ABSTRACT:

BASF Method 432 (**Reference 1**) was originally developed as a LC-MS residue method for the analysis of BAS 500 F and its metabolites BF 500-3, BF 500-6, and BF 500-7 in soil. This method was modified (as Method D9812) and validated at BASF Corporation, Research Triangle Park, N.C 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 in soil using LC-MS. The method validation was carried out as BASF Study No. 98130. The purpose of the study is to determine recovery efficiency for the above analytes in soil. BAS 500 F is the active ingredient and BF 500-3, BF 500-4, BF 500-5, BF 500-6, and BF 500-7 are the major metabolites found in several environmental fate studies.

A 50 g soil sample was extracted twice with acetonitrile. A trace amount of triethylamine was added to the combined extract and was concentrated to 10 mL. The extract was then rediluted with a buffer solution (water-acetonitrile, 70:30, v/v with 0.1 % formic acid and 10 mM ammonium formate) for LC-MS determination.

BASF Method D9812 was further modified (as Method D9812/1) and validated in clay type soils at BASF Corporation, Research Triangle Park, N.C to determine the residues of BF 500-5 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 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 near to approximately 40-50 mL and was added to the dry residue obtained after evaporation of ethyl acetate extracts. The combined extract was then concentrated to 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.

Both methods have a limit of quantitation of 0.01 mg/kg for each analytes in soil.

VALIDATION OF BASF METHOD No. D9812:

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

I. INTRODUCTION AND SUMMARY

A. PURPOSE OF STUDY

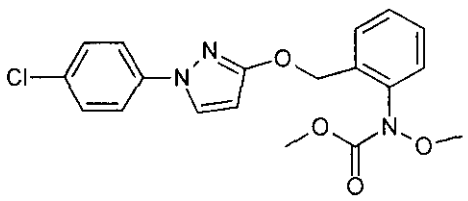
This study was conducted to validate BASF Analytical Method D9812. Recovery ranges and standard deviations were determined from fortified control soil samples. Recoveries of BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6, and BF 500-7 were determined in four soil types. The method No. D9812 allows the determination of BAS 500 F and its metabolites with the required limit of quantitation (0.01 ppm) in soil.

II. MATERIALS/METHODS

A. TEST AND REFERENCE SUBSTANCE

Fortification Standards

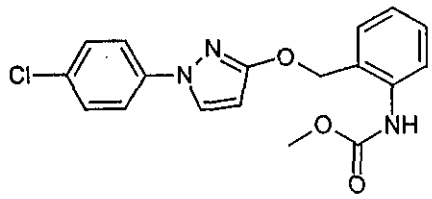
BASF Code Name:	BAS 500 F
BASF Registry Number:	304 428
Chemical Name:	Methy-N-[[[1-(4-chlorophenyl)pyrazol-3-yl]-oxy]-o-tolyl]-N-methoxycarbamate
Molecular Formula:	$C_{19}H_{18}ClN_3O_4$
Molecular Weight:	387.83
Appearance:	White powder
Water Solubility:	1.9 mg/L (at pH 9, 2.3 mg/L)
Lot No.:	00937-128
Purity:	99.8 %
Stability:	Expected to be stable at least 2 years in the refrigerator
Structural Formula:	



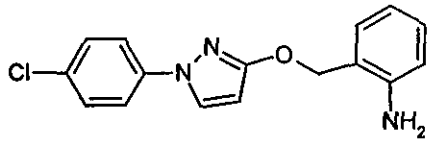
II. MATERIALS (Continued)

A. TEST AND REFERENCE SUBSTANCES (Continued)

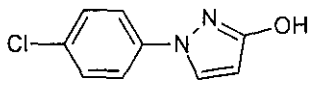
BASF Code Name: BF 500-3
BASF Registry Number: 340 266
Molecular Formula: $C_{18}H_{15}ClN_3O_3$
Molecular Weight: 357.8
Lot No.: 00937-272
Purity: 99.0 %
Stability: Expected to be stable at least 2 years in the refrigerator
Structural Formula:



BASF Code Name: BF 500-4
BASF Registry Number: 358 672
Molecular Formula: $C_{16}H_{14}ClN_3O$
Molecular Weight: 299.76
Lot No.: 01183-26
Purity: 99.3 %
Stability: Expected to be stable at least 2 years in the refrigerator
Structural Formula:



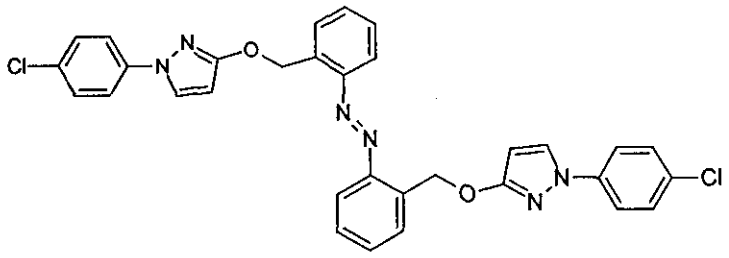
BASF Code Name: BF 500-5
BASF Registry Number: 298 327
Molecular Formula: $C_9H_7ClN_2O$
Molecular Weight: 194.6
Lot No.: 00937-275
Purity: 99.9
Stability: Expected to be stable at least 2 years in the refrigerator
Structural Formula:



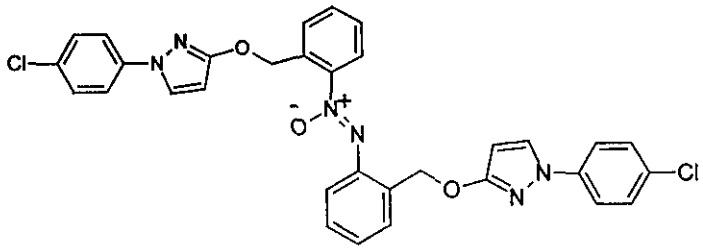
II. MATERIALS (Continued)

A. TEST AND REFERENCE SUBSTANCES (Continued)

BASF Code Name: BF 500-7
BASF Registry Number: 369 315
Molecular Formula: $C_{32}H_{24}Cl_2N_6O_2$
Molecular Weight: 595.5
Lot No.: 01185-022
Purity: 99.9%
Stability: Expected to be stable at least 2 years at room temperature
Structural Formula:



BASF Code Name: BF 500-6
BASF Registry Number: 364 380
Molecular Formula: $C_{32}H_{24}Cl_2N_6O_3$
Molecular Weight: 611.5
Lot No.: 01185-025
Purity: 99.8 %
Stability: Expected to be stable at least 2 years at room temperature
Structural Formula:



II. MATERIALS (Continued)

A. TEST AND REFERENCE SUBSTANCES (Continued)

Reference Standards (used for calibration)

Same as fortification compounds.

