Cover Sheet for

Environmental Chemistry Method

Pesticide Name: Pyraclostrobin

MRID#: 451187-07

Matrix: Soil

Analysis: LC/MS

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Draft Technical Procedure: Modification Number 1

Analytical Method No. D9812/1

THE DETERMINATION OF BAS 500 F AND ITS METABOLITES, BF 500-3, BF 500-4, BF 500-5, BF 500-6 AND BF 500-7 IN SOIL USING LC-MS

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ABSTRACT

BASF Method D9812 (Reference 1) was further modified at BASF Corporation, Research Triangle Park, N.C to determine the residues of BF 500-5 in clay soils using LC-MS determination. The modified method could also be used to determine residues of BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6, and BF 500-7 in soil.

A 50 g soil sample aliquot was extracted twice with acetonitrile. The soil marc was re-extracted once with 0.1 N NaOH. The extracts in acetonitrile and in 0.1 N NaOH were collected separately. The alkaline extract was acidified to pH ~ 2 and extracted twice with ethyl acetate. The combined ethyl acetate layer was evaporated to dryness. A trace amount (0.1 mL) of triethylamine was added to the combined acetonitrile extract which was concentrated to approximately 40-50 mL and was added to the dry residue obtained after evaporation of the ethyl acetate extracts. The combined extract was then concentrated near to a volume of approximately 10 mL and was rediluted with a buffer solution (water-acetonitrile, 70:30, v/v with 0.1 % formic acid and 10 mM ammonium formate) for HPLC-MS determination.

The method has a limit of quantitation of 0.01 mg/kg in soil for each analyte.

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1. INTRODUCTION

1.1 Scope of the method

The broader scope of the modified method is that it allows the determination of BF 500-5 in clay soil with acceptable recoveries. In general, the method D9812 will be used to determine the residues of BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-5, BF 500-6, and BF 500-7 with the required limit of quantitation (0.01 ppm) in soil. If the procedural recoveries for BF 500-5 are lower than 65 % in a particular analysis set, the modified method D9812/1 should be used for the re-extraction/reanalysis.

1.2 Principle of the method

Residues of BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6,and BF 500-7 were extracted from soil by initial shaking with acetonitrile. The remaining soil marc was further extracted with 0.1N NaOH to obtain the residues of BF 500-5. The residues of BF 500-5 were then isolated from the alkaline extract into ethyl acetate by liquid-liquid partition at pH~1-2 and subsequent concentration of the ethyl acetate layer. The residues were determined by LC-MS using selected ion monitoring. A flow chart of the analytical method is provided in **Figure 1** of this section of the report. The limit of quantitation is 0.01 ppm for each analyte.

2. MATERIALS

2.1 Test and reference substances are the same as mentioned in D9812 (**Appendix C**).

2.2 **Equipments** are the same as mentioned in D9812 (**Appendix C**) except the following.

Method Step	Equipment	Size, Description	Manufacturer/ Supplier	Catalog Number
3.2.4	Pipeter, Automatic		VWR	
3.2.5	Separatory funnel	125 and 250 mL	VWR	

NOTE: Other general laboratory glassware and equipment may be needed. Equipment with equivalent performance may be used, as required.

2.3 Reagents and Chemicals -- Suggested Sources

2.3.1 Chemicals are the same as mentioned in D9812 **(Section 2.3.1; Appendix C)** except the following.

Chemical	Grade	Manufacturer/ Supplier	Catalog Number
Ethyl acetate	High Purity	B & J	100-4
Hydrochloric acid (36.5-38%)	Reagent grade	J.T. Baker	
Methanol	High Purity	B & J	230-4
Sodium chloride	99 %	E.M Science	SX0425-3
Sodium hydroxide (pellet)	ACS grade	Fisher Scientific	
Water	High Purity	B & J	365-4

NOTE: Equivalent reagents and chemicals from other suppliers may be substituted.

2.3.2 Solvent Mixtures and their Preparation

Solvent Mixtures are preapared in the same way as mentioned in Method D9812 **(Section 2.3.2; Appendix C)** except the following.

Solvent Mixtures	Method Step
0.1 N NaOH: Dissolve 4 grams of sodium hydroxide in 800 mL of water. Cool solution and dilute to 1.0 liter.	3.2.4
2 N HCl: Add slowly 20 mL of Conc. HCl (12 N) into 100 mL of distill water with stirring and mix well to ensure complete homogeneous solution.	3.2.5
Solvent I: Water with 0.1 % formic acid and 10 mM ammonium formate	3.3
Solvent II: Acetonitrile- **Solvent I**, 70:30, v/v : Add 700 mL of acetonitrile into a 1L graduated cylinder and dilute to the mark with **Solvent I** . Pour the solution to a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.	3.3

2.4 Standard Solutions:

Same as described in D9812 **(Section 2.4.1 to 2.4.3; Appendix C)**

3. ANALYTICAL PROCEDURE

3.1 Sample Preparation

Same as described in D9812 **(Section 3.1; Appendix C)**

3.2 Fortification and Extraction

3.2.1 For the fortification samples, add an appropriate volume of BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7 standard solution to the respective control samples by volumetric pipet. For example, for a 0.01 ppm fortification sample, pipet 1 mL of the 0.5 µg/mL mixed standard solution of BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7 into a control sample.

3.2.2 Add 75 mL of acetonitrile to the centrifuge bottle containing the soil and shake at 300 RPM for thirty minutes. Centrifuge at about 3500-4000 rpm for 10 minutes at 20° C. Attach a glass funnel fitted with a filter paper (Whatman #1) into a 250 mL standard taper flat-bottom flask and transfer the supernatant by decantation through the funnel and collect.

NOTE: Centrifugation must be continued until the solid residue forms a compact pellet.

3.2.3 Add another aliquot of 75 mL of acetonitrile to the soil marc. Sonicate and vortex to loosen the soil and allow to mix to consistency. Repeat the extraction step above (3.2.2) for 30 minutes. Centrifuge for 10 minutes and transfer the supernatant into the above 250 mL flat-bottom flask by decantation through the funnel. **Extract is stored in acetonitrile until the completion of Section 3.2.5. Proceed as indicated in Section 3.2.6**

3.2.4 Add 40 mL of 0.1 N NaOH to the soil marc. Sonicate and vortex to loosen the soil and allow to mix to consistency. Repeat the extraction step above (3.2.2) for 30 minutes. Centrifuge for 10 minutes and transfer the supernatant into a 125 mL flat-bottom flask by decantation through the funnel.

