VALIDATION OF BASF METHOD No. D0503:

I. INTRODUCTION AND SUMMARY

A. PURPOSE OF STUDY

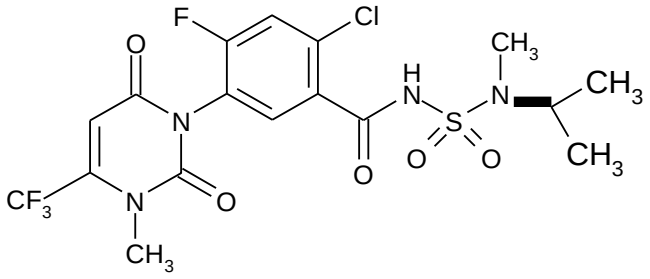
BAS 800 H is a new herbicide that will be used for cotton, cereal and other crops in the U.S. For registration of this herbicide and for establishing the DT50/90 values from field dissipation studies for these use patterns, a residue analytical method with a limit of quantitation of 0.01 mg/kg for the active ingredient and its metabolites in soil was developed. This study was conducted to validate BASF Analytical Method D0503. Recovery ranges and standard deviations were determined from fortified control soil samples. Recoveries of all analytes were determined in four different soil types.

II. MATERIALS/METHODS

A. TEST AND REFERENCE SUBSTANCES

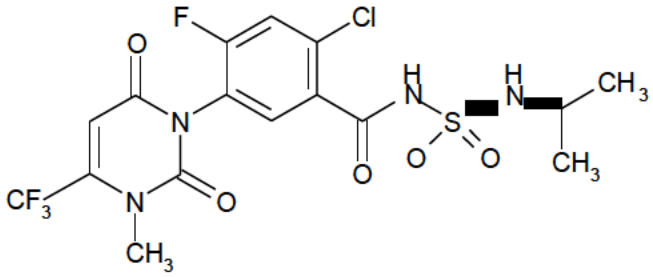
Fortification Compound

BASF Code Name:	BAS 800 H
BASF Registry Number:	4054449
CAS Number:	372137-35-4
Molecular Formula:	$C_{17}H_{17}ClF_4N_4O_5S$
Molecular Weight:	500.9
Lot No.:	L67-140
Purity: Expiration	99.9%
date: Structural	July 01, 2008
Formula:	

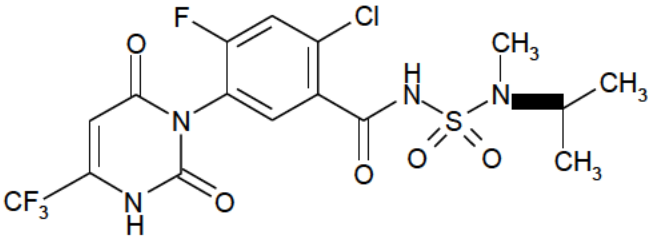


II. MATERIALS/METHODS (Continued)

BASF Code Name:	M800H01
BASF Registry Number:	4118561
Molecular Formula:	$C_{16}H_{15}ClF_4N_4O_5S$
Molecular Weight:	486.8
Lot No.:	L74-62
Purity: Expiration	98.8%
date: Structural	February 1, 2008
Formula:	

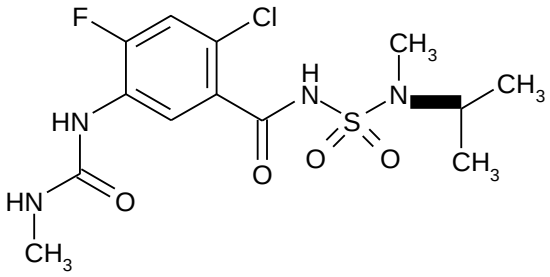


BASF Code Name:	M800H02
BASF Registry Number:	4118416
Molecular Formula:	$C_{16}H_{15}ClF_4N_4O_5S$
Molecular Weight:	486.8
Lot No.:	L67-186
Purity: Expiration	99.2%
date: Structural	March 1, 2009
Formula:	

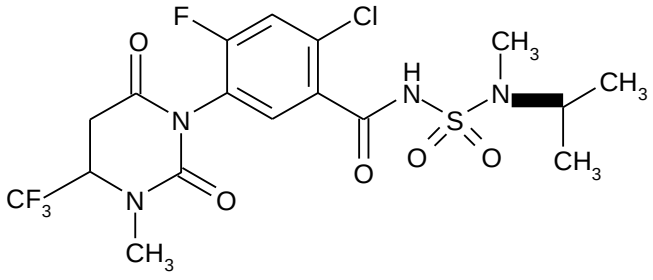


II. MATERIALS/METHODS (Continued)

BASF Code Name: M800H07
BASF Registry Number: 4775453
Molecular Formula: $C_{13}H_{18}ClFN_4O_4S$
Molecular Weight: 380.8
Lot No.: L67-196
Purity: Expiration 95.4%
date: Structural March 1, 2009
Formula:



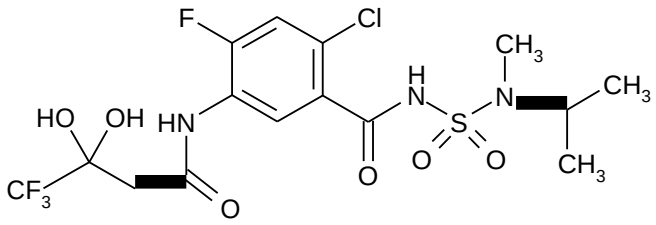
BASF Code Name: M800H08
BASF Registry Number: 4773881
Molecular Formula: $C_{17}H_{19}ClF_4N_4O_5S$
Molecular Weight: 502.9
Lot No.: L74-66
Purity: Expiration 97.2%
date: Structural April 1, 2008
Formula:



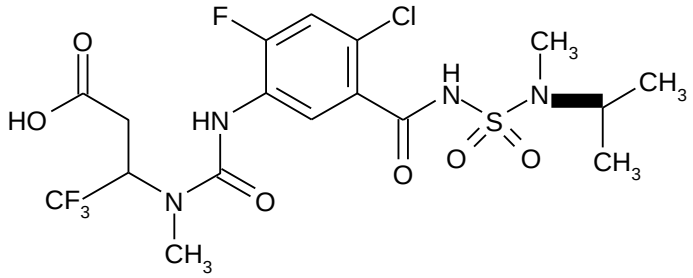
II. MATERIALS/METHODS (Continued)