Standard substances except BF 500-6 and BF 500-7 are stored in a refrigerator (~ 4°C) until use. BF 500-6 and BF 500-7 is stored in ambient temperature until use. Characterization, purity and stability were determined prior to use for this study. Details of these determinations are available to BASF and are located atLandwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

Test and reference substance solutions were refrigerated during their use in this study. Stock solutions (1 mg/mL) were made fresh every three months and further diluted to proper concentration. Dilutions of stock standards for fortifications were made fresh every month. During the course of this study, the stability of fortification and LC-MS standard solutions were examined. Solutions were stored in a refrigerator at 4°C. The following table shows the stability of the analytes in various solvent system used within the method.

SOLUTION	STABILITIES (DAYS)
Stock solutions of BAS 500 F, BF 500-3, BF 500-4, BF 500-6, and BF 500-7, in Toluene	110
Stock solution in methanol (BF 500-5)	*110*
Fortification solutions in Acetonitrile for all analytes	42
LC-MS injection standards in Solvent II[1]	12

[1] Solvent II: Acetonitrile- Solvent I (70: 30, v/v)
Solvent I: water with 0.1 % formic acid and 10mM ammonium formate

B. TEST SYSTEM

The test system consisted of untreated soil samples obtained from trial sites of soil dissipation studies (BASF Studies 98017 and 95024) conducted in the US and Canada. Different soil types and depths (0-3, 30-36, 36-42 and 42-48 inch) were used to validate this method. Soil samples were obtained from California, New Jersey (Study 98017) and Manitoba (Study 95024) sites and were identified as BASF Residue Control Number (RCN) 98093, 98090 and 95012). Soil characterization data for soil samples used in this study are summarized in **Table VII**.

II. MATERIALS (Continued)

C SAMPLE STORAGE AND HANDLING

The soil samples were homogenized to a consistency suitable for analysis. Bulk soil samples received from the field are homogenized using a blender or mill. Homogenized soil samples are stored frozen (<-5°C) before analysis.

D. EXPERIMENTAL DESIGN

To determine recoveries of BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6, and BF 500-7, control soil samples were fortified by applying standard solutions directly to the soil prior to extraction. Samples were fortified with 0.01, 0.1 and 1.0ppm each of BAS 500 F and BF 500-3, BF 500-4, BF 500-6, BF 500-6, and BF 500-7 and subsequently analyzed with the method. At least one set consisting of three fortification levels was analyzed for each soil type. Initially a total of four sample sets were conducted. Due to poor recovery of BF 500-5 in clay soil, the method was modified and subsequently validated using five extra sample sets. The modified method (D9812/1) was used in clay soil and/or in soil with high water content.

E. METHOD OF ANALYSIS

BASF Analytical Method D9812 was developed to determine the residues of BAS 500 F and its metabolite BF 500-3, BF 500-4, BF 500-5, BF 500-6, and BF 500-7 in soil matrices using LC-MS. The method was designed to determine the residues as an individual analytes. Both methods D9812 and D9812/1 (modified method) were used for the residue analysis of soil samples collected for soil dissipation studies.

The technical procedures of the methods D9812 and D9812/1 are attached to this report as **Appendix C** and **Appendix D** respectively. Due to different soil types, some other modifications were made to the original validated method during residue analysis [Study protocol C98016 (**Reference 2**)]. These modifications are provided in **Appendix E.**

A brief description of these methods is provided below:

Method D9812: BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-5, BF 500-6, and BF 500-7 were extracted from soil (50 g) with acetonitrile. A trace amount (0.1 mL) of triethylamine was added to the combined extract and the extract was then concentrated near to a volume of approximately 10 mL which 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.

D9812/1: A 50 g soil sample aliquot was extracted twice with acetonitrile. The soil marc was re-extracted once with dilute base (0.1 N NaOH). The extracts in acetonitrile and base were collected separately. The alkaline extract was acidified and re-extracted with

II E. METHOD OF ANALYSIS (Continued)

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 to 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 flow charts of the analytical procedures D9812 and D9812/1 are provided in **Figure 1 and 2** respectively.

Specific chromatographic conditions are listed with each analysis set. Typical chromatographic parameters are provided in **Appendix C and D** (Section 3.5). Typical standard curves and chromatograms of standard, and soil samples are provided in **Appendix A (Figure A.1 through A.18)**.

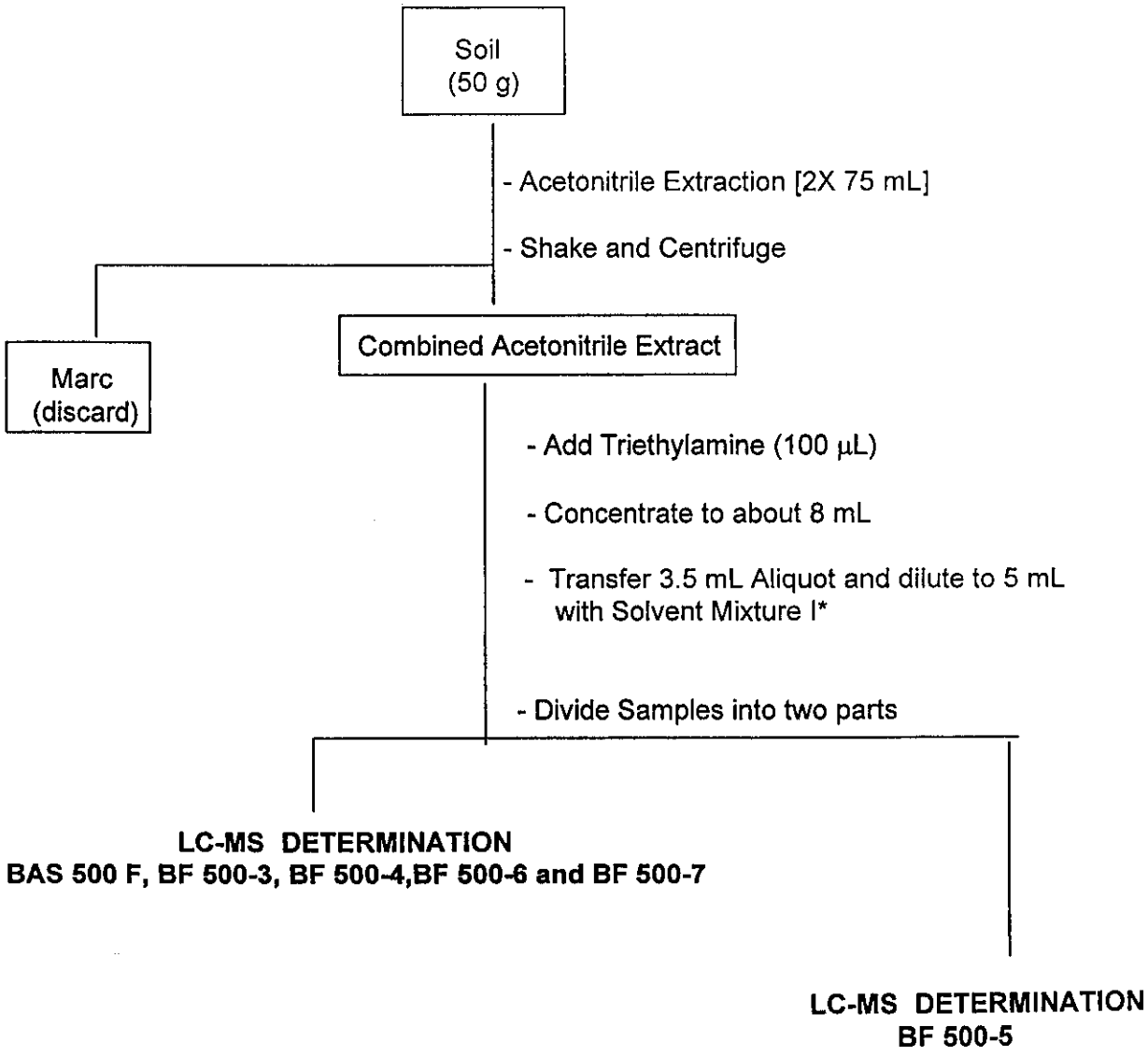
Typical recovery calculations for the LC-MS quantitation are shown in **Figure 3.**

