VALIDATION OF BASF METHOD No. D0301:

I. INTRODUCTION AND SUMMARY

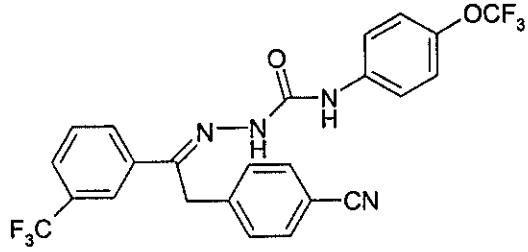
A. PURPOSE OF STUDY

BAS 320 I is a new insecticide that will be used for cotton and potato in the US and Europe. For registration of the insectiicide and for establishing the DT50/90 values from field dissipation studies in 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 D0301. Recovery ranges and standard deviations were determined from fortified control soil samples. Recoveries of BAS 320 I and its its metabolites, p-[m-(Trifluoromethyl)-phenacyl]benzonitrile (M320I04), p-Cyanobenzoic acid (M320I06) and 4-[5-hydroxy-3-oxo-4-{4-(trifluoromethoxy)phenyl}-6-{3-(trifluoromethyl)phenyl}-2,3,4,5-tetrahydro-1,2,4-triazin-5-yl]benzonitrile (M320I23) were determined in four soil types. The method No. D0301 allows the determination of BAS 320 I and its metabolites, M320I04, M320I06, and M320I23 with the required limit of quantitation (0.01 ppm) in soil.

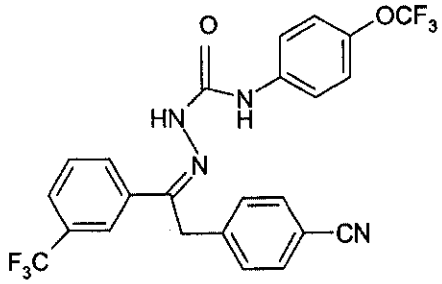
II. MATERIALS/METHODS

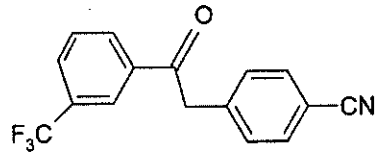
A. TEST AND REFERENCE SUBSTANCES

Fortification Compounds

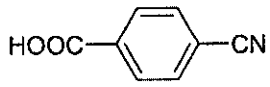
BASF Code Name:	BAS 320 I (E-isomer)
BASF Registry Number:	4102472 (CL 814027)
CAS Number:	139968-49-3
Chemical Name:	2-[2-(4-Cyanophenyl)-1-[3-trifluoromethyl)-phenyl]-ethylidene]-N-[4-(trifluoromethoxy)-phenyl]-hydrazinecarboxamide (E)
Molecular Formula:	$C_{24}H_{16}F_6N_4O_2$
Molecular Weight:	506.4
Appearance:	White powder
Water Solubility:	0.57 µg/L
Lot No.:	AC12145-19C
Purity:	98.7%
Structural Formula:	

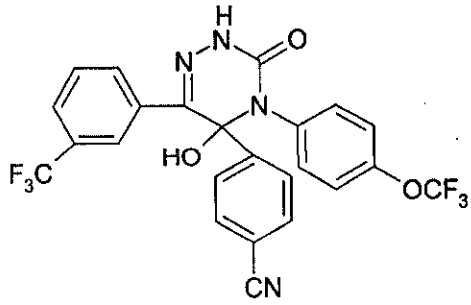
II. MATERIALS/METHODS (Continued)

BASF Code Name:	BAS 320 I (Z-isomer)
BASF Registry Number	4102572 (CL 399260)
CAS Number:	139970-56-2
Chemical Name:	2-[2-(4-Cyanophenyl)-1-[3-trifluoromethyl)-phenyl]-ethylidene]-N-[4-(trifluoromethoxy)-phenyl]-hydrazinecarboxamide (Z)
Molecular Formula:	$C_{24}H_{16}F_6N_4O_2$
Molecular Weight:	506.4
Appearance:	White powder
Water Solubility:	1.22 µg/L
Lot No.:	AC12705-150-P2
Purity:	96.9%
Structural Formula:	