BASF Code Name:	M800H015
BASF Registry Number:	5264357
Molecular Formula:	$C_{15}H_{18}ClF_4N_3O_6S$
Molecular Weight:	479.9
Lot No.:	L74-80
Purity:	94.5%
Expiration date:	June 1, 2008

Structural Formula:



BASF Code Name:	M800H022
BASF Registry Number:	5216337
Molecular Formula:	$C_{17}H_{21}ClF_4N_4O_6S$
Molecular Weight:	520.9
Lot No.:	L74-56
Purity: Expiration	94.1%
date: Structural	March 1, 2008
Formula:	



Reference Standards (used for calibration)

Same as fortification compounds (Section II A)

Standard substances are stored in a freezer (<-5^{0}C) 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 at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

Standard Solution Stability

During the course of dissipation study (**Reference 1**), the stability of fortification and LC-MS/MS calibration standard solutions was examined and the summary of the results are provided in this report. Test and reference substance solutions were stored in a refrigerator at 4°C and 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. The following table shows the stability of the analytes in various solvent system used within the method.

SOLUTION	STABILITY (DAYS)
Stock solutions of BAS 800 H and its metabolites, M800H01, M800H02, M800H07, M800H08, M800H15, M800H22 in methanol	90
Fortification solutions and LC-MS/MS calibration standards of BAS 800 H and its metabolites, M800H01, M800H02, M800H07, M800H08, M800H15, M800H22 in acetonitrile-water (70:30, v/v)	35

Soil Extract Stability

During the course of several residue studies (**Reference 1**), the stability of the test and reference substances in the soil extracts were examined and the summary of the results are provided in this report. To demonstrate the stability of all analytes in soil matrix extracts, two samples from fortified matrices were used. The samples were analyzed at the day of extraction and were stored in a refrigerator. These extracts were re-analyzed using method D0503 after certain days of storage (see table below). The results were compared to the initial analysis (zero day) to establish the stabilities. The following table shows the stability of the analytes in soil extracts in different solutions used within the method.

Extracts	STABILITY (DAYS) [1]
Soil extract containing BAS 800 H and its metabolites, M800H01, M800H02, M800H07, M800H08, M800H15, M800H22 in acetonitrile-water (70:30, v/v)	7

II. MATERIALS/METHODS (Continued)

B. TEST SYSTEM

The test systems consisted of untreated soil samples obtained from trial sites of various field dissipation studies conducted in the U.S. These soils were used to validate method D0503. The test systems and their residue control numbers (RCN) and the locations are listed below.

RCN (Location)	Sample Number	Sample Description	Characterization
R05227 (WA)	0891	Untreated plot, 30DALA, Rep A, 0-1 "	Loamy Sand
R05229 (CA)	0841	Untreated plot, -T1,Rep A, 0-1 "	Loam
R05229 (CA)	0847	Untreated plot, -T1,Rep A, 24-30 "	Clay Loam
German 2.2	Lufa soil		Sandy loam

C. SAMPLE STORAGE AND HANDLING

Bulk soil samples received from the field are homogenized with dry ice using a Fitzmill (hammermill). An aliquot of the homogenized soil samples are further homogenized in Retsch Ultra Centrifugal mill equipped with a 1.0 mm screen and stored frozen (<-5°C) before analysis.

D. EXPERIMENTAL DESIGN

Control soil samples were fortified by applying standard solutions directly to the soil prior to extraction with a volumetric pipet. The fortified control samples were analyzed to determine the recoveries of each analytes.

Initially, a total of 4 validation sets were conducted with 4 different types of soil. An additional set was added at a later point by an amendment. Each validation set consisted a reagent blank, two unfortified matrix controls, 5 matrix control samples fortified at the LOQ (see table below) and 5 matrix control samples fortified at 10 times LOQ (a total of 13 samples per matrix type). All of these sets were subsequently analyzed with the method.

II. MATERIALS/METHODS (Continued)

E. METHOD OF ANALYSIS

BASF Analytical Method D0503 was developed to determine the residues of BAS 800 H, M800H01, M800H02, M800H07, M800H08, M800H15, and M800H22 in soil matrices using LC-MS/MS. The method was designed to determine the residues as individual analyte and will be used for the residue analysis of soil samples collected from soil dissipation studies.

The technical procedure of this method is attached to this report as **Appendix A**. A brief description of the method is provided below:

A 0.1 g soil sample aliquot was extracted by shaking twice with acetonitrile followed by mixture of acetonitrile and water (70:30, v/v) and the residues are determined using HPLC/MS/MS.

The transitions monitored for quantitation ion are: m/z 501.0 → 348.9, 487.0 → 365.9, 487.0 → 335.0, 381.0 → 229.0, 503.2→ 351.1, 480.0 → 420.1, and 521.00 → 369.0 for analytes BAS 800 H, M800H01, M800H02, M800H07, M800H08, M800H15 and M800H22, respectively.

The transitions monitored for secondary ion (confirmation purposes) are: m/z 501.00 → 459.0, 487.0 → 445.0, 487.0 → 445.0, 381.0 → 338.96, 503.6 → 197.9, 480.0 → 310.0, and 521.00 → 172.0 for analytes BAS 800 H, M800H01, M800H02, M800H07, M800H08, M800H15 and M800H22, respectively.

The confirmatory ions used for the analysis of analytes M800H01 and M800H02 were the same (m/z 487.0 → 445.0). Therefore, an additional chromatographic method was used to conduct the analysis of M800H01 and M800H02 to separate these two analytes for the confirmation purposes (see **Method B** in the technical procedure, **Appendix A**).

For the quantitation of metabolite M800H08, a correction of observed MRM transition 503.2 to 351.1 should be made due to the contribution of the chlorine isotope ^{37}Cl from the parent BAS 800 H, since these two analytes are not chromatographically separated. See detail explanations and calculations in **Appendix B**. This correction is only applied for the quantitation of unknown treated samples. For procedural fortification samples, all analytes are fortified in equal amount and the changes in recoveries are insignificant while a wide range (70 -120%, according to the guideline) of recoveries are acceptable.