This report describes analytical methods to measure the residues of BAS 500 F and its five major metabolites in sandy and clay soil. Four different types of soil were used as substrates for the validation study. These soil samples represent the types of soils on which BAS 500 F is customarily used. The soil characterization data are provided in **Appendix F** and summarized in **Table VII.**

The analyses were conducted with a HPLC-Mass Selective Detector using Selected Ion Monitoring. LC-MS was operated in the APCI positive ion mode. LC-MS parametersfor each analyte were optimized using a Flow Injection Analysis (FIA) procedure. Full scan mass spectra exhibited highest response of molecular ion plus one ($M^+ + H$) for every analyte of interest **(Appendix A; Figure A.19 through Figure A.22)**. Therefore LC-MS quantitation was based on extracted ions of molecular ion plus one ($M^+ + H$), which are m/z 388 (BAS 500 F), m/z 358 (BF 500-3), m/z 300 (BF 500-4), m/z 195 (BF 500-5), m/z 611 (BF 500-6) and m/z 595 (BF 500-7).

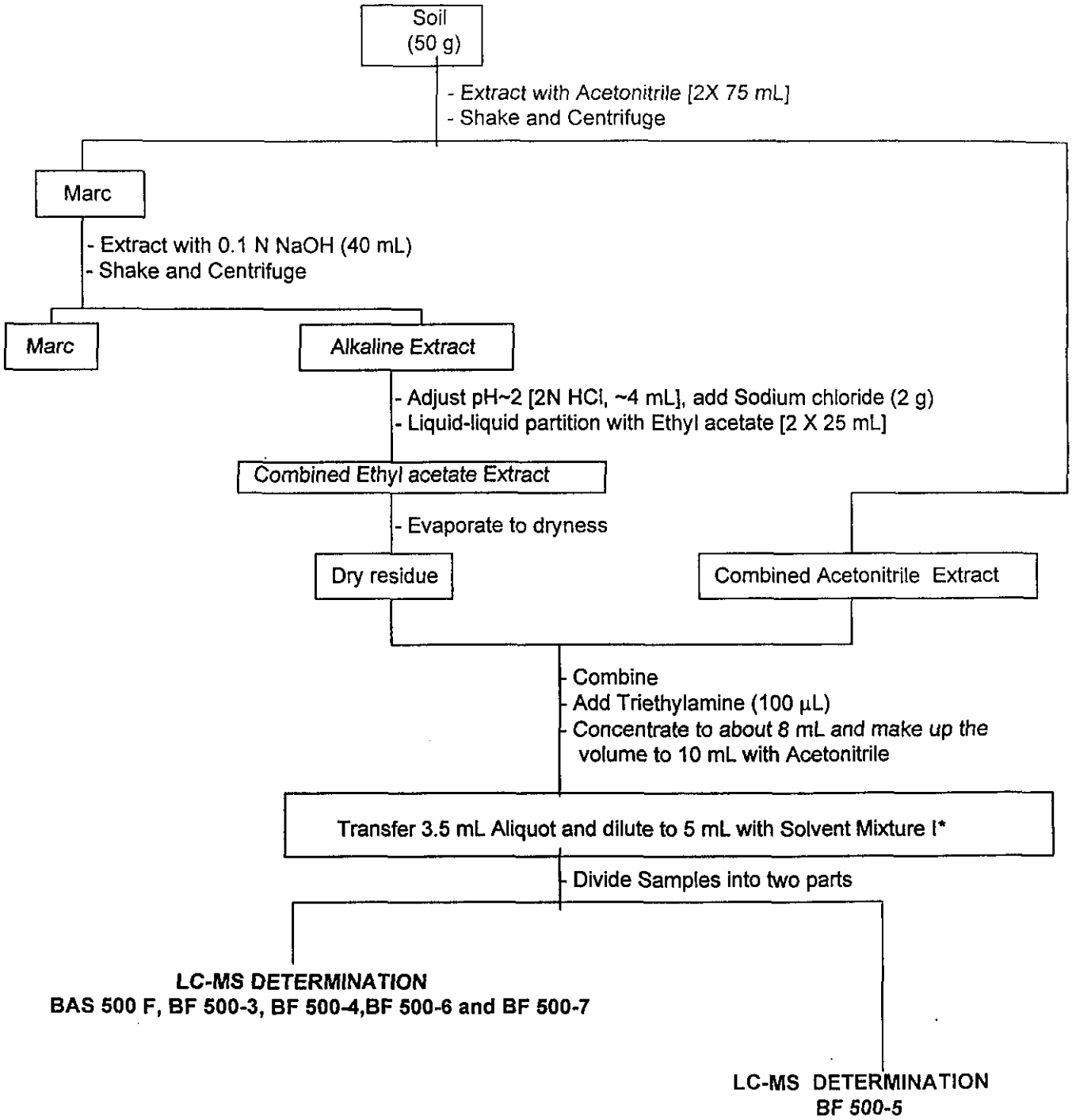
During the validation of method D9812, low recoveries were observed for the metabolite BF 500-5 in clay soil with high water content. A modification of the method was required to obtain acceptable recoveries (>65 %) for BF 500-5. The modified method was validated in clay and loamy soils. In general, during the routine residue analysis Method D9812 and its modification (Change No. 2 and 4, Amendment C98016-1, **Appendix** E) were 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

