I. Introduction

An analytical procedure (enforcement residue method) was developed and used for residue determination of MON 4660 (parent only) in soil. The procedures are specific for analysis of the safener and are separate from the determination of the herbicide acetochlor.

Soil samples are extracted with acetonitrile on a mechanical shaker, filtered and evaporated to dryness. The residue is passed through a 6 mL disposable Florisil® column and analyzed by GC-ECD. The methods were validated on corn matrices and soil taken from a field not treated with MON 4660. Analytical recoveries were determined from samples fortified with known amounts of MON 4660.

The lower limit of method validation (LMV) and lowest fortification level on soil is 0.01 ppm of MON 4660. No residue values are reported below the LMV.

II. Materials/Methods

The following materials, equipment and reagents are required to perform the analysis. Appropriate substitution for certain items is left to the discretion of the analyst. Glassware and other equipment should be cleaned in a manner which avoids contamination of future samples.

A. Equipment

Explosion proof blender, Fisher No. 14-509-33

Blender jars: Fisher No. 14-509-11A

8 oz. French square Bottles: Northwestern Bottle Co., St. Louis, MO

Polyethylene-lined caps for 8 oz. French square Bottles: Northwestern Bottle Co.

Mettler balance, Model PC-440 or equivalent

Mettler balance, Model AE163 or equivalent

85 mm Buchner funnel: Fisher No. 10-356C

7 cm glass fiber filter: Fisher No. 9-873F

Splash guard adapters 24/40 joints; 250 mL size: Aldrich No. Z-14, 779-6

Vacuum filtration adapter 24/40 joint; 38 mm i.d. Aldrich Z-11, 563-0.

Calab rotary evaporator

250 mL separatory funnel: Fisher No. 10-437-10E

Kraft shaker: Model S-500

500 mL Round bottom flask: Fisher No. 10-060-2E

250 mL Round bottom flask: Fisher No. 10-060-2C

100 mL Round bottom flask: Fisher No. 10-060-2B

10 mL Graduated centrifuge tubes: Fisher No. K45201-10

5 mL Graduated centrifuge tubes: Fisher No. 10-437-10C

Pasteur pipette 9" length: Fisher No. 13-678-6B

Pasteur pipette 5 3/4" length: Fisher No. 13-678-6A

Serological pipettes from 0.1 through 5 mL: Fisher No. 13-676-28A, B, C, D, E, F

Volumetric Pipets from 1.0 through 10 mL: Fisher No.13-650B

Bottle-top dispenser 50 mL: Fisher No. 13-688-7

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

Vacuum manifold: P. J. Cobert No. 944011

Glass Wool: Fisher No. 11-388

Glass chromatographic column: 1.2 x 22 cm (1.0 cm ID), topped with a 3 x 10 cm reservoir and fitted with a Teflon stopcock with a 1.5 cm delivery tip.

Varian 3700 or VISTA 6000 gas chromatograph equipped with a ^{63}Ni electron capture detector and automatic sampler

Fisher Recordall Series 5000 strip chart recorder

1.8 mL Autosampler vials with Teflon lined resealable septa and phenolic caps: Varian No. 96-000095-00

GLC Column: J & W fused silica capillary column (Durabond); Liquid phase: DB-5; (Non-extractable bonded phase); Film thickness, 0.25 µM; column dimensions, 30 M x 0.32 mm

Tracor Mark III (Model 6881) Liquid Scintillation Counter (LSC) manufactured by TM Analytic, Inc.

Assorted standard laboratory equipment

B. Reagents and Standards

Solutions which must be prepared for use should be made in sufficient quantity to perform a complete analysis. If additional reagents must be prepared during an analysis, differences in reagent composition may result in background variations in the chromatography.

Acetonitrile: Fisher No. A-998.

Deionized water from a Milli-Q-water purification system (Millipore Co.). This system consists of an activated carbon cartridge for the removal of organics in series with two mixed-bed ion exchange cartridges for the removal of ionic species.

