soil samples were dried, homogenized and stored frozen. The test systems chosen for these experiments is representative of the systems on which this method will be used.

1. Sample Preparation

Control soil was air dried and then homogenized using a mill. Because the water samples to which this method is applicable were taken from the standing irrigation water in flooded rice fields, a simulated standing water sample was prepared by mixing ~100 g of control soil and 2 L of water in an Erhlenmyer flask. The flask was shaken by hand for several minutes and then centrifuged to settle the soil. Aliquots of the "dirty" water were used for method validation.

C. Reference Substances

The reference substances for this study were clomazone, FMC 55657, and FMC 65317. Reference substances were received from FMC Agricultural Products Group (FMC APG), Princeton, NJ on April 25, 1995. The reference substances were stored at approximately -10°C. The chemical names, CAS numbers, structures, purities and reference numbers are listed in Section XI, Table 2. Documentation of the stability, solubility, as well as chemical and physical characterization of the reference substances are maintained by FMC APG.

1. Preparation of Standards

Stock solutions of approximately 1000 µg/mL of clomazone, FMC 55657, and FMC 65317 were prepared individually by dissolving 10 mg of the analytical standard in 10 mL acetonitrile using a 10 mL volumetric flask. A fortification solution of approximately 10 µg/mL was prepared by mixing 100 µL aliquots of each stock solution and diluting to 10 mL with acetonitrile. Dilutions of the 10 µg/mL fortification solution were prepared in acetonitrile to produce individual calibration solutions of 0.05, 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50 µg/mL. The stock and standard solution were stored at ≤-10°C when not in use. Information on the reference solutions is in Section XI, Table 3.

D. Calculations

1. Evaluation of Accuracy and Precision

The method accuracy was assessed by measuring the recovery obtained for the fortified control soil and water samples. Recovery was calculated by dividing the obtained value of the fortified sample by the fortified expected value and multiplying that ratio by 100. The method was considered valid if the average recoveries were between 70% and 120%.

The method precision was assessed by measuring the standard deviation (SD) and the relative standard deviation (RSD) of the values for the fortified samples in each analytical run. The method was considered valid if the RSDs were ≤20% at each fortification level for each matrix, and ≤15% RSD for all fortifications for each matrix.

2. Evaluation of Standard Curve Linearity

A least squares linear regression analysis was performed comparing the concentration of the analytes in the calibration standards to the chromatographic response (peak area). The y-intercept, slope, and correlation coefficient for the regression analysis were determined. The standard curve linearity was evaluated by monitoring the slopes and the correlation coefficients for each calibration. The analyses were considered valid if the correlation coefficients were ≥0.95 for the curves. For sample sets with more than one curve, the slopes should be similar.

E. Interferences

Blank control soil or water were analyzed for the presence of interfering peaks at the retention time of clomazone, FMC 55657, and FMC 65317. Small interference peaks, representing approximately 10% of LOQ, were observed in the target ion chromatograms for each compound. These interference peaks did not contain the qualifier peaks in the correct ratios, if at all, indicating that they were not the analytes of interest. To determine accurate fortified sample recoveries, the peak areas of the interference peaks were subtracted from the analyte peak areas in fortified sample extract chromatograms.

F. Confirmatory Techniques

Mass spectrometry in the selected ion mode served as the confirmatory technique. Five ions were monitored. Three of the ions are fragments characteristic of an individual analyte (m/z 204 for clomazone, m/z 176 for FMC 55657, and m/z 302 for derivatized FMC 65317). The other two ions (m/z 125 and 127) are present in all three analytes and if these ions co-maximize with the characteristic ion and have the correct relative intensities, they can confirm the presence of a particular analyte.

G. Radio-Validation

The method was further evaluated by using the method to analyze an aged sediment sample which had been dosed with ^{14}C-labeled clomazone as part of an aerobic aquatic metabolism study. The data obtained were compared to the data obtained in the metabolism study (Section XIII, Appendix C).

XI. TABLES

TABLE 2

TEST AND REFERENCE SUBSTANCES

Common Name	Chemical Name/Structure	CAS Number	FMC Reference Number	Purity
Clomazone	2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone	81777-89-1	E6788:76	99.7%
FMC 55657	N-[(2-chlorophenyl)methyl]-2-methyl-propanamide	NA	C9698:133	95.0%
FMC 65317	N-[(2-chlorophenyl)methyl]-3-hydroxy-2,2-dimethyl propanamide	NA	E2028:104	99.0%

TABLE 3

REFERENCE SOLUTIONS

Name	Analyte(s)	Solution Solvent	Nominal Concentration (ng/µL)	Validation Solution ID #*
Stock	Clomazone	Acetonitrile	1000	9508-43-01
Stock	FMC 55657	Acetonitrile	1000	9508-43-03
Stock	FMC 65317	Acetonitrile	1000	9508-43-02
Fortification Solution	Clomazone, FMC 55657, and FMC 65317	Acetonitrile	10	9508-43-04
Calibration Solutions	Clomazone, FMC 55657, and FMC 65317	Acetonitrile	0.05	9508-44-07
			0.25	9508-44-06
			0.50	9508-44-05
			0.75	9508-44-04
			1.00	9508-44-03
			1.25	9508-44-02
			1.50	9508-44-01

* Prepared on 1/17/96 and stored at approximately -20°C when not in use.