Figure 1. Flow Diagram for Analytical Method No. D9812 in Soil (BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7)



*Solvent Mixture I: Water with 0.1 % formic acid and 10 mM ammonium formate
Solvent Mixture II: Acetonitrile- Solvent Mixture I
For 1.0 and 0.1 ppm level samples, dilute 1:10 and 1:50 respectively with Solvent Mixture II

Figure 2. 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

Figure 3. Typical Recovery Calculation for BASF Method D9812

Sample Number 98130/1374-05-20: Control Soils Fortified with 0.01 ppm of BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6, and BF 500-7. Calculations are shown only for BAS 500 F.

A = ng found per injection

= Amount of analyte (BAS 500 F) calculated from calibration curve

Standard curve: $ng = \frac{\text{Peak Area} - \text{intercept}}{\text{slope}} = \frac{11859 - (-1083.812)}{19.077} = 0.678$

mg injected $= \frac{\text{Sample weight (g) extracted} \times \mu\text{L injected}}{\text{Final dilution volume (mL)}}$

$= \frac{50.0\text{ (g)} \times 20\text{ (µL)}}{14.29\text{ (mL)}} = 69.97$

Final dilution volume = 10 mL $\times \frac{5\text{ mL}}{3.5\text{ mL}} = 14.29$ mL

Residue in ppm $= \frac{\text{ng found per injection}}{\text{mg injected}} \times$ Molecular weight conversion factor

$= \frac{(0.678) \times 1}{69.97} = 0.00969$ [molecular weight conversion factor = 1]

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

$= \frac{0.00969 - 0.0000}{0.01} = 97\ \%$

[The corresponding control sample (BASF Sample 98130/1374-05-10) contained 0.00 ppm of BAS 500 F residue].

Similar calculations were used for all other analytes. Total area are obtained for BF 500-7 by adding the areas of Z and E isomers.

Protocol Changes:

The following changes were incorporated into the study protocol (98130) during method validation:

1. **Change to Protocol:**

   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).**

   Reason for Change:

   During the validation of method D9812, low recoveries were observed for the metabolite BF 500-5 in clay soil with high water content. It was required to modify the existing method to obtain the acceptable recoveries (>65 %) for BF 500-5. In general, during the routine residue analysis, 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, modified method D9812/1 will be used for the re-extraction/reanalysis.

2. **Change to Protocol:**

   A total of nine sample sets were performed to complete the method validation.

   Reason for Change:

   Although the protocol originally stated that four sample sets should be conducted, it was necessary to analyze five extra sample sets to improve the recovery of BF 500-5 for the different soil type.

ABSTRACT

BASF Method 432 (Reference 1) was originally developed as a LC-MS residue method for the analysis of BAS 500 F and its metabolites BF 500-3, BF 500-6, and BF 500-7 in soil. This method was modified at BASF Corporation, Research Triangle Park, N.C 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 in soil using LC-MS.

A 50 g soil sample was extracted twice with acetonitrile. A trace amount of triethylamine was added to the combined extract and was concentrated to approximately 10 mL. The extract was then 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.

1. INTRODUCTION

1.1 Scope of the method

BAS 500 F is a new fungicide is developed for use on Turf in the US and for several other crops (vineyard, row crops, grapes, peanuts and potatoes) in the US, Canada and Europe. For registration of the fungicide and for establishing the DT50/90 values from field dissipation studies in different use patterns, a residue analytical method with a limit of quantitation of 0.01 mg/kg for the active ingredient and its metabolites in soil was developed. Two different chromatographic methods (LC-MS Detection) of the final extracts were performed to determine BAS 500 F and its metabolites in soil. Method A (section 3.5) was used to analyze BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6, and BF 500-7. Method B (section 3.5) was used to analyze BF 500-5. The method No D9812 allows the determination of BAS 500 F and its metabolites with the required limit of quantitation in soil.

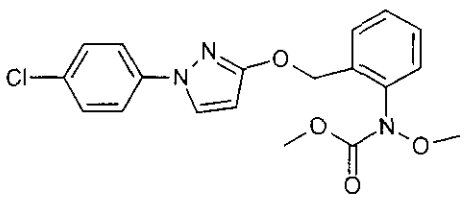
2. MATERIALS

Standard substances are stored in a freezer (<-5°C) until use. Information on the characterization of these substances is available from BASF and is located at the Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

2.1 Test and Reference Substances

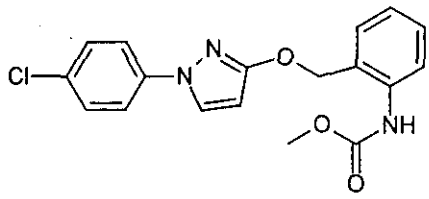
2.1.1 Fortification Compounds

BASF Code Name:	BAS 500 F
BASF Registry Number:	304 428
Chemical Name:	Methy-N-[[[1-(4-chlorophenyl)pyrazol-3-yl]-oxy]-o-tolyl]-N-methoxycarbamate
Molecular Formula:	$C_{19}H_{18}ClN_3O_4$
Molecular Weight:	387.83
Appearance:	White powder
Water Solubility:	1.9 mg/L (at pH 9, 2.3 mg/L)
Lot No.:	00937-128
Purity:	99.8
Stability:	Expected to be stable at least 2 years
Structural Formula:	

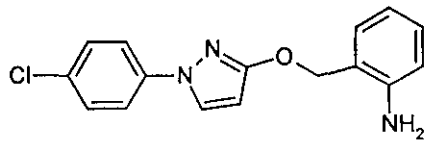


2.1 Test and Reference Substances (Continued)

BASF Code Name:	BF 500-3
BASF Registry Number:	340 266
Molecular Formula:	$C_{18}H_{16}ClN_3O_3$
Molecular Weight:	357.8
Lot No.:	00937-272
Purity:	99.0
Stability:	Expected to be stable at least 2 years
Structural Formula:	

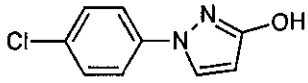


BASF Code Name:	BF 500-4
BASF Registry Number:	358 672
Molecular Formula:	$C_{16}H_{14}ClN_3O$
Molecular Weight:	299.76
Lot No.:	01183-26
Purity:	99.3
Stability:	Expected to be stable at least 2 years
Structural Formula:	