3.2.5 Adjust the pH of the solution about to 1-2 with 2 N HCl (4 mL) and swirl the flask gently to mix. Add sodium chloride (2gm) into the acidified water and swirl the flask to dissolve majority of the salt. Transfer the contents from the 125 mL flat bottom flask into a 125 mL separatory funnel with a glass stopper. Rinse the flask with 5 to 10 mL of distilled water and add it to the separatory funnel. Add 25 mL of ethyl acetate into the separatory funnel. Shake vigorously for 2 minutes and wait for 5 minutes for phase separation. Use a 10 mL disposable pipette with an automatic pipetter and remove all but about 5 mL of ethyl acetate (top layer) and transfer to a 125 mL flat bottom flask. **[Note: Take precaution not to transfer all the ethyl acetate layer to prevent transfer of trace amounts of water].**

3. ANALYTICAL PROCEDURE (Continued)

Repeat the above extraction step with another 25 mL aliquot of ethyl acetate and transfer the ethyl acetate layer into the above 125 mL flat bottom flask.

Evaporate the combined ethyl acetate layer to dryness using a rotary evaporator with the water bath temperature set at approximately 50°C (set vacuum initially at about 250 mbar until removal of all ethyl acetate and then gradually decrease the vacuum to about 35 to 45 mbar). Use a gentle stream of nitrogen to remove trace moisture and **proceed as indicated in Section 3.2.6.**

3.2.6 Add 0.1 mL of triethylamine using a graduated disposable pipet to the combined acetonitrile extract in 250 mL flat-bottom flask **(Section 3.2.3)** and **mix it well to obtain a homogeneous extract.** Evaporate the extract carefully to **about 50 mL** using a rotary evaporator with the water bath temperature set approximately at 40°C (set vacuum initially at about 200 mbar and then gradually reduce to 100 mbar). Swirl and sonicate the extract to dissolve the dry residue from the side of the 250 mL flat-bottom flask and transfer the extract into the above 125 mL flat-bottom flask containing the dry residue from **Section 3.2.5.** Rinse the 250 mL flat-bottom flask with 5 to 10 mL of acetonitrile for complete transfer. Evaporate the extract very carefully to about 5-8 mL using a rotary evaporator with the water bath temperature set approximately at 40°C (set vacuum initially at about 200 mbar and then gradually reduce to 100 mbar).

NOTE:

- **It is absolutely necessary to use a 125 mL flat bottom flask to avoid a large surface area and not to allow the samples to go dryness. BF 500-4 sticks to the glass upon solvent evaporation and causes low recovery. If the sample goes to dryness, do not proceed to the next step. Start over with a new soil sample.**

- **To determine how much 5-8 mL of solution represents in a 125 mL flask during rotary evaporation, it is suggested that the analyst add 8 mL of water into an empty flask prior to conducting step 3.2.6 and compare. This will give the analyst a " picture" of how much 8 mL of solution is and prevent over evaporation.**

3.2.7 Sonicate and vortex to ensure complete dissolution of residues from the side of the 125 mL flask. Transfer the extract to a 10 mL volumetric flask with a disposable glass pipet. Rinse the flask thoroughly with acetonitrile. Use about 1 mL acetonitrile twice to ensure complete transfer of the solution and then dilute to the mark with acetonitrile (if needed). Sonicate and vortex to ensure a homogeneous solution. Proceed to sample preparation for LC-MS determination.

3.3 **Preparation for LC-MS Analysis**

Same as described in D9812 **(Section 3.3; Appendix C)**

3. Analytical Procedure (Continued)

A flow chart of the analytical procedure is presented in **Figure 1**.

3.4. Moisture Determinaion

Results of soil analysis are reported on a "dry weight" basis for residue determination. Therefore soil sample weights must be corrected for moisture content by accepted methodology. Procedural recoveries will not be corrected for moisture content of the sample. An example of a moisture determination procedure will be provided in the validation report (**Section IV**; page 18).

3.5. Instrumentation: Suggested LC-MS Operating conditions

Same as described in D9812 **(Section 3.3; Appendix C)**

3.6 Calibration Procedures

Same as described in D9812 **(Section 3.6; Appendix C)**

3.7 Limit of Quantitation and Limit of Detection

The limit of quantitation is defined as the lowest fortification level successfully tested. The limit of quantitation is 0.01 ppm for BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7. The limit of detection has not been determined, but the lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

4. CALCULATION OF RESULT

Same as described in D9812 **(Section 3.6; Appendix C)**

5. TIME REQUIRED FOR ANALYSIS

The time required for a set of 8 samples (either 6 fortified and 2 controls or 5 treated samples, 2 fortified and 1 control) is approximately 12 person-hours, or 1.5 calendar day, provided that no special problems arise, such as matrix interference.

6. CONFIRMATORY TECHNIQUE

The method allows for the determination of BAS 500 F and its metabolites using LC-MS which is a highly selective and self-confirmatory detection technique. Therefore, no confirmatory technique is required.