A flow diagram of the analytical procedure is provided in **Figure 1**.

Typical recovery calculations for the LC/MS/MS quantitation are shown in **Figure 2**. Specific chromatographic conditions are listed with each analysis set. Typical chromatographic parameters are provided in the technical procedure **(Appendix A).**

Typical standard curves and chromatograms of standard, and soil samples are provided in **Appendix C** (**Figure C.1 through C.22**).

Soil characerization data for each soil type used in the validation study is presented in the **Appendix D.**

Figure 1. Flow Diagram of Analytical Method No. D0503 in Soil

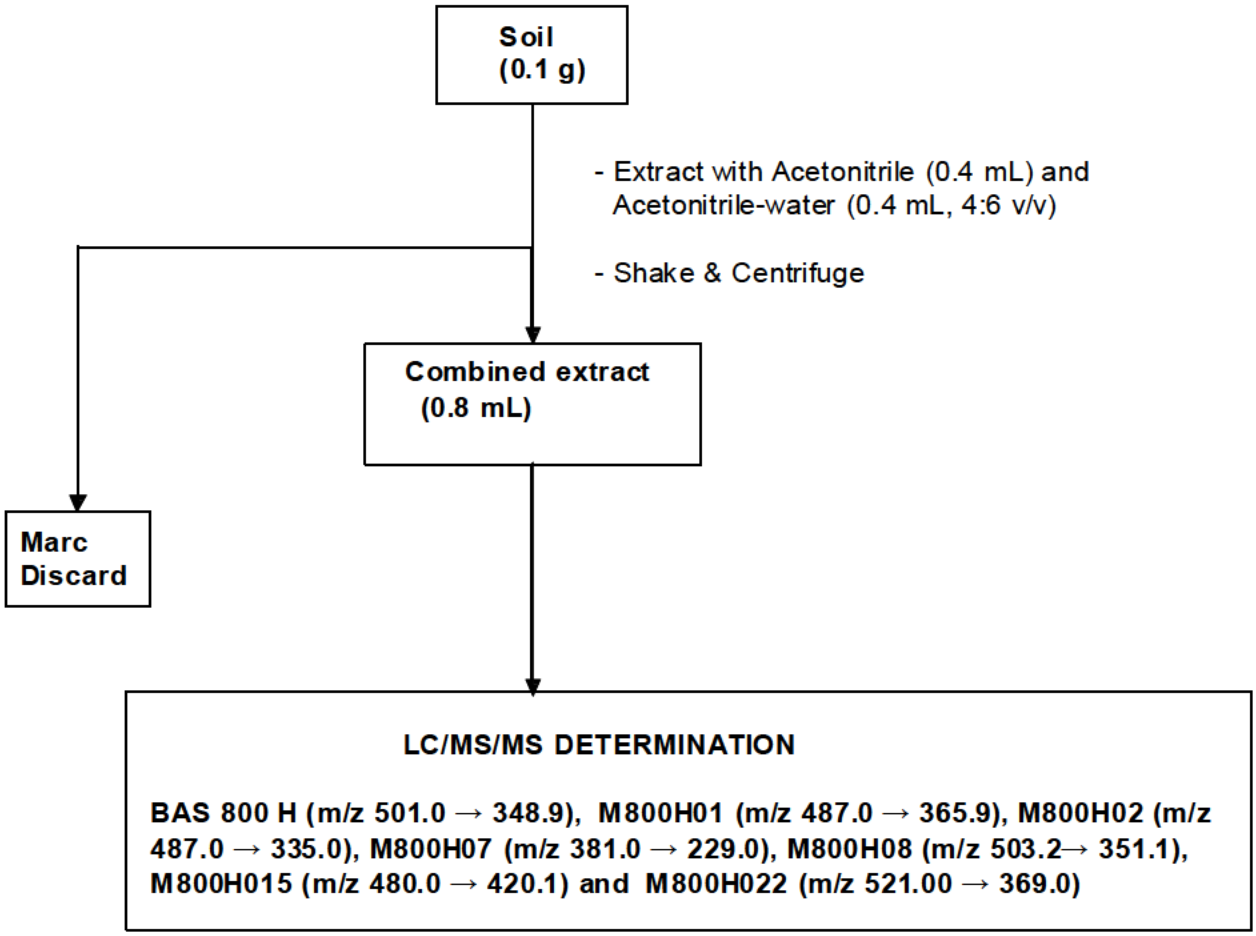


Figure 2. Typical Recovery Calculation (BASF Method D0503)

Sample Number 132659-1F Control soil samples fortified with 0.01 ppm of BAS 800 H, and its metabolites, M800H01, M800H02, M800H07, M800H08, M800H15, M800H22

Sample Number 132659-1B, C: Controls unfortified. Average (ppm) of two controls were used to subtract the control value for fortifications.

Calculations are shown only for BAS 800 H.

Following equations were used to calculate procedural recoveries (%):

Residue calculation for BAS 800 H for the Sample Number 132659-1F**:**
Slope (m): 4,878.8
Intercept (b): 121.5
correl. coeff. = 0.9965

$$\text{BAS 800 H Found (ng/mL)} = \frac{\text{peak area - intercept}}{\text{Slope}}$$

Peak Area = 6,100.0; Intercept = 121.5470; Slope = 4,878.7719

$$\text{BAS 800 H Found (ng/mL)} = \frac{6{,}100.0 - 121.5470}{4{,}878.7719} = 1.2254$$

Residue (ppm of BAS 800 H) =

$$\frac{\text{BAS 800 H Found (ng/mL) x Final Volume (mL)}}{\text{Sample Weight (g) x } A_F \text{ x 1000 (to convert to ppm)}}$$

Sample Weight = 0.1 g
Final Volume = 0.8 mL
Aliquotation Factor (A_F) = 100%

$$\text{Residue (ppm of BAS 800 H) (wet weight basis)} = \frac{1.2254 \text{ ng/mL} \times 0.8 \text{ mL}}{0.1000 \text{ g} \times 100\% \times 1000} = 0.0098 \text{ ppm}$$

Net Residue (ppm of BAS 800 H) = Residue (ppm of BAS 800 H) - Residue in Control (ppm)

$$\text{Recovery of BAS 800 H (\%)} = \frac{\text{Residue (ppm of BAS 800 H) - Residue in Control (ppm)} \times 100}{\text{Amount Fortified (ppm)}}$$

Amount Fortified = 0.01 ppm
Residue in Control = none detected

$$\text{Recovery of BAS 800 H (\%)} = \frac{0.0098 \times 100}{0.01} = 98\%$$

Full computer/calculator precision in any intermediate calculations is used and the final values are only rounded for reporting purpose. Percent recoveries of all other analytes were calculated in similar fashion.