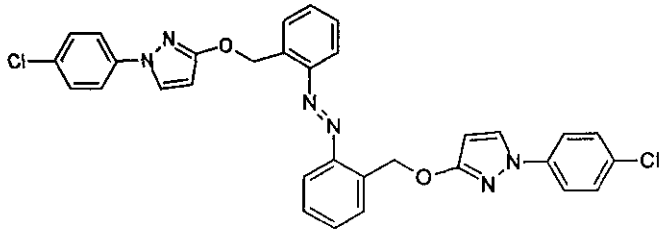


2.1 Test and Reference Substances (Continued)

BASF Code Name:	BF 500-5
BASF Registry Number:	298 327
Molecular Formula:	$C_9H_7ClN_2O$
Molecular Weight:	194.6
Lot No.:	00937-275
Purity:	99.9
Stability:	Expected to be stable at least 2 years
Structural Formula:	

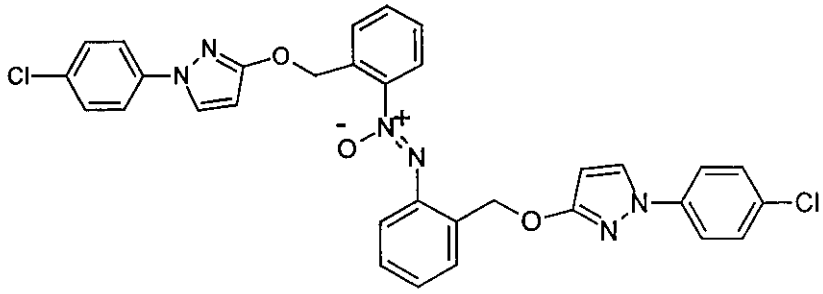


BASF Code Name:	BF 500-7
BASF Registry Number:	369 315
Molecular Formula:	$C_{32}H_{24}N_6Cl_2O_2$
Molecular Weight:	595.5
Lot No.:	01185-022
Purity:	99.9
Stability:	Expected to be stable at least 2 years
Structural Formula:	



2.1 Test and Reference Substances (Continued)

BASF Code Name:	BF 500-6
BASF Registry Number:	364 380
Molecular Formula:	$C_{32}H_{24}N_6Cl_2O_3$
Molecular Weight:	611.5
Lot No.:	01185-025
Purity:	99.8
Stability:	Expected to be stable at least 2 years
Structural Formula:	



2.1.2 Reference Standards (used for calibration)

Same as fortification compounds (section 2.1.1)

2.2 Equipment -- Suggested Sizes/Suppliers, Manufacturers

Method Step	Equipment	Size, Description	Manufacturer/ Supplier	Catalog Number
2.4.2, 2.4.3	Balance, Analytical	Model AT100	Mettler	
Various	Balance, Top Loading	Model PM 4800	Mettler	
Various	Bar, Magnetic Stirring	2 inch lengths	Various	
2.4	Bottle, Amber glass	2 oz, 4 oz and 8 oz Teflon®-lined screw cap	Qorpak	
3.2.1-3.2.3	Centrifuge	Refrigerated Centrifuge Model CS-6KR	Beckmann	
3.2.1-3.2.3	Centrifuge Bottles, plastic (Conical wide mouth)	175 mL	Nalgene	3143-0175
3.2.1-3.2.3	Centrifuge Adapter (rotary adapter)	for 174 mL bottles	VWR (Nalgene	21020-250
Various	Flask, Erlen meyer, 24/40	250, 300, 500 and 1000 mL	Various	
Various	Flask, Volumetric	2,5, 10, 25, 100, and 200mL	Various	
3.2	Flask, Flat Bottom	125 and 250 mL	Various	
Various	Glass funnel, short stem;	60 mm i.d.	Various	
Various	Graduated cylinders:	25 - 2,000 ml	Various	
Various	Hot Plate, Magnetic Stirring	Cimarec 1	Thermolyne	VWR 33921-867
	Millex-FH_{13}; Millipore (Fluoropore)	0.5 μm	Sigma	Z 22, 746-3
Various	Pipet, Volumetric	0.5, 1-10 mL, 25, 50, 100 mL	Various	
Various	Pipet, Borosilicate, disposable	1 and 5 mL	Various	
Various	Rotary Evaporator	Buchi RE 111 or R-124 or Labconco 78892-00	Brinkmann (VWR)	
Various	Rotary Evaporator Trap	100 mL, 24/40	Kimble-Kontes	570200-0124

2.2 Equipment -- Suggested Sizes/Suppliers, Manufacturers (Continued)

Method Step	Equipment	Size, Description	Manufacturer/ Supplier	Catalog Number
	Laboratory Shaker	Model HS501-D	Janke and Kunkel	
Various	Spatula		Various	
Various	Stopper, Teflon®	24/40	Various	
	Syringes, plastic, disposable	1 and 3 mL	Various	
Various	Ultrasonic Bath	Model FS-14	Fisher Scientific Co.	
Various	Vacubrand vacuum pump/controller	Model HS501-D	Elnik Systems, Inc.	
	Vials, HPLC			
	Votex mixer	Genie	Fisher Scientific Co	
	Whatman Filter paper	#1, 150 mm	VWR	
3.5	LC-MSD with HP 1100 Series HPLC		Hewlett Packard	

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

Chemical	Grade	Manufacturer/ Supplier	Catalog Number
Acetonitrile	High Purity	B & J	015-4
Ammonium Formate	MicroSelect >99%	Fluka	09735
Formic Acid	98%	EM/VWR	FX0440-7
Methanol	High Purity	B & J	230-4
Triethylamine	Reagent grade	J.T Baker	W635-07
Toluene	High Purity	B & J	
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	Method Step
Solvent I: Water with 0.1 % formic acid and 10 mM ammonium formate	3.2
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.	3.2
LC-MS Mobile Phase A: Water with 0.1 % formic acid	3.5
LC-MS Mobile Phase B: Acetonitrile with 0.1 % formic acid	3.5
LC-MS Mobile Phase C: Water with 0.1 % formic acid and 5 mM ammonium formate	3.5

2.4 Standard Solutions

2.4.1 Standard Solution Storage and Stability

Standard solutions are kept refrigerated. The storage stability of standard solutions made in acetonitrile and any other solvent were established during the course of the study (see page). Stock solutions (1 mg/mL) were made fresh every three months. Fortification solutions were made fresh every months and injection standards were made fresh every weeks.