2,2,4-Trimethylpentane (iso-Octane): Fisher No. O296-4

Ethyl Acetate: Fisher No. E195-4

Sodium Sulfate (Na_2SO_4) anhydrous: Fisher No. S-421

Florisil 60-100 mesh (chromatographic grade), Fisher No. F-100

Disposable Florisil columns (1000 mg) J.T. Baker Chemical Co., No 7213-07

Carbon, Decolorizing: Fisher No. C17C

20% (v/v) Water/Acetonitrile: Using graduated cylinder, add 400 mL of distilled deionized water to 1600 mL of acetonitrile and mix thoroughly

5% Sodium Sulfate Solution: Weigh 50 g of sodium sulfate anhydrous into 950 mL of distilled deionized water and mix thoroughly

2% Ethyl Acetate/Iso-octane (v/v): Measure 392[illegible] mL iso-octane into a suitable container (empty solvent jug), add 80 mL ethyl acetate, shake well to insure complete dissolution.

5% Ethyl Acetate/Iso-octane (v/v): Measure 380[illegible] mL iso-octane into a suitable container and add 20[illegible] mL of ethyl acetate. Shake well.

10% Ethyl Acetate/Iso-octane (v/v): Measure 36[illegible] mL iso-octane into a suitable container and add 40[illegible] mL of ethyl acetate. Shake well.

5% Deactivated Florisil: Weigh 285 grams of Florisil into a container and pipette 15 mL of distilled deionized water onto the Florisil. Place the container on a mechanical shaker for 30 minutes.

5% Deactivated Florisil/2% Decolorizing Charcoal Weigh 294 g of the 5% deactivated Florisil into a bottle and add 6 g of decolorizing charcoal to the Florisil. Place the mixture on a mechanical shaker for 30 minutes. Rotate the bottle and shake mixture for an additional 15 minutes.

1-oxa-4-azaspiro[4.5]decane-4-(dichloroacetyl) acetamide: synthesized by Starks Associates, Inc., Lot #MS2-61-1, MON 4660, purity >99%.

The purity of MON 4660 was verified by elemental analyses and GC/MS. Results of the analyses and MS data are found in Appendix A.

Isotopically labeled ^{14}C-MON 4660 was prepared by B.V. Mischke (Monsanto Co.; NBP 3711311). The ^{14}C - labeled material was determined to have a specific activity of 9.40 mCi/mM after HPLC purification. The radiochemical purity was 98.[illegible]% based on GC and HPLC analysis. The chemical purity of this material was also determined to be >99% by GC and HPLC analysis. The carbon isotopic label was at the C-2' position.

Fortification Spiking Solutions:

Weigh to four significant figures 0.25[illegible] gram of analytical grade MON 4660 into a 100 mL volumetric

flask and dilute to volume with 20% water/acetonitrile, mix well to insure complete dissolution. This solution contains 2500 µg/mL of MON 4660.

Pipet 10 µL of the 2500 µg/mL solution into a 100 mL volumetric flask, dilute to volume with 20% water/acetonitrile and mix well. This solution contains 0.25 µg/mL of MON 4660.

Pipet 10 µL of the 2500 µg/mL solution into a 100 mL volumetric flask, dilute to volume with 20% water/acetonitrile and mix well. This standard contains a 2.5 µg/mL of MON 4660.

Pipet 1000 µL of the 2500 µg/mL solution into a 100 mL volumetric flask and dilute to volume with 20% water/acetonitrile. This solution contains 25.0 µg/mL of MON 4660.

Store all standards refrigerated in amber bottles.

Detector Calibration Standards:

Weigh to four significant figures 0.2500 gram of MON 4660 into a 100 mL volumetric flask and dilute to volume with 2% ethyl acetate/iso-octane and mix well. This solution contains 2500 µg/mL of MON 4660.

Pipet 1000 µL of the 2500 µg/mL solution into a 100 mL volumetric flask, dilute to volume with 2% ethyl acetate/iso-octane and mix well. This solution contains 25 µg/mL of MON 4660.