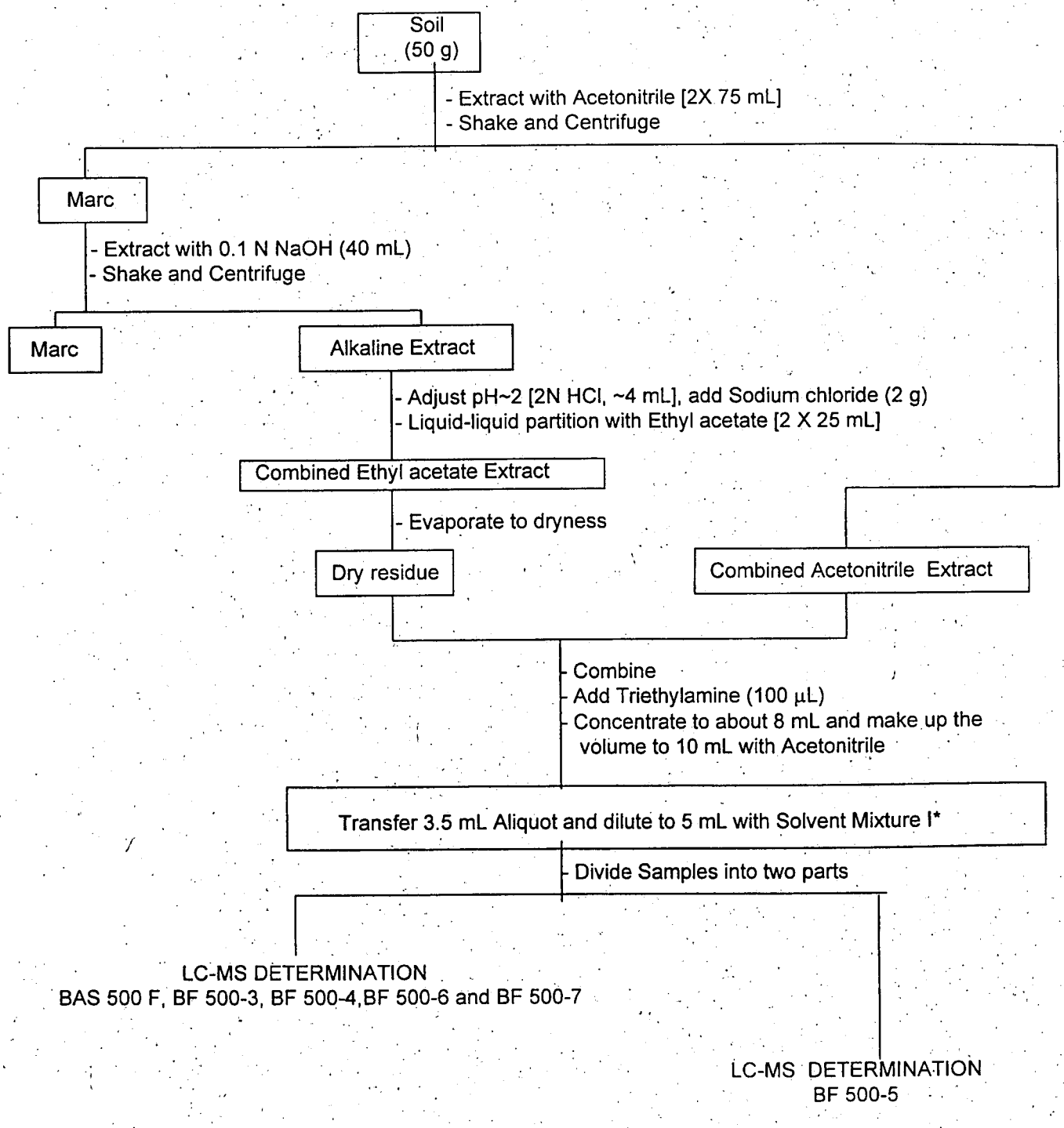
7. POTENTIAL PROBLEMS

Low recoveries for BF 500-4 were observed if the extract goes to dryness upon rotary-evaporation (steps 3.2.5 to 3.2.7). Low recoveries are always observed if samples were stored in acetonitrile for more than a week. During residue analysis, it was observed that the LC-MS system (such as ion source, peek tubing, column etc.) needs frequent cleaning with acetonitrile and with the buffer used for the gradient. Otherwise after injection of several sample sets, lower recoveries were observed for BF 500-3 and BF 500-4. Typically these routine maintenance are required in about every 1000 – 2000 injections.

8. SAFETY AND HEALTH CONSIDERATION

All procedures involving organic solvents should be performed in a well-ventilated hood. Personal protective equipment (gloves, lab coats) should would worn while performing this method. Read all label statements and precautions.

Figure 1. Flow Diagram of Method D9812/1



Solvent Mixture I: Water with 0.1 % formic acid and 4 mM ammonium formate
Solvent Mixture II: Acetonitrile-water (70:30, v/v) containing 0.1 % formic acid and 4 mM ammonium formate
*For 0.1 and 1.0 ppm level samples, dilute 1:10 and 1:100 respectively with Solvent M

APPENDIX E

METHOD MODIFICATIONS

This section of the report summarizes all of the changes made to the original method D9812

Modification No. 1:

During the validation of method D9812, low recoveries were observed for the metabolite BF 500-5 in clay soil with high water content. The existing method requird modification to obtain the acceptable recoveries (>65 %) for BF 500-5. An alternative method (D9812/1) was developed and validated to determine the residues of BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-5, BF 500-6, and BF 500-7 **(Appendix D).** The change was incorporated into the study protocol (98130) during method validation.

Modification No. 2:

The following changes were made to the method D9812 and/or D9812/1 to expedite the residue analyses (reduce run time, high sample throughput). The changes were incorporated to the analytical phase protocol (C98016) during the residue analysis **(Reference 4).**

Modification No. 2.1

Different LC-MS conditions were used to analyze the samples. LC-MS Analyses were performed using the following modification of the method (D9812) in section 3.5:

Solvent A = water with 0.1% formic acid
Solvent B = acetonitrile with 0.1% formic acid
Solvent C = water with 0.1% formic acid and 5 mM ammonium formate

Analysis of BAS 500 F, BF 500-3 and BF 500-4:
Isocratic 50 % Solvent A + 50 % Solvent B

Analysis of BF 500-6 and BF 500-7:
Isocratic 75 % Solvent B + 25 % Solvent C

Analysis of BF 500-5: Gradient

Time (min.)	Composition
0.0	50% A + 50% B
3.0	50% A + 50% B
3.1	30% A + 70% B
5.0	30% A + 70% B
5.1	50% A + 50% B
7.1	50% A + 50% B

Run every 7 minutes

Method Modifications; Modification No. 2.1 (Continued)

Analytes	BAS 500 F	BF 500-3	BF 500-4	BF 500-5	BF 500-6	BF 500-7 (A-Isomer)	BF 500-7 (B-Isomer)
Expected Retention times (min.)	11.86	10.82	7.73	3.06	5.76	5.76	7.61

Modification No. 2.2:

In order to expedite the residue analyses (reduce run time, high sample throughput) further, a different instrument (LC-MS/MS detection) was used to analyze the samples for residue analyses. There were also some minor changes in different sections of the method to transfer the chromatographic method from LC-MSD (Hewlett Packard) to LC-MS/MS (Sceix API 300/365, Perkin Elmer). A flow diagram of this modified method is provided in **Figure 1** of this section. Method modifications as well as the instrument conditions (modification of the method D9812 in section 3.5) are described below:

Section 3.2: **Extractions** were performed using the following modifications of the method D9812 in Section 3.2:

3.2.1 Same as mentioned in Method D9812.

3.2.2 Add 75 mL of acetonitrile to the centrifuge bottle containing the soil and shake at 300 RPM for thirty minutes. Centrifuge at 3000 rpm for 10 minutes at about 20° C. Attach a glass funnel fitted with a filter paper (Whatman #1) into a 100 mL volumetric flask and transfer the supernatant by decantation through the funnel and collect.