2.4.2 Standard Solutions of BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7 for Fortifications

2.4.2.1 Stock Solution (mg/mL) of BAS 500 F

Prepare a 1.0 mg/mL BAS 500 F stock solution by weighing an appropriate amount of BAS 500 F into a volumetric flask. Dissolve with toluene and dilute to mark. For example, to prepare a 25 mL stock solution, place 25.0 mg of BAS 500 F into a 25 mL volumetric flask. Dissolve and dilute to mark with toluene.

2.4.2.2 Stock Solution (mg/mL) of BF 500-3

Prepare a 1.0 mg/mL BF 500-3 stock solution by weighing an appropriate amount of BF 500-3 into a volumetric flask. Dissolve with toluene and dilute to mark. For example, to prepare a 25 mL stock solution, place 25.0 mg of BF 500-3 into a 25 mL volumetric flask. Dissolve and dilute to mark with toluene.

2.4 Standard Solutions (Continued)

2.4.2.3 Stock Solution (mg/mL) of BF 500-4

Prepare a 1.0 mg/mL BF 500-4 stock solution by weighing an appropriate amount of BF 500-4 into a volumetric flask. Dissolve with toluene and dilute to mark. For example, to prepare a 25 mL stock solution, place 25.0 mg of BF 500-4 into a 25 mL volumetric flask. Dissolve and dilute to mark with toluene.

2.4.2.4 Stock Solution (mg/mL) of BF 500-5

Prepare a 1.0 mg/mL BF 500-5 stock solution by weighing an appropriate amount of BF 500-5 into a volumetric flask. Dissolve with methanol and dilute to mark. For example, to prepare a 25 mL stock solution, place 25.0 mg of BF 500-5 into a 25 mL volumetric flask. Dissolve and dilute to mark with methanol.

2.4.2.5 Stock Solution (mg/mL) of BF 500-6

Prepare a 1.0 mg/mL BF 500-6 stock solution by weighing an appropriate amount of BF 500-6 into a volumetric flask. Dissolve with toluene and dilute to mark. For example, to prepare a 25 mL stock solution, place 25.0 mg of BF 500-6 into a 25 mL volumetric flask. Dissolve and dilute to mark with toluene.

2.4.2.6 Stock Solution (mg/mL) of BF 500-7

Prepare a 1.0 mg/mL BF 500-7 stock solution by weighing an appropriate amount of BF 500-7 into a volumetric flask. Dissolve with toluene and dilute to mark. For example, to prepare a 25 mL stock solution, place 25.0 mg of BF 500-7 into a 25 mL volumetric flask. Dissolve and dilute to mark with toluene.

2.4.2.7 Mixed Standards for Fortifications

Prepare a 50 µg/mL mixed standard solution for fortification by combining 5.0 mL of each of the BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7 stock solutions into a 100 mL volumetric flask. Dilute to mark with acetonitrile. Prepare serial dilutions of this combined solution as needed. Suggested concentrations of mixed standards for fortifications are 50 µg/mL, 5.0 µg/mL, and 0.5 µg/mL, in acetonitrile.

2.4.3 Injection Standard Solutions BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7 for LS-MS Analysis (Calibration Standards): 200, 100, 50, 25 and 12.5 pg/µL in Solvent mixture II

Prepare a 200 pg/µL mixed injection standard solution by transferring an appropriate amount of the 5.0 µg/mL fortification solutions (2.4.2.7) with a volumetric pipette into a volumetric flask (typically 1.0 mL of the 5.0 µg/mL solution in a 25 mL volumetric flask). Dilute to the mark with **Solvent mixture II**.

NOTE: Use amber bottles with Teflon®-lined screw caps as storage containers for standard solutions. Suggested standard concentrations are listed above. A different concentration scheme may be used and additional standards may be prepared as needed.

3. ANALYTICAL PROCEDURE

3.1 Sample Preparation

Bulk soil samples received from the field are homogenized using a blender or mill. Homogenized soil samples are stored frozen (<-5°C) before analysis. Weigh a 50 g (± 0.1 g) aliquot of the soil sample into a 175 mL centrifuge bottle.

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 sample 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 3000 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.

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 and transfer the supernatant into the above 250 mL flat-bottom flask by decantation through the funnel.

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

3.2.4 Add 0.1 mL of triethylamine using a graduated disposable pipet to the combined extract and **mix well to obtain a homogeneous extract.**

3. ANALYTICAL PROCEDURE (Continued)

3.2.5 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).

3.2.6 Swirl and sonicate the extract to dissolve the dry residue from the side of the250 mL flat-bottom flask and transfer the extract to a standard taper flat-bottom flask (125 mL). Rinse the 250mL flat-bottom flask with 5 to 10 mL of acetonitrile for complete transfer of the residue. 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 this 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 3.2.7 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 the125 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

3.3.1 Transfer 3.5 mL of the extract (3.2.7) to a 5 mL volumetric flask and dilute to the mark with **solvent mixture I**. Sonicate and vortex to ensure a homogeneous solution. Two different chromatographic methods (LC-MS Detection) of the final extracts were performed to determine BAS 500 F and its metabolites in soil. Method A (section 3.5) was used to analyze BAS 500 F and its metabolites BF 500-3, BF 500-4, BF 500-6, and BF 500-7. Method B (section 3.5) was used to analyze BF 500-5. The following procedures are typically used to prepare the samples for analysis:

For control and 0.01 ppm fortifications, filter the solution through a syringe filter (a 0.5µ Fluoropore disc fitted to an 1.0 mL disposable plastic syringe). Transfer the sample

3. ANALYTICAL PROCEDURE (Continued)

solution (3.3.1) with a glass disposable pipette to the syringe, discard the initial 300- 400 µL of the filtrate and collect the filtrate (about 1-2mL) into two injection vial.

For 0.1 ppm fortifications, take 1 mL of the sample solution (3.3.1) and dilute to 10 mL with **solvent mixture II**. Sonicate and vortex to ensure a homogeneous solution. Filter the solution into the injection vial using the procedure above.

For 1.0 ppm fortifications, take 0.5 mL of the sample solution (3.3.1) and dilute to 25 mL with **solvent mixture II**. Sonicate and vortex to ensure a homogeneous solution. Filter the solution into the injection vial using the procedure above.

The samples are ready for injection.