Prepare the GC calibration standards in 25 mL volumetric flasks according to the following scheme:

Volume of 2500 µg/mL MON 4660 Standard	Standard Dilution	Final Concentration
25 µL	25 mL	2.5 µg/mL

Volume of 25 µg/mL MON 4660 Standard	Standard Dilution	Final Concentration
7.5 µL	25 mL	0.0075 µg/mL
12.5 µL	25 mL	0.0125 µg/mL
25 µL	25 mL	0.025 µg/mL
40 µL	25 mL	0.04 µg/mL
50 µL	25 mL	0.05 µg/mL
75 µL	25 mL	0.075 µg/mL
100 µL	25 mL	0.10 µg/mL
250 µL	25 mL	0.25 µg/mL
750 µL	25 mL	0.75 µg/mL

Each of the calibration standards is diluted to the 25 mL final volume with 10% ethyl acetate/iso-octane.

Standard solutions are stored in properly cleaned and labeled amber glass bottles and stored refrigerated. Extreme care should be taken to prevent cross contamination of the lower standards with the higher concentration standards.

C. Analytical Procedure for Soil

1. Sample Preparation for Soil

Soil samples are taken in the field with a soil probe which uses a plastic liner to hold the soil core in place. The soil sample in the plastic liner is properly labelled and frozen immediately for shipment to the Residue section at Monsanto. The frozen soil core is then cut into the appropriate depth segments. Replicate soil samples from the same plot at a given sampling time are combined and mixed thoroughly to obtain a homogeneous sample. After mixing, the samples are stored frozen until analyzed.

2. Extraction Efficiency

Extraction efficiency and recovery experiments with different solvents were carried out. Check soil samples were spiked with MON 4660 and extracted. The treated soil samples were taken 1 week after chemical treatment with a mixture of acetochlor/^{14}C labeled MON 4660. Carbon-14 radioactivity level present in the soil was determined by combustion analyses before extraction.

3. Extraction

Weigh 25.0 ± 0.04 grams of the previously prepared soil sample into an 8 oz french square glass bottle. Fortify appropriately at this stage. Example: Pipet 0.5 mL of the 0.5 µg/mL MON 4660 standard in 20% water/acetonitrile directly onto the sample matrix for a 1.0 ppm fortification. Add approximately 15 grams of Na_2SO_4 and 100 mL of acetonitrile to the bottle. Cap the bottle tightly and shake on a reciprocating shaker for 15 minutes.

Prepare an 8.5 cm Buchner funnel with 7.0 cm glass fiber filter paper (Whatman 934-AH) and rinse with about 25 mL of acetonitrile under vacuum. Filter the extract through the Buchner funnel directly into a 250 mL round bottom flask by means of an adapter suitable for applying vacuum to the system. Rinse the bottle and contents of the funnel with small portions of acetonitrile. Collect the rinses in the same round bottom flask.

Evaporate the extract to dryness using a rotary film evaporator with the flask immersed in a room temperature water bath. Raise the water bath temperature gradually to about 40°C.

Place approximately 0.5 to 1.0 gram of Na_2SO_4 and 3 mL of 2% ethyl acetate in iso-octane (v/v) into the flask and swirl the flask to dissolve the analyte in the solvent system.

4. Clean-Up

Place 6 mL (1000 mg) Baker disposable Florisil columns in a vacuum manifold. Pre-wash the columns with 10 mL of 10% ethyl acetate in iso-octane followed by 10 mL iso-octane. Quantitatively transfer the contents of the round bottom flask to the pre-washed Florisil column. Allow the 3 mL to drip through the columns. Apply additional 2 x 3 mL 2% ethyl acetate in iso-octane flask rinses, allowing each rinse to clear the head of column before the next is applied. Discard all eluates collected to this point. Place a 10 mL graduated centrifuge tube inside the vacuum manifold beneath the column.

Quantitatively rinse the round bottom flask with 3 mL of 10% ethyl acetate/iso-octane and transfer the rinse to the column. After the rinse has completely entered the column bed, apply an additional 2 x 3 mL 10% ethyl acetate/iso-octane.

Remove sample from the vacuum manifold and dilute to 10 mL with iso-octane and mix well. The samples are ready for GC separation and subsequent quantitation.

5. Soil Moisture Determination

The calculation of the concentration of MON 4660 is made with respect to the dry soil mass analyzed, therefore, the percent moisture of each sample must be determined.