3.2.3 Add another aliquot of 75 mL of acetonitrile to the soil marc. Sonicate and vortex to loosen the soil and allow to mix to consistency. Repeat the extraction step above (3.2.2) for 30 minutes. Centrifuge at 3000 rpm for 10 minutes at about 20° C and transfer about 25 mL of the supernatant by decantation through the funnel (Note: Make sure not to add the extract to the mark at this point). Attach the same glass funnel with the filter paper (section 3.2.2) into a 50 mL volumetric flask and transfer the remainder of the supernatant by decantation through the funnel. Add acetonitrile with a disposable pipette to dilute the extract in both 50 mL and 100 mL volumetric flasks to the mark. Pour the extracts from both 50 mL and 100 mL volumetric flasks into a Qorpak bottle (8 oz, amber glass Teflon®-lined screw cap). Sonicate and vortex to obtain a homogeneous solution. Proceed for LC-MS or LC-MS/MS determination.

Method Modifications; Modification No. 2.2 (Continued)

3.2.4 – 3.2.7 Not used

Section 3.3: Different aliquot amounts of the extract in acetonitrile (Section 3.2.3) were used to determine the residues of BAS 500 F and its metabolites in soil. Analyses were conducted using two chromatographic methods in two different instruments.

LC-MS/MS determination was used to analyze BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6, and BF 500-7. LC-MS determination was used to analyze BF 500-5.

The following procedures were used to prepare the samples for analysis:

3.3.1 Sample preparation for the analysis of BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6, and BF 500-7

3.3.1.1 Transfer 7.0 mL of the extract (Section 3.2.3), measuring with a volumetric pipette, into a 10 mL volumetric flask and dilute to the mark with **solvent mixture I**. Sonicate and vortex to ensure a homogeneous solution.

3.3.1.2 **For control and 0.01 ppm fortifications**, filter the solution through a syringe filter (a 0.5 µ Fluoropore disc fitted to an 1.0 mL disposable plastic syringe). Transfer the sample solution (3.3.1.1) with a glass disposable pipette to the syringe, discard the initial 100 - 200 µL of the filtrate and collect the filtrate (about 1-2 mL) into an injection vial.

For 0.1 and 1.0 ppm fortifications, take 1 mL of the sample solution (3.3.1.1) and dilute with **solvent mixture II** to 10 mL and to 25 mL respectively. Sonicate and vortex to ensure a homogeneous solution. Filter the solution into the injection vial using the procedure above.

3.3.2 Sample preparation for the analysis of metabolites BF 500-5

3.3.2.1 Transfer 10 mL of the extract (3.2.3), measuring with a volumetric pipette, into a 50 mL glass conical centrifuge tube (VWR Cat. No.21020-695). Carefully evaporate the extract to dryness using a gentle stream of nitrogen at 50-60°C.

NOTE: It is recommended to immerse the centrifuge tube as deeply as possible to achieve faster evaporation and to avoid condensation along the side of the tube. Do not allow samples to remain dry longer than necessary. Proceed immediately to the next evaporation step which usually takes 35-40 minutes.

Method Modifications; Modification No. 2.1 (Continued)

Add 2.0 mL (or any amount depending of the sensitivity of the instrument) of **solvent mixture II,** measuring with a volumetric pipette, to the dry residue. Attach a cap (VWR Cat. No.16198-915) and sonicate and vortex to dissolve the residue from the side of the centrifuge tube to ensure a homogeneous solution.

Use the same procedure as mentioned in Section 3.3.1.2 for a sample preparation for LC-MS or LC-MS/MS detection.

Section 3.5: Instrumentation

Analysis of BAS 500 F, BF 500-3, BF 500-4, BF 500-6, and BF 500-7:
LC-MS/MS Analyses were performed using the following conditions:

Instrument:	PE Sciex API 300/365 Biomolecular Mass Analyzer
Inlet [HPLC System]:	PE Series 200 Micro Pump system with Series 200 Autosampler
Data System:	MassCrom 1.1
Column:	HP Zorbax 3.5µ, SB-C8, 30 X 2.1 mm, [P/N 873700-906]
Injection volume	10 µL
Flow Rate:	300 µL/minute
Mobile Phase:	Solvent A = Water with 0.1% formic acid and 4 mM ammonium formate Solvent B = Methanol with 0.1% formic acid and 4 mM ammonium formate
Isocratic:	20% Solvent A + 80 % Solvent B
Ionization Mode:	positive ion for all analytes; Turbo Ion Spray (Electrospray)
Turbo Temperature	300 °C (not applicable without Turbo)

Analytes	**BAS 500 F**	**BF 500-3**	**BF 500-4**	**BF 500-6**	**BF 500-7 (A-Isomer)**	**BF 500-7 (B-Isomer)**
Expected Retention times	33.8 seconds	32.8 seconds	28.7 seconds	2 minutes 03 seconds	1 minutes 44 seconds	3 minutes 37 seconds
Transitions:	388→163	358→132	300→106	611→417	595→207	595→207
Q1/Q3 Masses:	388/163±0.2	358/132±0.2	300/106±0.2	611/417±0.2	595/207±0.2	595/207±0.2

Method Modifications; Modification No. 2.1 (Continued)

Analysis of BF 500-5: LC-MS Analyses were performed using the following conditions:

Gradient	Time (min.)	Composition
	0.0	50% A + 50% B
	3.0	50% A + 50% B
	3.1	30% A + 70% B
	5.0	30% A + 70% B
	5.1	50% A + 50% B
	7.0	50% A + 50% B
		Run every 7 minutes

Expected Retention times: 3.06 minutes.