Tables I through X: Procedural Recovery Data for BAS 800 H, and its metabolites, M800H01, M800H02, M800H07, M800H08, M800H15, M800H22 in differet soil types

Table XI: Summary of Standard Data

Unless otherwise noted, the following parameters were used for all analyses:

Sample size: 0.1 g

- Extraction volume = 0.8 mL
- Injection volumes: 20 µL
- Final volume (LOQ) = 0.8 mL
- Aliquot factor = 100%

- Dilution factor = 1/10 = 0.1 for 10 times LOQ samples
- Amount of analyte (pg) found = (peak area - intercept)/slope [obtained from calibration curve]
- Values in these tables may have been rounded off for reporting purpose, but not for further calculation. Rounding occurs only in the final result, the “percent recovery” column
- If no signal was detected, ND was reported
- Calculations used to obtain "mg injected", "ppm calculated" and percent recoveries are shown in page 21 (**Figure1**).
- Recoveries were corrected for control background, if present, the average of two control values were used. If no signal or signal with amount less than LOD (20% of the LOQ) was detected for control samples, not applicable (NA) was reported in the recovery column.
- Sample Number: Master Sheet Number (Study Number-Sequential Number for master sheet – sequential number) for the samples
- Reagent blank: Has shown no interference peaks at retention time of all analytes

- Source and description of the soil used in the study (**Reference 1 and 2);** Master sheet 1 through 5:
  - Master sheet 1: Loamy Sand soil for Washington, RCN R05227, soil depth 0-1”
  - Master sheet 2: German soil 2.2
  - Master sheet 3 and 5: Loamy soil from California, RCN R05229, Soil depth 0-1”
  - Master sheet 4: Clay Loam soil from California, RCN R05229, Soil depth 24-30”

1. INTRODUCTION

1.1 Scope of the method

BAS 800 H is a new herbicide that will be used for cotton, potato and other crops in the US and Europe. For registration of this herbicide and for establishing the DT50/90 values from field dissipation studies for these 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.

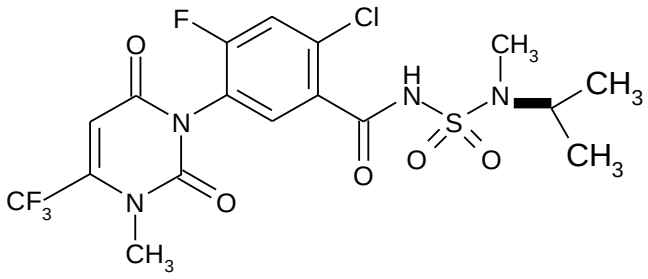
2. MATERIALS

Standard substances are stored in a freezer 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 Substance

2.1.1 Fortification Compound

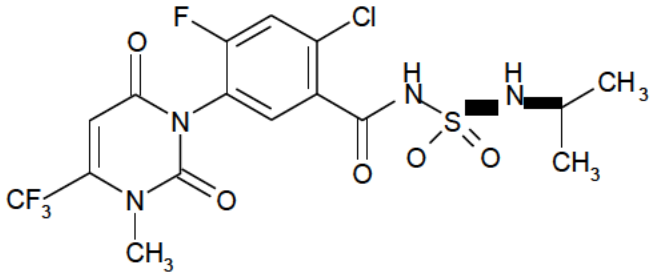
BASF Code Name:	BAS 800 H
BASF Registry Number:	4054449
CAS Number:	372137-35-4
Molecular Formula:	$C_{17}H_{17}ClF_4N_4O_5S$
Molecular Weight:	500.9
Lot No.:	L67-140
Purity: Expiration	99.9%
date: Structural	July 01, 2008
Formula:	



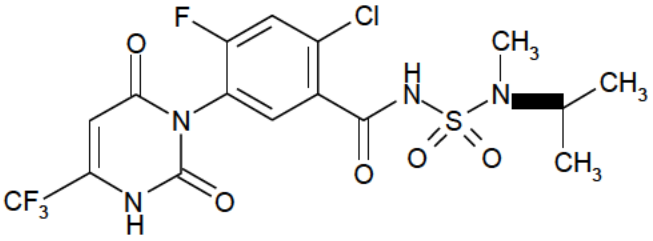
BASF Code Name:	M800H01
BASF Registry Number:	4118561
Molecular Formula:	$C_{16}H_{15}ClF_4N_4O_5S$

2. Materials (Continued)

Molecular Weight:	486.8
Lot No.:	L74-62
Purity: Expiration	98.8%
date: Structural	February 1, 2008
Formula:	



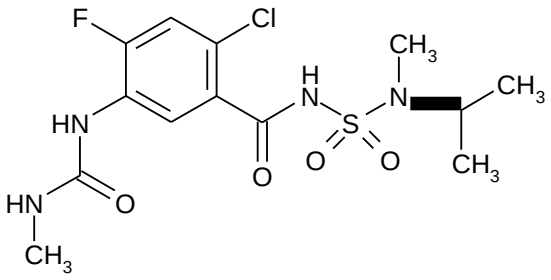
BASF Code Name:	M800H02
BASF Registry Number:	4118416
Molecular Formula:	$C_{16}H_{15}ClF_4N_4O_5S$
Molecular Weight:	486.8
Lot No.:	L67-186
Purity: Expiration	99.2%
date: Structural	March 1, 2009
Formula:	