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

3.4 Moisture Determination

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

Instrument:	HP LC-MSD
Inlet (HPLC System):	HP 1100 Series with Quarternary pump
Data System:	HP ChemStation
Column:	Inertsil C4, 5µ, 150 X 3.0 mm, [MetaChem Technologies Inc., P/N 0298-150X030]
Injection volume:	20 µL
Mobile Phase: (Gradient)	A = Water with 0.1% Formic Acid B = Acetonitrile with 0.1% Formic Acid C = Water with 0.1% Formic Acid and 5 mM Ammonium formate

3. ANALYTICAL PROCEDURE (Continued)

Column Temperature	30° C			
Flow Rate:	500 µL/minute			
Ionization Mode:	APCI with positive ion for all analytes			
Corona Current: 5 µA	Vaporizer Temperature: 325° C	Drying gas Temperature: 300° C	Nebulizer pressure: 25 psi	Drying gas Flow: 6 L/minute

Method A:
Gradient used for the Analysis of BAS 500 F, BF 500-3, BF 500-4, BF 500-6 and BF 500-7

Time (min.)	Composition	
0.0	50% A + 50% B	
12.0	50% A + 50% B	
13.0	75% B + 25% C	
22.0	75% B + 25% C	
23.0	50% A + 50% B	
30.0	50% A + 50% B	Post time 5.0 minutes and inject every 35 minutes

Method B: Gradient used for the Analysis of BF 500-5

Time (min.)	Composition	
0.0	60% A + 40% B	
4.0	40% A + 60% B	
4.5	25% A + 75% C	
7.5	25% A + 75% B	
8.0	60% A + 40% B	
12.0	60% A + 40% B	Post time 2.0 minutes and inject every 14 minutes

3. ANALYTICAL PROCEDURE (Continued)

LC-MS parameters used for the Analysis of BAS 500 F, BF 500-3, BF 500-4, BF 500-6 and BF 500-7 (Method A)						
Capillary Voltage	At zero minute 3000 and at 14 minute changed to 2000					
	BAS 500 F	**BF 500-3**	**BF 500-4**	**BF 500-6**	**BF 500-7**	
					A-Isomer	**B-Isomer**
Fragmentor	20	20	20	25	25	
Gain (Use low resolution)	3	3	5	3	3	
Expected Retention Times (minutes)	10.2	9.4	7.0	19.5	19.5	21.2
	BAS 500 F	**BF 500-3**	**BF 500-4**	**BF 500-6**	**BF 500-7**	
Ion monitored (SIM):	388.0	358.0	300.0	611.0	595.0	

LC-MS parameters used for the Analysis of BF 500-5 (Method B)			
Capillary Voltage	Kept constant at 3000	Ion monitored (SIM): (Use low resolution)	195.0
Fragmenter	40	Expected Retention Times (minutes)	4.3
Gain (Use low resolution)	1.0		

NOTES:

1. The equipment listed was used for method development and validation. Other equivalent hardware may be used. The use of a guard column is optional.
2. The recommended instrument parameters and chromatographic systems were found to be optimal for the instrument used for the method validation. The exact values used must be optimized for each instrument. Different chromatographic systems might be necessary to be developed for different type of instrument.

3. ANALYTICAL PROCEDURE (Continued)

This method could conceivably be validated on another instrument manufacturer's platform. If this is the case, instrument parameters and flow splits will be different; however, the basic principles of analysis by LC-MS will remain the same.

3.6 Calibration Procedures

Calculation of results is based on peak area measurements using a calibration curve. The standard curve is obtained by direct injection of 20 µL of the mixed BAS 500 F, BF 500-3, BF 500-4, BF 500-6 and BF 500-7 standards for LC-MS in the range of 12.5 pg/µL to 200 pg/µL. A similar standard curve is obtained by direct injection of 20 µL of the BF 500-5 standards for LC-MS in the range of 12.5 pg/µL to 200 pg/µL. In a given injection run, the same volume is used for all samples and standards. Typical standard amounts injected on-column range as follows: 250 pg, 500 pg, 1000 pg, 2000 and 4000 pg.

Prepare calibration curves by plotting the peak area (selected ion monitoring m/z 588, 358, 300, 195, 611 and 595 for BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7 respectively) versus the weight of BAS 500 F, BF 500-3, BF 500-4, BF 500-5, BF 500-6 and BF 500-7 respectively using a linear least squares working curve in the form $y = mx + b$.

Establish the stability of the detection response by injecting several concentrations of standards. For analysis, alternate samples and standards. For each injection set, the set should begin and end with standard injections, and each standard level should be injected at least in duplicate.

Note: It is advisable to "stabilize" the on column retention time of the analytes before injecting the first sample of an analytical series.

3.7 Limit of Quantitation and Limit of Detection

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 (**Section III D**). The limit of detection has not been determined, but the lowest standard for each analyte in calibration curve has good detectability (signal to noise ratio greater than 3:1).

4. CALCULATION OF RESULT

Calculation of results is based on peak area measurements. A typical calculation to determine the residues of BAS 500 F and its metabolites are shown in page 22 of this report.

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 8 person-hours, or 1 calendar day, provided that no special problems arise, such as matrix interference.

6. CONFIRMARY 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 BF500-3 and BF500-4. 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.

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.

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.

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

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) = Aliquot (mL) taken from final extract in acetonitrile / 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 (%) = [Residue (ppm) for fortified sample - Residue (ppm) for control sample] / 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**