Section 4.2:
CALCULATIONS: The recoveries and residues of BAS 500 F and its metabolites in mg/g (ppm) are calculated with the following formulas:

Moisture content (ratio) = $\frac{\text{Dry Sample Weight (g)}}{\text{Wet Sample Weight (g)}}$

Residue in ppm (Dry Sample Weight) = $\frac{\text{Wet Sample Weight (ppm)}}{\text{moisture content}}$

Residue in ppm (Wet Sample Weight) = $\frac{\text{ng found per injection}}{\text{mg injected}}$

ng found per injection = Amount of analyte calculated from Standard curve

Standard curve: $\text{ng} = \frac{\text{Peak Area - intercept}}{\text{Slope}}$

[For BF500-7, calibration curve is built in Excel; total area is obtained by adding the area of A and B- isomers].

mg injected = $\frac{\text{Sample weight (g) extracted}}{\text{Fv}} \times \text{µL injected} \times \text{F1} \times \text{F2}$

For the analysis of BAS 500F, BF 500-3, BF 500-4, BF 500-6 and BF 500-7 (LC-MS/MS Detection):

Fv = Final volume (mL) of the extract in acetonitrile (section 3.2.3) = 150 mL
F1 (First dilution factor) = $\frac{\text{Aliquot (mL) taken from final extract in acetonitrile}}{\text{Dilution volume (mL)}}$ = 7/10 = 0.7 (Section 3.3.1)

Method Modifications; Modification No. 2.1 (Continued)

For the analysis of BF 500-5 (LC-MS Detection):

Fv = Final volume (mL) of the extract in acetonitrile (section 3.2.3) = 150 mL

F1 (First dilution factor) = $\frac{\text{Aliquot (mL) taken from final extract in acetonitrile}}{\text{Dilution volume (mL)}}$ = 10/2 = 5 (Section 3.3.2)

F2 (Second dilution factor): Equals 1, 0.1 and 0.02 for 0.01, 0.1 and 1.0 ppm fortification samples respectively

Percent recovery (%) = $\frac{\text{[Residue (ppm) for fortified sample - Residue (ppm) for control sample]}}{\text{Amount (ppm) fortified}}$ X 100

Typical Standard Concentrations for Standard Curve:

For the analysis of BAS 500F, BF 500-3, BF 500-4, BF 500-6 and BF 500-7 (LC-MS/MS Detection): 1.0, 2.5, 5.0 and 10.0 pg/ µL

For the analysis of BF 500-5 (LC-MS/MS Detection): 6.25,12.5 and 50.0 pg/ µL

Modification No. 2.2

The initial attempt to analyze soil samples from RCN 98087 (high clay content; **Reference 4**) using method described in **Modification No. 2.1** yielded poor recovery for BF 500-5. It was also necessary to use sodium hydroxide to extract BF 500-5 from clay soil. This extraction also brought much more matrix which was sufficient to contaminate the LC-MS/MS system and to absorb the analytes with active NH groups. This was due to heavy matrix load on to LC-MS/MS for repeated analysis. This problem was eliminated by using a separate aliquot of the acetonitrile extract for the analysis of BF 500-5 as described below (**Section 3.2.4**). A flow diagram of this modified method is shown in **Figure 2** of this section. Method modifications as well as the instrument conditions (modification of the method D9812 in **Section 3.5**) are described below:

Section 3.2: **Extractions** were performed using the following modifications of the method D9812/1 in Section 3.2:

3.2.1- 3.2.3 **Same as described in Modification No. 2.1**

3.2.4 Add 40 mL of 0.1 N NaOH to the soil marc and shake at 300 RPM for thirty minutes. Sonicate and vortex to loosen the soil and allow to mix to consistency. Centrifuge for 10

Method Modifications; Modification No. 2.2 (Continued)

minutes and transfer the supernatant into a 50 mL volumetric flask by decantation through the funnel. Add 0.1 N NaOH to dilute to the mark. Sonicate and vortex to mix well and to obtain a homogeneous solution.

Transfer 10 mL of the alkaline extract in to a 50 mL Teflon centrifuge tube (VWR, Cat. No. 21009-477) with a 10ml volumetric pipet and add 2 N HCl (1.0 mL) to adjust the pH of the solution to about 1-2. Swirl the centrifuge tube gently to mix. Add sodium chloride (5 g) and ethyl acetate (10 mL) into the acidified extract and attach a screw cap to the centrifuge tube. Vortex vigorously (about 3-5 minutes) to dissolve majority of the salt. Centrifuge at about 2000 -3000 rpm for 5 minutes at room temperature for phase separation. Use a 10 mL disposable pipette with an automatic pipetter and remove all but about 1 mL of ethyl acetate (top layer) and transfer into a 50 mL glass centrifuge tube (VWR, Cat. No. 21020-695). **[Note: Take precaution not to transfer all the ethyl acetate layer to prevent transfer of trace amounts of water]**

Repeat the above extraction step with another 10 mL aliquot of ethyl acetate and transfer the ethyl acetate layer into the above 50 mL glass centrifuge tube. Carefully evaporate the combined extract to dryness using a nitrogen evaporator at 50-60°C under mild positive flow of nitrogen. Add 5 mL of acetonitrile and sonicate and vortex to dissolve the residue from the sides and to ensure a homogeneous solution. Transfer the entire solution in to a Qorpak bottle (4 oz, amber glass Teflon®-lined screw cap) and add 30 mL of the acetonitrile extract obtained from Section 3.2.3 with a 30 ml volumetric pipet. **Sonicate for 5-10 minutes and vortex to mix well and to obtain a homogeneous solution.** Proceed for LC-MS determination of BF 500-5.

3.2.5 – 3.2.7 Not used

Section 3.3: Same as described in **Modification No. 2.1,** except for the BF 500-5 analysis.

For BF 500-5 analysis, follow Section 3.3.2 as described in **Modification No. 2.1** except transfer the aliquot from Section 3.2.4 (extract obtained after sodium hydroxide extraction) for evaporation to analyze BF 500-5.