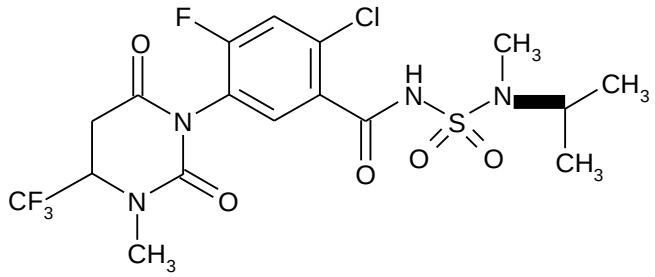
BASF Code Name:	M800H07
BASF Registry Number:	4775453
Molecular Formula:	$C_{13}H_{18}ClFN_4O_4S$
Molecular Weight:	380.8
Lot No.:	L67-196
Purity:	95.4%
Expiration date:	March 1, 2009

2. Materials (Continued)

Structural Formula:

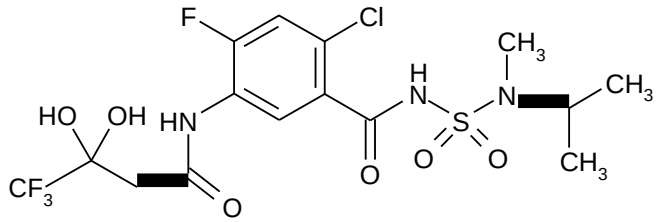


BASF Code Name: M800H08
BASF Registry Number: 4773881
Molecular Formula: $C_{17}H_{19}ClF_4N_4O_5S$
Molecular Weight: 502.9
Lot No.: L74-66
Purity: Expiration 97.2%
date: Structural April 1, 2008
Formula:



BASF Code Name: M800H015
BASF Registry Number: 5264357
Molecular Formula: $C_{15}H_{18}ClF_4N_3O_6S$
Molecular Weight: 479.9
Lot No.: L74-80
Purity: 94.5%
Expiration date: June 1, 2008

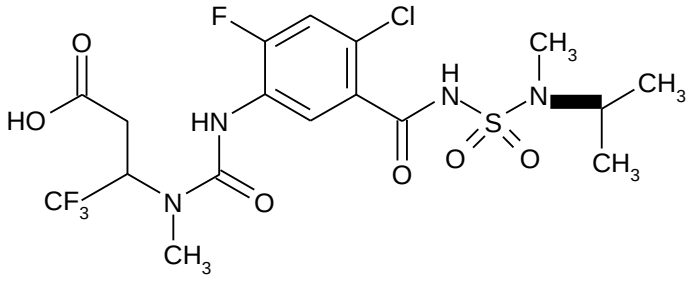
Structural Formula:



BASF Code Name: M800H022
BASF Registry Number: 5216337

2. Materials (Continued)

Molecular Formula:	$C_{17}H_{21}ClF_4N_4O_6S$
Molecular Weight:	520.9
Lot No.:	L74-56
Purity: Expiration	94.1%
date: Structural	March 1, 2008
Formula:	



2.1.2 Reference Standard (used for calibration)

Same as fortification compound (section 2.1.1)

BASF has retained a reserve sample of these chemicals, and has documentation at the BASF Agricultural Products Center, Research Triangle Park, North Carolina.

2. Materials (Continued)

2.2 Equipment -- Suggested Sizes/Suppliers, Manufacturers

Method Step	Equipment	Size, Description	Manufacturer / Supplier	Catalog Number
2.3, 2.4	Balance, Analytical	Model AT100	Mettler	
Various	Balance, Top Loading	Model PM 4800	Mettler	
Various	Bar, Magnetic Stirring	2 inch lengths	Various	
2.4, 3.2.	Bottle, Amber glass	Qorpak , 2 oz, 4 oz and 8 oz with Teflon®-lined screw cap	Qorpak	
3.1	Centrifuge	Refrigerated Centrifuge Model CS-6KR	Beckmann	
Various	Cylinder, Graduated	Various sizes	Various	
Various	Flask, Erlenmeyer, 24/40	1000 mL	Various	
Various	Flask, Volumetric	100, 50, 25 ,10 and 5 mL	Various	
3.3	Liquid Handling System	Quadra96®, Model 320 or Quadra 3_{NS} ®	Tomtec Inc.	
3.3	MicroMan pipettes	10-1000 µL	Gilson	M-25,M-50,M-250, M-1000
3.3	Multitube Vortexer	VX-2500	VWR	58816-116
Various	Pipet, Volumetric	0.5, 1-10, 25 mL	Various	
Various	Pasteur Pipet, disposable	various size	VWR	
Various	Pipet tips	polypropylene	Matrix Inc.	196-205
Various	Reagent reservoir	Dimpled polypropylene	Tomtec Inc.	
Various	Spatula		Various	
Various	Stopper, Teflon®	24/40	Various	
Various	Ultrasonic Bath	Model FS 7652H	Fisher Scientific	

2. Materials (Continued)

Method Step	Equipment	Size, Description	Manufacturer / Supplier	Catalog Number
Various	Vials, Amber Borosilicate	8 and 40 mL	VWR	224984 and 15900-018
Various	Vortex mixer	Genie 2	Fisher Scientific Co	12-812
3.3	Well Plates (Storage block)	1.4 mL AlphaNumeric Tubes, 2.2 mL polypropylene with 1200 μL glass tubes	Matrix Inc., Hirschmann Laborgerate	4211, 4253, 924 05 96
3.3	Well Plate caps/seals	SepraSeal	Matrix Inc.	4463
3.5	LC/MS/MS	API 3000 Biomolecular Mass Analyzer	PE Sciex	

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%	E.M. Science	FX0440-7
Methanol	High Purity	B & J	230-4
Water	High Purity	B & J	365-4