Procedure:

Weigh a glass container, such as a 150 mL beaker, and record this weight to the nearest hundredth of a gram. Next, weigh out an aliquot of the soil sample to be analyzed (between 25.00 and 26.00 g) and record this weight to the nearest hundredth of a gram. Total the weight of the container plus the weight of the soil and record this weight. Place the container plus soil in a dry heat oven set at 130°C for at least 24 hours. After this period, remove the container and allow to cool.

Reweigh the container plus the dry soil and record this weight. The amount of moisture contained in the original soil sample is the difference between the combined (container & soil) weight before drying and the combined weight after drying. The percent soil moisture is determined by dividing the amount of soil moisture by the weight of wet soil before drying times 100.

This calculation is illustrated as follows:

$$\frac{\text{g of undried soil - g of dried soil}}{\text{grams of undried soil}} \times 100 = \% \text{ moisture}$$

Determine the weight of a 25.00 gram soil sample corrected for % moisture as follows:

$$\frac{25.00\ g \times (100 - \%\ moisture)}{100} = \text{dry weight of soil}$$

D. Instrumentation

1. Separation and Quantitation of MON 4660

The analyte MON 4660 in corn matrices and soil is separated and quantitated by capillary gas chromatography using the following conditions.

(Be aware that different instruments may require modification of these parameters in order to achieve satisfactory detectability and separation of MON 4660 from co-extracted species. Follow the procedures recommended by the manufacturer of the instrument regarding operation and optimization.)

Detector ^{63}Ni; Temperature 300°C

Injector Temperature: 250°C

Column Temperature Program: 150°C for 10 min. program at 10°C/min. to 230°C and hold at 230°C for 5 min.

Column: J & W DB-5 fused silica capillary column; Film thickness 0.25 micron; 30 m x 0.32 mm.

Detector range: 1

Detector Attenuation: 64

Chart Speed: 0.5 inch/min.

Injection Volume: 3 µL

Split: 10/1

Nitrogen carrier: 2.0 mL/min.

Make-up gas: 28.0 mL/min.

2. Detector Calibration

A linear calibration curve is generated for every set of samples run. Several levels of MON 4660 standards are used in the range of 0.0020 to 0.080 µg/mL. These standards are periodically placed among the analytical samples and injected into the instrument. The calibration curve is generated by plotting the peak height of the detector response against the concentration of each calibration standard of MON 4660.

Linear least squares estimates of the data points are then used to define the calibration curve.

The response of any given sample must not exceed the response of the most concentrated standard. If this occurs, dilution of the sample will be necessary.

E. Interferences

Analyses of reagents, solvents and labware should be conducted to assure a minimum contribution of interferences to actual sample analyses.

1. Sample Matrices

The analytical procedure described has been used and validated for the determination of MON 4660 in soil from the three sites examined.

2. Other Pesticides

Detailed interference studies of other herbicides and safeners have not been performed. However, in all cases untreated check samples were free from interferences. Relative retention times for parent acetochlor and alachlor were determined to not interfere with MON 4660.

3. Solvents

When using pesticide or HPLC grade solvents, no interferences have been observed.

4. Labware

Glassware used was rinsed with deionized water and acetone. When handling high levels of standards, the glassware is always discarded after use. Therefore no interferences have been observed.

F. Confirmatory Techniques

As previously discussed, experiments were performed using ^{14}C-labeled MON 4660 to determine method extraction and column recovery efficiencies. Additionally, mass spectral confirmation was performed on field samples. This data is presented in Appendix J.

G. Analysis Time

A set of 24 samples can be completed in 3 working days for corn matrices samples and 2 working days for soil samples. For 6 corn or soil samples, 1 working day is needed.

H. Problem Areas

It is necessary to determine the elution profile of MON 4660 on the purchased cleanup columns (each lot) before proceeding with the methods. This is carried out by eluting the column with 5 mL fractions and injecting each fraction into the GC.

I. Method of Calculation

The amount of MON 4660 is determined based upon external standard calibration. A non-weighted linear least squares estimate of the calibration curve is used to calculate the amount of MON 4660 in the unknowns. The response of any given sample must not exceed the response of the most concentrated standard. If this occurs, dilution of the sample will be necessary.