Section 3.5: **Instrumentation:** Same as described in **Modification No. 2.1**

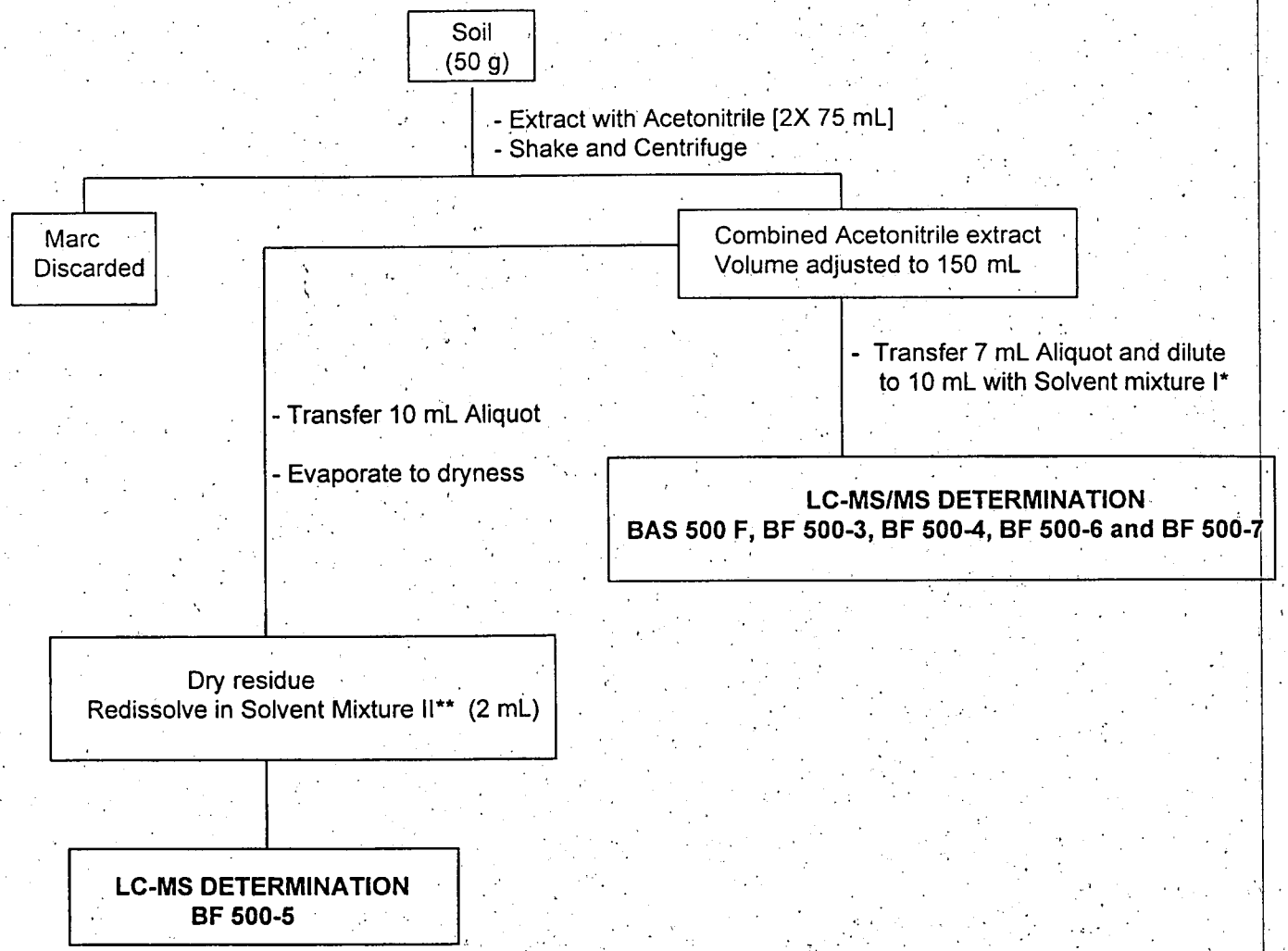
Section 4.2: Calculation: Same as described in **Modification No. 2.1, except the following:**

For the analysis of BF 500-5 (LC-MS Detection):