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

2. Materials (Continued)

2.3.2 Solvent Mixtures and their Preparation

Solvent Mixtures	Method Step
Solution I: Acetonitrile-water, 40:60, v/v Add 400 mL of acetonitrile and 600 mL of water into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.	3.3.2
Solution II: Acetonitrile-water, 70:30, v/v Add 700 mL of acetonitrile and 300 mL of water into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution. **Note:** Significant amount of cool may occur while mixing.	3.3.1, 3.3.2 and 2.4.2.3
LC/MS/MS Mobile Phase A: Water with 0.1 % formic acid 4 mM ammonium formate Add 1.0 mL of formic acid (98 %) and 252 mg of ammonium formate into a 1L volumetric flask. Mix well to ensure complete dissolution of the ammonium formate. Dilute to the mark with water and mix well to ensure a complete homogeneous solution.	3.5
LC/MS/MS Mobile Phase B: Methanol with 0.1 % formic acid 4 mM ammonium formate Add 1.0 mL of formic acid (98 %) and 252 mg of ammonium formate into a 1L volumetric flask. Mix well to ensure complete dissolution of the ammonium formate. Dilute to the mark with methanol and mix well to ensure a complete homogeneous solution.	3.5

LC-MS Mobile Phase A: Water with 4 mM ammonium formate: Add 252 mg of ammonium formate into a 1L volumetric flask. Mix well to ensure complete dissolution of the ammonium formate. Dilute to the mark with water and mix well to ensure a homogeneous solution.

2.4 Standard Solutions and their Storage Stability

2.4.1 Standard Solution Storage Stability

Standard solutions are kept refrigerated. The storage stability of standard solutions made in methanol and any other solvent will be established during the course of the method validation study. BASF recommends that stock solutions (1 mg/mL) in methanol be made fresh every three months. Dilution of stock solutions should be stored refrigerated no longer than one month or according to their established storage stability in a particular solvent.

2. Materials (Continued)

2.4.2 Standard Solutions

2.4.2.1 Stock solution of BAS 800 H, M800H01, M800H02, M800H07, M800H08, M800H015 and M800H022 (1 mg/mL):

Prepare a 1.0 mg/mL stock solution individually by weighing an appropriate amount of each analyte into a volumetric flask. Dissolve with appropriate solvent as described below and dilute to mark.

For example, to prepare a 10 mL of 1.0 mg/mL stock solution of BAS 800 H in methanol, weigh 10 mg BAS 800 H into a 10 mL volumetric flask. Dissolve and dilute to mark with methanol. Sonicate and vortex to ensure a complete homogeneous solution. The stock solutions for all other analytes are made in a similar fashion. The solvents used for stock solutions for individual anlaytes are described below.

Stock solution solvent for M800H01, M800H02, M800H07, M800H08, M800H15, and M800H22: Methanol

2.4.2.2 Mix Standards for Fortifications

Prepare mixed standard solution for fortification by combining stock solutions of each analyte (2.4.2.1) in a volumetric flask using the following scheme. Dilute to the mark with appropriate solvents as specified in the table below and vortex to ensure a complete homogeneous solution.

Preparation of fortification solutions:

A**Analytes**	**Take Solution (μg/mL)**	**Volume (mL)**	**Dilute to a final volume of (mL) (Solvent)**	**Concentration of each analyte (μg/mL)**
BAS BAS 800 H, M800H01, M800H02, M800H07, M800H08, M800H15, and M800H22	1000	0.5 (each)	50 (Acetonitrile-water, 70:30, v/v)	10
	10	5	50 (Acetonitrile-water, 70:30, v/v)	1.0
	1	5	50 (Acetonitrile-water, 70:30, v/v)	0.1

2. Materials (Continued)

2.4.2.3 Calibration Standard Solutions for LC/MS/MS Analysis

Prepare mixed calibration solution for LC/MS/MS analysis by combining solutions that were prepared in Section 2.4.2.2 in volumetric flasks using the following scheme in the table below. Dilute to the mark with appropriate solvents as specified and vortex to ensure a complete homogeneous solution.

Preparation of calibration standard solutions:

Volume (mL) taken/ Solution used	Dilute to a final volume of (mL) (Acetonitrile-water, 70:30, v/v)	Concentration
5.0 mL of 0.1 μg/mL of mix solution in Acetonitrile	50	10 ng/mL
25 mL of 0.01 μg/mL of mix solution in Acetonitrile-Water	50	5 ng/mL
25 mL of 5 ng/mL of mix solution in Acetonitrile-Water	50	2.5 ng/mL
20 mL of 2.5 ng/mL of mix solution in Acetonitrile-Water	50	1 ng/mL
25 mL of 1.0 ng/mL of mix solution in Acetonitrile-Water	50	0.5 ng/mL

NOTE:
- Use amber bottles with Teflon®-lined screw caps as storage containers for standard solutions.
- Suggested standard concentrations are listed here. 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 with dry ice using a Fitzmill (hammermill). An aliquot of the homogenized soil samples are further homogenized in Retsch Ultra Centrifugal mill equipped with a 1.0 mm screen and stored frozen (<-5°C) before analysis.

3.2 Weighing and Fortification

Weigh a 100 mg or to the nearest tenth of a milligram aliquot of the soil sample into a 1.4 mL AlphaNumeric well plate tube (Matrix).

3. Analytical Procedures (Continued)

For the fortification samples, add volumetrically an appropriate volume of standard solution to the respective control sample by a micro pipet. For example, for a 0.01 ppm fortification sample, pipet 10 µL of the 0.1 µg/mL standard fortification solution (2.4.2.2) onto 100 mg of control sample.

3.3 Extraction

3.3.1 Add 0.4 mL of acetonitrile to the well tubes containing soil (Section 3.2) using a single or multi-channel automatic pipeter. Firmly cap the well tubes with Matrix SepraSeal cap. Vortex the capped well-plate tubes containing the soil samples upside down using Multitube vortexer at about 2400 rpm for 1.0 minute to mix the solvent to the soil. Repeat the vortexing the capped well plate tubes containing the soil samples upright position using Multi-tube vortexer at about 2400 rpm for 1.0 minute. Centrifuge samples at about 3000 rpm for 5 minutes in a swinging bucket centrifuge. Detach the SepraSeal cap from the well-plate tubes containing the soil samples.