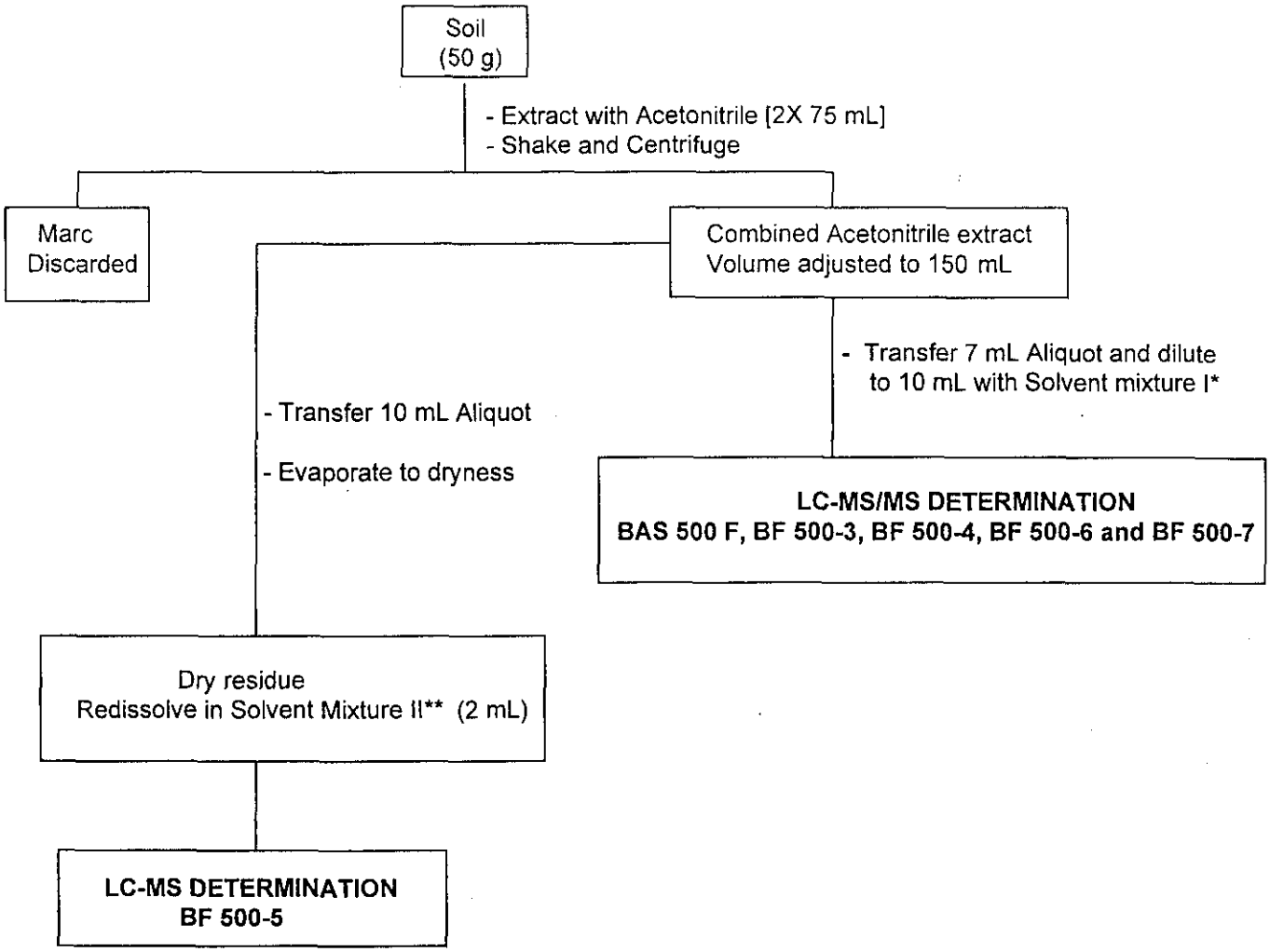
Section 4.2: Calculation: Same as described in **Modification No. 2.1, except the following:**

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

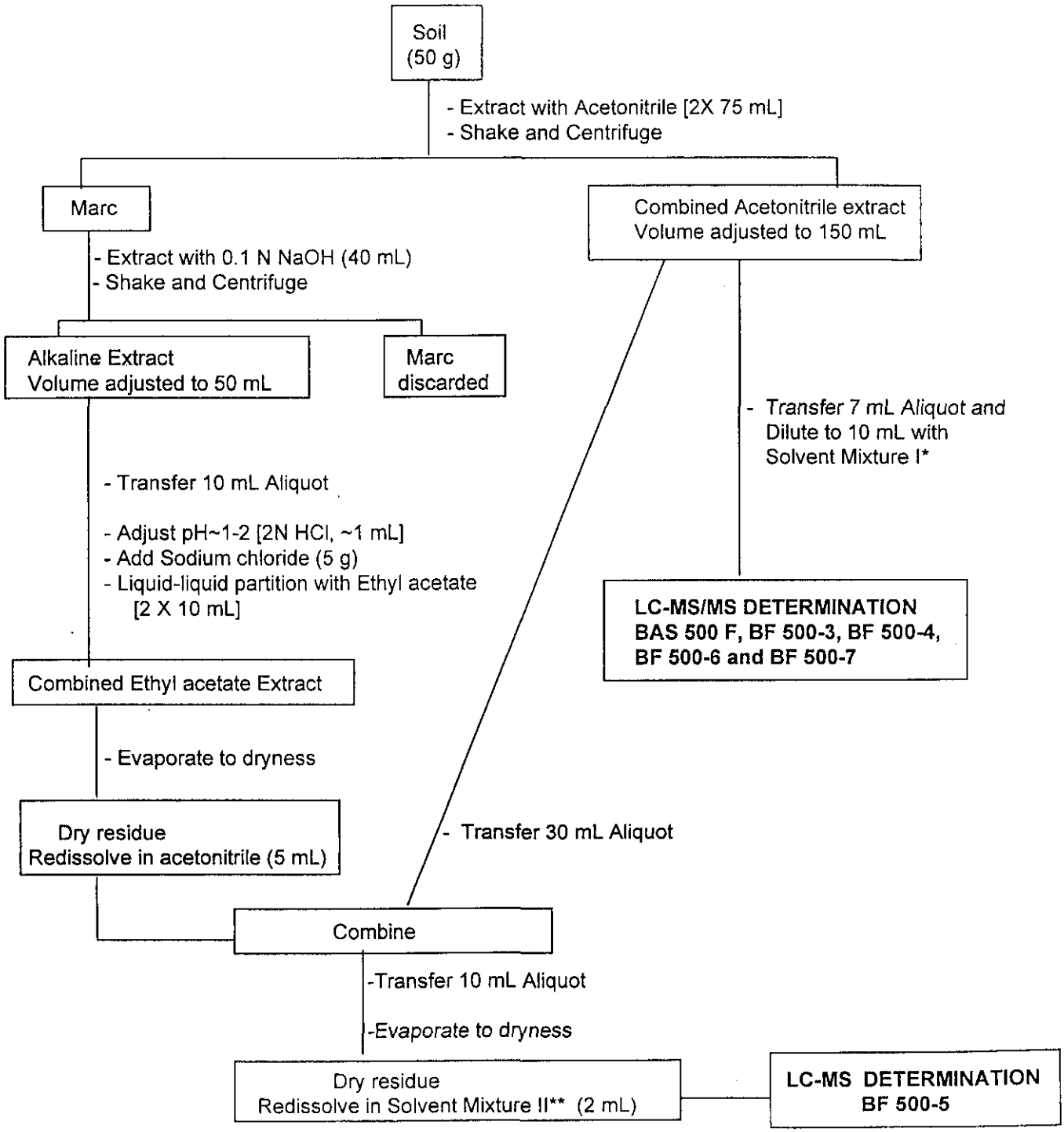
F1 (First dilution factor) = $\frac{\text{Aliquot (mL) taken from final extract in acetonitrile}}{\text{Dilution volume (mL)}}$ = 10/2 = 5 (Section 3.3.2)

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.