Fv = Final volume (mL) of the extract in acetonitrile (section 3.2.4) = 35 mL

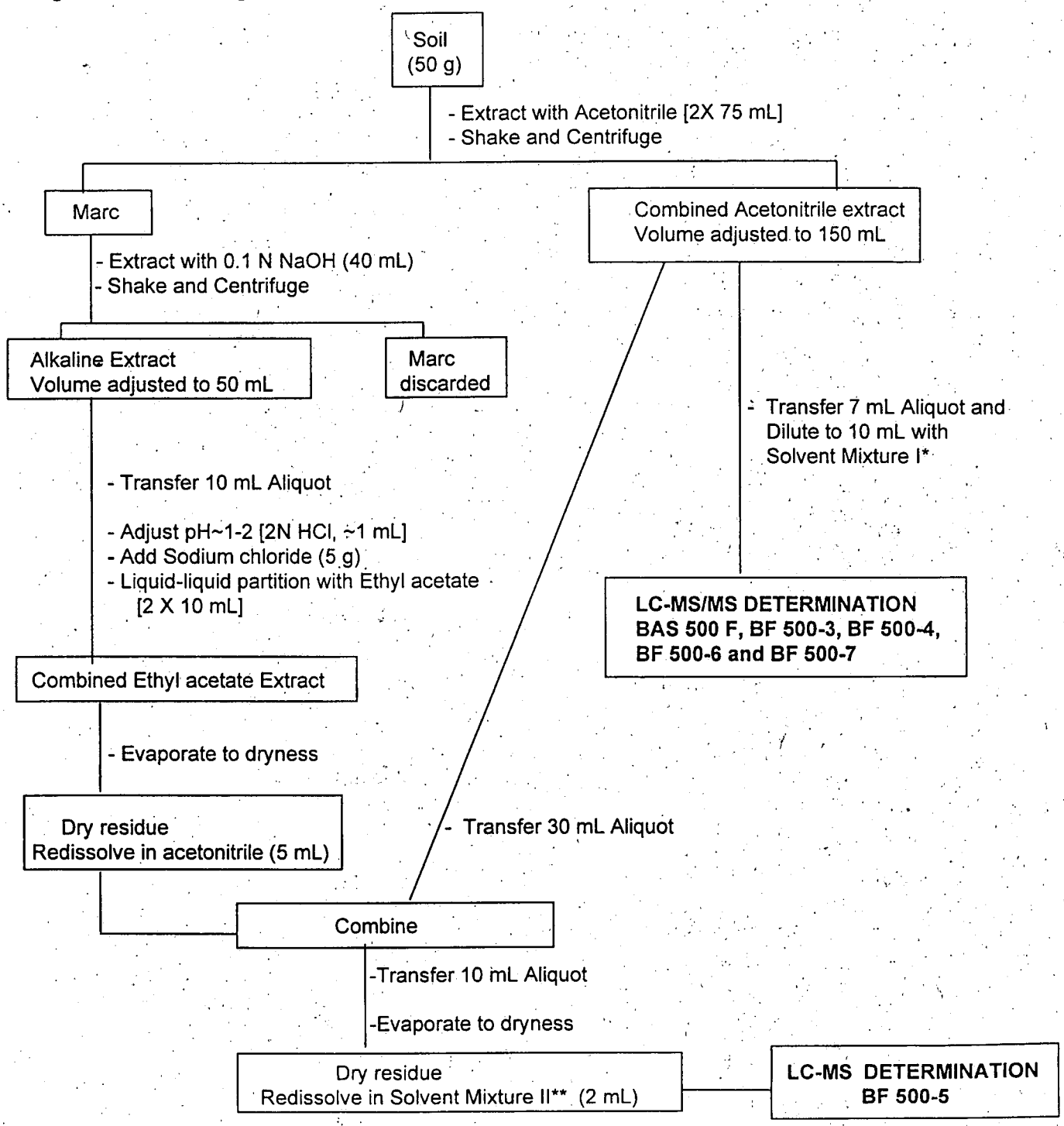
F1 (First dilution factor) = $\frac{\text{Aliquot (mL) taken from final extract in acetonitrile}}{\text{Dilution volume (mL)}}$ = 10/2 = 5 (Section 3.3.2)

Figure 1: Flow diagram of the method D9812 with modification (Modification No. 2.1)



*Solvent Mixture I: Water with 0.1 % formic acid and 4 mM ammonium formate
**Solvent Mixture II: Acetonitrile-water (70:30, v/v) containing 0.1 % formic acid and 4 mM ammonium formate

Figure 2: Flow diagram of the method D9812/1 with modification (Modification No. 2.2)



*Solvent Mixture I: Water with 0.1 % formic acid and 4 mM ammonium formate
**Solvent Mixture II: Acetonitrile-water (70:30, v/v) containing 0.1 % formic acid and 4 mM ammonium formate

Method Modifications (Continued)

Modification No. 4:

A different instrument (LC-MS/MS detection) was used during the **Independent Laboratory Validation (ILV)**. The validation was conducted using same procedure as described in method **Modification No. 2.2** except the instrumentation used for the study (Section 3.5 of **Modification No. 2.2)**

Instrument conditions as well as the parameters are described below:

Instrumentation: **Suggested LC-MS/MS Operating condition:**

Instrument:	VG/Fisons Quattro II	
Inlet (HPLC System):	Hewlett Packard Model 1100	
Data System:	VG/Fisons MassLynx v. 3.2	
Column:	Hewlett Packard Zorbax SB-C8 Rapid Resolution Cartridge, 2.1 x 30 mm, 3.5 µ, [P/N 873700-906]	
Injection Volume:	10 µL	
Mobile Phase	A = water with 4 mM ammonium formate and 0.1% formic acid B = methanol with 4 mM ammonium formate and 0.1% formic acid	
LC conditions for the analysis of BF 500-5 : **(Gradient)**	**Time (min.)**	**Composition**
	0.0	50% A + 50% B
	3.0	50% A + 50% B
	3.1	30% A + 70% B
	5.0	30% A + 70% B
	5.1	50% A + 50% B
	7.0	50% A + 50% B Run every 15 minutes
LC conditions for all other analytes: **(Isocratic)**	20% A + 80% B	Run every 7 minutes
Flow Rate: 300 µL/minute	**Ionization Mode:** Electrospray positive ion for all analytes	

Method Modifications; Modification No. 4: (Continued)

Analytes	BAS 500 F	BF 500-3	BF 500-4	BF 500-5	BF 500-6	BF 500-7	
						A-Isomer	B-Isomer
Expected Retention Times (min.)	0.52	0.52	0.45	7.58	1.66	1.40	2.85
Transitions:	388→163	358→132	300→106	195 MS Only	611→417	595→207	595→207

Modification No. 5:

During residue analysis of RCN 98243 (Study No. 98046; **Reference 6**), it was observed that more acetonitrile was required for the extraction of BAS 500 F, BF 500-5 and BF 500-4. The method used for the analyses were same as described in **Modification No. 2.1,** except 2 X 150 mL acetonitrile (Section 3.2) were used for the extraction. The soil type from RCN 98243 was Loam and with high organic mater. The modification was required only for 0-5 cm turf soil to obtain acceptable recoveries for the above analytes.

Chromatographic Conditions for BAS 500-5:

LC/MS/MS PESciex API 365, APCI
Column: Inertsil C4, 5u, 150mm X 3.0mm
Column Temperature: 30°C
Injection Volume: 20 uL
Mobile Phases: A: Water + 0.1% Formic Acid
B: ACN + 0.1% Formic Acid

Pump Timetable:

Time (min.)	%A	%B	Flow Rate (mL/min)
0.0	50	50	0.5
3.0	50	50	
3.1	30	70	
5.0	30	70	
5.1	50	50	
9.0	50	50	

Experiment Information:

Mass Range: Q1 195.1 Q3 195.1
Dwell(msec): 500.000
Pause Time: 5.000 mse
Retention Time: 3.27 min.

Summary:

In general, during the routine residue analysis, a majority of the soil samples were analyzed using the method D9812 with modification described in **Modification No. 2.1.** If the procedural recoveries for BF 500-5 were lower than 60-65 % in a particular analysis set, modified method D9812/1 with modification described in **Modification No. 2.2** were used for the re-extraction/reanalysis. Although majority of BF 500-5 analysis were conducted in LC-MS (HP LC-MSD), LC-MS/ MS (Sciex API 300/365 or VG/Fisons Quattro II) analysis could also be used as described in **Modification No. 4 and 5.** Following table summarizes the soil types and method to be used for the analysis of BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7.