3.3.2 Add 0.4 mL of acetonitrile-water (40:60, v/v) to the well tubes containing soil with acetonitrile (Section 3.3.4.1) using a single or multi-channel automatic pipeter. Firmly cap the well tubes with Matrix SepraSeal cap. Vortex the capped well-plate tubes containing the soil samples upside down using Multitube vortexer at about 2400 rpm for 1.0 minute to mix the solvent to the soil. Repeat the vortexing the capped well plate tubes containing the soil samples upright position using Multi-tube vortexer at about 2400 rpm for 1.0 minute. Centrifuge samples at about 3000 rpm for 5 minutes in a swinging bucket centrifuge. Detach the SepraSeal cap from the well-plate tubes containing the soil samples. Remove the supernatant to a Matrix AlphaNumeric Tubes and Proceed to Section 3.4.

NOTE:
- In case of sandy soil (fluffy), centrifugation may be conducted at about 0^{o}C to compact the soil
- In case of some soil types, it may be necessary to increase the number of vortex cycles or to further agitate on a mechanical shaker for 5 minutes. The shaking step, if needed, should be performed in between vortex steps and document in the master sheet.

3.4 Sample Preparation for LC/MS/MS Analysis

3.4.1 For control and 0.01 ppm fortifications samples at this level were directly analyzed.

3.4.2 For 0.1 ppm fortifications, take 1 mL of the sample solution (3.4.1 and dilute to 10 mL with Solution II (Acetonitrile-water, 70:30, v/v).

3.4.3 All residue samples are diluted at the limit of quantitation level (0.01) with Solution II (Acetonitrile-water, 70:30, v/v). Any further dilutions are made with Solution II (Acetonitrile -water, 70:30, v/v).

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

3. Analytical Procedures (Continued)

3.5 Method Automation

The method extraction and clean-up procedures can be automated with the use of an automated liquid handling system. See Appendix A for examples of the automated liquid handling system programs. The instrument parameters (e.g. stage height, air gap, blow out, etc) that do not impact the data, may be changed, if needed. It is the responsibility of the analyst to ensure that the automated liquid handler delivers the correct volumes. Using automated liquid handling system, the analyst should print out the program that documents the parameters and keep this with the study file.

3.6 Moisture Determination

The procedural recoveries will not be corrected for moisture content of the sample. Results of soil analysis are reported on a "dry weight" basis for residue determination. Therefore field treated soil sample weights must be corrected for moisture content by any method the laboratory customarily uses. The moisture determination will be conducted for the treated samples with residue value above LOD. An example of a moisture determination procedure is provided below:

Weigh 5 ± 0.1 g of wet soil accurately into a tared glass petri dish or other container to obtain Wet Sample Weight (g) and place into a 100°C oven for at least 16 hours (overnight). Remove the petri dish or other container from the oven quickly and allow it to cool down to room temperature in a desiccator. Working quickly, remove the cool petri dish or other container from the desiccator and weigh accurately to obtain the Dry Sample Weight (g).

Wet Sample Weight (g) = [Tare + Wet Sample Weight (g)] – Tare

Dry Sample Weight (g) = [Tare + Dry Sample Weight (g)] – Tare

$$\text{Percent Moisture} = \frac{\text{Wet Sample Weight (g) - Dry Sample Weight (g)}}{\text{5.0 (g)}} \times 100$$

Residue in ppm (Dry residue) = Wet Sample Residue (ppm) / (100 – "Percent Moisture") / 100

3. Analytical Procedures (Continued)

3.7. Instrumentation

Suggested LC/MS/MS Operating condition:

Method A: (Used for method validation and residue analysis for soil dissipation studies)

Instrument:	PE Sciex API 3000 Biomolecular Mass Analyzer
Inlet [HPLC System]:	PE Series 200 Micro Pump system with Series 200 Autosampler
Software Version:	Analyst 1.1
Column:	Phenomenex Columbus C18, 5 μ, 100 X 2.1 mm, [P/N 00D-4108-B0]
Injection:	Typically 20 μL

Mobile Phase:	A = water with 0.1 % formic acid and 4 mM ammonium formate B = methanol with 0.1 % formic acid and 4 mM ammonium formate		
[Gradient]	**Time (minute)**	**Composition (%)**	
		A	**B**
	0.0	95	5
	2.0	20	80
	4.0	20	80
	4.1	95	5
	6.0	95	5
	Run every 6 minutes		
Flow Rate:	400μL/minute		

3. Analytical Procedures (Continued)

Analytes	Expected Retention Times (minutes)	Transitions (m/z):	
		Quantitation ion	Secondary ion
BAS 800 H	3.26	501.0 → 348.9	501.00 → 459.0
M800H01	3.21	487.0 → 365.9	487.0 → 445.0
M800H02	3.23	487.0 → 335.0	487.0 → 445.0
M800H07	3.10	381.0 → 229.0	381.0 → 338.96
M800H08	3.26	503.2→ 351.1	503.6 → 197.9
M800H015	3.14	480.0 → 420.1	480.0 → 310.0
M800H022	3.21	521.00 → 369.0	521.00 → 172.0
Ionization Mode:	Positive ion; Turbospray (450^{o}C)		

NOTE:

1. The LC/MS/MS instrument and 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 were found to be optimal for the instrument used for the method validation. The exact values used must be optimized for each instrument.
3. The recommended chromatographic systems were found to be optimal for the types of instrument used for the method validation. Different chromatographic systems might be necessary to be developed for a different type of instrument.

Method B: (Used for the Separation of M800H01 and M800H02 for the Confirmatory method)

Instrument:	PE Sciex API 3000 Biomolecular Mass Analyzer		
Inlet [HPLC System]:	PE Series 200 Micro Pump system with Series 200 Autosampler		
Software Version:	Analyst 1.1		
Column:	Inertsil ODS-3, 5 μ, 150 X 2.1 mm, [GL Science]		
Injection:	Typically 10 μL		
Mobile Phase:	A = water with 0.1 % formic acid and 4 mM ammonium formate B = methanol with 0.1 % formic acid and 4 mM ammonium formate		
[Gradient]	**Time**	**Composition (%)**	
	(minute)	**A**	**B**
	0.0	95	5
	1.0	95	5
	3.0	75	25
	5.0	75	25
	7.0	10	90
	9.0	95	5
	9.1	95	5
	9.1	95	5
Flow Rate:	500μL/minute		