SOIL TYPE	METHOD USED FOR THE ANALYSIS OF BAS 500 F, BF 50 BF 500-4, BF 500-6, BF 500-7 and BF 500-5
Sandy Loam, Loam with high sand content	Method D9812; Modification No. 2.1
Clay, high silt and clay, soil with high water content	Method D9812/1; Modification No. 2.2, Modification No. 4
Loam and with high organic mater	Modification No. 5

APPENDIX F

SOIL CHARACTERIZATION DATA

AGVISE LABORATORIES
P.O. Box 510, HWY 15
PH. (701) 587-6010
FAX: (701) 587-6013
NORTHWOOD
NORTH DAKOTA
58267-0510

AGVISE Soil Characterization Report

Submitting firm = GRAYSON RESEARCH/BASF
Protocol or Study No = 95024/RCN95012
Sample ID. = SC 0-15CM
Trial ID. = NA
Date Received = 6-7-95
Date Reported = 06-19-1995

AGVISE Lab No	95- 1333
Percent Sand	44
Percent Silt	36
Percent Clay	20
USDA Textural Class (hydrometer method)	Loam
Bulk Density (disturbed) gm/cc	1.01
Cation Exchange Capacity (meq/100 g)	36.7
% Moisture at 1/3 Bar	43.2
% Moisture at 15 Bar	18.2
Percent Organic Matter	3.9
pH	8.1

Base Saturation Data

Cation	Percent	ppm
Calcium	79.1	5800
Magnesium	13.2	580
Sodium	0.4	32
Potassium	3.2	463
Hydrogen	4.1	15

These tests were completed in compliance of 40 CFR Part 160.

Robert Deutsch
Soil Scientist

6/19/95
Date

AGVISE LABORATORIES
Highway 15
P.O. Box 510
Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013

AGVISE Soil Characterization Report

Submitting firm = CMS INC.
Protocol or Study No = A98017
Sample ID. = 98090-1(0190) 0-6"
Trial ID. = RCN98090
Date Received = 5-19-98
Date Reported = 05-26-1998

AGVISE Lab No	98- 1257
Percent Sand	32
Percent Silt	46
Percent Clay	22
USDA Textural Class (hydrometer method)	Loam
Bulk Density (disturbed) gm/cc	1.10
Cation Exchange Capacity (meq/100 g)	9.1
% Moisture at 1/3 Bar	27.4
% Moisture at 15 Bar	10.2
Percent Organic Matter	2.3
pH	6.3

Base Saturation Data

Cation	Percent	ppm
Calcium	49.6	900
Magnesium	11.9	130
Sodium	1.6	34
Potassium	2.5	87
Hydrogen	34.4	31

These tests were completed in compliance of 40 CFR Part 160.

Robert Deutsch
Soil Scientist

5/26/98
Date

AGVISE LABORATORIES
Highway 15
P.O. Box 510
Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013

AGVISE Soil Characterization Report

Submitting firm = CMS INC.
Protocol or Study No = A98017
Sample ID. = 98090-6(0195) 30-36"
Trial ID. = RCN98090
Date Received = 5-19-98
Date Reported = 05-26-1998

AGVISE Lab No	98- 1262
Percent Sand	28
Percent Silt	30
Percent Clay	42
USDA Textural Class (hydrometer method)	Clay
Bulk Density (disturbed) gm/cc	1.12
Cation Exchange Capacity (meq/100 g)	9.2
% Moisture at 1/3 Bar	27.6
% Moisture at 15 Bar	18.6
Percent Organic Matter	0.1
pH	5.8

Base Saturation Data

Cation	Percent	ppm
Calcium	32.7	600
Magnesium	27.3	300
Sodium	1.6	33
Potassium	2.7	97
Hydrogen	35.8	33

These tests were completed in compliance of 40 CFR Part 160.

Robert Deutsch
Soil Scientist

5/26/98
Date

AGVISE LABORATORIES
Highway 15
P.O. Box 510
Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013

AGVISE Soil Characterization Report

Submitting firm = CMS INC.
Protocol or Study No = A98017
Sample ID. = 98090-8(0197) 42-48"
Trial ID. = RCN98090
Date Received = 5-19-98
Date Reported = 05-26-1998

AGVISE Lab No	98- 1264
Percent Sand	30
Percent Silt	30
Percent Clay	40
USDA Textural Class (hydrometer method)	Clay Loam
Bulk Density (disturbed) gm/cc	1.10
Cation Exchange Capacity (meq/100 g)	9.9
% Moisture at 1/3 Bar	29.3
% Moisture at 15 Bar	19.0
Percent Organic Matter	0.1
pH	5.4

Base Saturation Data

Cation	Percent	ppm
Calcium	35.3	700
Magnesium	25.2	300
Sodium	1.4	33
Potassium	2.6	102
Hydrogen	35.5	35

These tests were completed in compliance of 40 CFR Part 160.

Robert Deutsch
Soil Scientist

5/26/98
Date

AGVISE LABORATORIES
Highway 15
P.O. Box 510
Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013

AGVISE Soil Characterization Report

Submitting firm = CMS INC.
Protocol or Study No = A98017
Sample ID. = 98090-7(0196) 36-42"
Trial ID. = RCN98090
Date Received = 5-19-98
Date Reported = 05-26-1998

AGVISE Lab No	98- 1263
Percent Sand	30
Percent Silt	28
Percent Clay	42
USDA Textural Class (hydrometer method)	Clay
Bulk Density (disturbed) gm/cc	1.09
Cation Exchange Capacity (meq/100 g)	9.7
% Moisture at 1/3 Bar	28.1
% Moisture at 15 Bar	18.7
Percent Organic Matter	0.1
pH	5.7

Base Saturation Data

Cation	Percent	ppm
Calcium	36.1	700
Magnesium	26.7	310
Sodium	1.6	35
Potassium	2.6	99
Hydrogen	33.0	32

These tests were completed in compliance of 40 CFR Part 160.

Robert Deutsch
Soil Scientist

5/26/98
Date