Analytes	Expected Retention Times (minutes)	Transitions (m/z):	
		Quantitation ion	Secondary ion
M800H01	7.86	487.0 → 365.9	487.0 → 445.0
M800H02	7.93	487.0 → 335.0	487.0 → 445.0
Ionization Mode:	Positive ion; Turbospray (500^{o}C)		

Method C : (Used for Independent Laboratory Validation)

Instrument:	Applied Biosystems API 4000 LC/MS/MS with Analyst 1.4.1 Software		
Inlet [HPLC System]:	Agilent 1200 HPLC System with Binary Pump, High Performance Autosampler and Heated Column Compartment set at 50ºC		
Column:	Phenomenex Columbus C18, 5 μ, 100 X 2.1 mm, [P/N 00D-4108-B0]		
Injection:	Typically 20 μL		
Mobile Phase:	A = water with 0.1 % formic acid and 4 mM ammonium formate B = methanol with 0.1 % formic acid and 4 mM ammonium formate		
[Gradient]	**Time (minute)**	**Composition (%)**	
		A	**B**
	0.0	95	5
	0.5	95	5
	3.0	10	90
	6.0	10	90
	6.1	95	5
	9.0	95	5
Flow Rate:	500μL/minute		

Analytes	Expected Retention Times (minutes)	Transitions (m/z):	
		Quantitation ion	Secondary ion
BAS 800 H	3.26	501.0 → 348.9	501.00 → 459.0
M800H01	3.21	487.0 → 365.9	487.0 → 445.0
M800H02	3.23	487.0 → 335.0	487.0 → 445.0
M800H07	3.10	381.0 → 229.0	381.0 → 338.96
M800H08	3.26	503.1→ 351.1	503.6 → 197.9
M800H015	3.14	480.0 → 420.1	480.0 → 310.0
M800H022	3.21	521.00 → 369.0	521.00 → 172.0
Ionization Mode:	Positive ion; Turbospray (500°C)		

3. Analytical Procedures (Continued)

3.8 Calibration Procedures

Calculation of results is based on peak area measurements using a calibration curve. The calibration curve is obtained by direct injection of BAS 800 H, M800H01, M800H02, M800H07, M800H08, M800H015 and M800H022 mix standards for LC/MS/MS in the range of 0.5 pg/μL to 5.0 pg/μL. In a given injection run, the same injection volume is used for all samples and standards. Typical standard amounts injected on-column range as follows: 10, 20, 50, and 100 pg.

Calibration curves are prepared by plotting the peak area versus the weight using a linear least squares working curve in the form of y = bx + c. The transitions monitored are m/z 501.0 → 348.9 (Quantitation ion), 487.0 → 365.9, 487.0 → 335.0, 381.0 → 229.0, 503.2→ 351.1, 480.0 → 420.1, and 521.00 → 369.0 for analytes BAS 800 H, M800H01, M800H02, M800H07, M800H08, M800H15 and M800H22, respectively.

A correction of observed MRM transition 503.2 to 351.1 for the quantitation of metabolite M800H08 should be made due to the contribution of the chlorine isotope ^{37}Cl from the parent BAS 800H, since these two analytes are not chromatographically separated. See detail explanations and calculations in **Appendix B**. This correction is only applied for the quantitation of unknown treated samples. For procedural fortification samples, all analytes are fortified in equal amount and the changes in recoveries are insignificant while a wide range (70 -120%, according to the guideline) of recoveries are acceptable.

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” on column retention time of the analytes before injecting the first sample of an analytical series.

3.9 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 each for all analytes. The limit of detection was estimated at 20% of the limit of quantitation, equivalent to 0.002 ppm each for all analytes. Therefore, at the LOQ, if the amount of analyte is 10 pg on column, the LOD is 2 pg on column. The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

4. CALCULATION OF RESULTS

4.1 Principle

Calculation of results is based on peak height or area measurements.

For the procedural recoveries, the sample weight will be considered 100 mg (0.100 g) in the final calculation of residues [μg/g (ppm)]. The method requires that the sample weight to be 100 ± 10 mg for fortification samples. The recovery is the percentage of the fortified amount (μg or ng), which is recovered through the method and the weights cancels out, as shown in the equation below, during the final calculation step.

The recoveries and residues of all analytes in μg/g (ppm) are calculated with the following formulas:

Residue in ppm = ng found per injection/ mg injected

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

ng found per injection = Amount of analyte calculated from calibration curve/1000

Standard curve: pg = $\frac{\text{Peak Area - intercept}}{\text{slope}}$

mg injected = $\frac{\text{Sample weight (0.1 g) extracted X μL injected X Dilution factor (F1)}}{\text{Final extraction volume (0.8 mL)}}$

Dilution Factor (F1) = 1, 0.1 and 0.01 for 0.01, 0.1 and 1.0 ppm fortification samples, respectively

5. TIME REQUIREMENT FOR ANALYSIS

The time required for a set of 13 samples (10 fortified, 2 controls and one reagent blank) is approximately 2 person-hours, provided that no special problems arise, such as matrix interference. The extraction using 96-well plates for more samples will not require additional time, except for weighing the samples (1 minute/sample).

6. CONFIRMATORY TECHNIQUES

The method of determination is LC/MS/MS, which is a highly selective and self-confirmatory detection technique. However, two ions were monitored during analysis for peak confirmation.

7. POTENTIAL PROBLEMS

In case of clay soil, the soil marc has to be broken completely after the first centrifugation in the extraction step in order to obtain acceptable recovery.

The glassware used for the method should be thoroughly rinsed with methanol to prevent contamination.

Peak enhancement could be a potential problem without sufficient sample clean-up. It is highly recommended to perform instrument check routinely during LC/MS/MS analysis for standard peak enhancement or suppression. The instrument check sample is basically prepared by adding known amount of standard to the control matrix at the limit of quantitation (0.01 ppm level). It is recommended to clean the LC/MS thoroughly, if peak enhancement or suppression has been observed. Some of the cleaning procedure included exhaustive cleaning of the hardware, such as skimmer, fused silica for sample introduction, and several gradient systems to wash the column.

8. SAFETY AND HEALTH CONSIDERATIONS

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

FIGURE 1: FLOW DIAGRAM OF ANALYTICAL METHOD NO. D0503 IN SOIL