BASF Code Name:	M320I04
BASF Registry Number:	4096485 (CL 397864)
CAS Number:	146653-56-7
Chemical Name:	p-[m-(Trifluoromethyl)-phenacyl]benzonitrile
Molecular Formula:	$C_{16}H_{10}F_3NO$
Molecular Weight:	289.3
Appearance:	Reddish brown powder
Lot No.:	AC12705-149-P
Purity:	97.0%
Structural Formula:	

II. MATERIALS/METHODS (Continued)

BASF Code Name:	M320I06
BASF Registry Number:	121464 (CL 25945)
CAS Number:	619-65-8
Chemical Name:	p-Cyanobenzoic acid
Molecular Formula:	$C_8H_5NO_2$
Molecular Weight:	147.13
Appearance:	White powder
Lot No.:	AC12859-10
Purity:	93%
Structural Formula:	

BASF Code Name:	M320I23
BASF Registry Number:	4984051
Chemical Name:	4-[5-hydroxy-3-oxo-4-{4-(trifluoromethoxy)phenyl}-6-{3-(trifluoromethyl)phenyl}-2,3,4,5-tetrahydro-1,2,4-triazin-5-yl]benzonitrile
Molecular Formula:	$C_{24}H_{14}F_6N_4O_3$
Molecular Weight:	520.39
Lot No.:	2059004
Appearance:	White powder
Purity:	98.8%
Structural Formula:	

Reference Standards (used for calibration)

Same as fortification compounds (section IIA)

Standard substances are stored in a freezer (<-5°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.

II. MATERIALS/METHODS (Continued)

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/MS standard solutions was 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	STABILITY (DAYS)
Stock solutions of BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 in methanol	93
Fortification solutions in methanol for all analytes	38
LC-MS calibration standards in Methanol-water, 80:20, v/v[1]	29
Soil extract containing BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 (Section 3.2.4)[1]	10
Soil extract containing BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 (Section 3.3.1)[1]	10

[1]Technical procedure of Method D0301 (Appendix A, page 58)

B. TEST SYSTEM

The test system consisted of untreated soil samples obtained from trial sites of Field dissipation study (BASF Study No. 67702) conducted in the US and Canada to validate method D0301. Different soil types and depths were used to validate this method. Soil samples were obtained from Idaho and California (**Reference 2a**) sites and were identified as BASF Residue Control Number (RCN) 2000503, and 2000504, respectively. German soil 2.2 was also used to validate this method (**Reference 2b**). Soil characterization data for soil samples used in this study are summarized in **Table VI**. The detailed soil characterization data is provided in **Appendix C**.

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 with dry ice. Homogenized soil samples are stored frozen (<-5°C) before analysis, allowing the dry ice to dissipate.

II. MATERIALS/METHODS (Continued)

D. EXPERIMENTAL DESIGN

Control soil samples were fortified by applying standard solutions directly to the soil prior to extraction with an aid of a volumetric pipet. The fortified control samples were analyzed to determine the recoveries of BAS 320 I and its metabolites, M320I04, M320I06 and M320I23. The validation sets consisted of a reagent blank, two unfortified controls, five controls fortified at 0.01 ppm (Limit of Quantitation) and five controls fortified with 0.1 ppm (ten times of Limit of Quantitation). All of these sets were subsequently analyzed with the method. Analyses of four sample sets (four soil different soil types) were conducted.

E. METHOD OF ANALYSIS

BASF Analytical Method D0301 was developed to determine the residues of BAS 320 I and its metabolites, M320I04, M320I06 and M320I23 in soil matrices using LC-MS/MS. The method was designed to determine the residues as individual analytes and was 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 10 g soil sample aliquot was extracted by shaking twice with methanol, followed by methanol-water, 50:50, v/v. The volume of the combined extract was adjusted to 100 mL. An aliquot of the extract (10%) was concentrated to a volume of 2-3 mL. The concentrated extract was then transferred to a 5 mL volumetric flask to adjust the volume with methanol for HPLC-MS/MS determination.

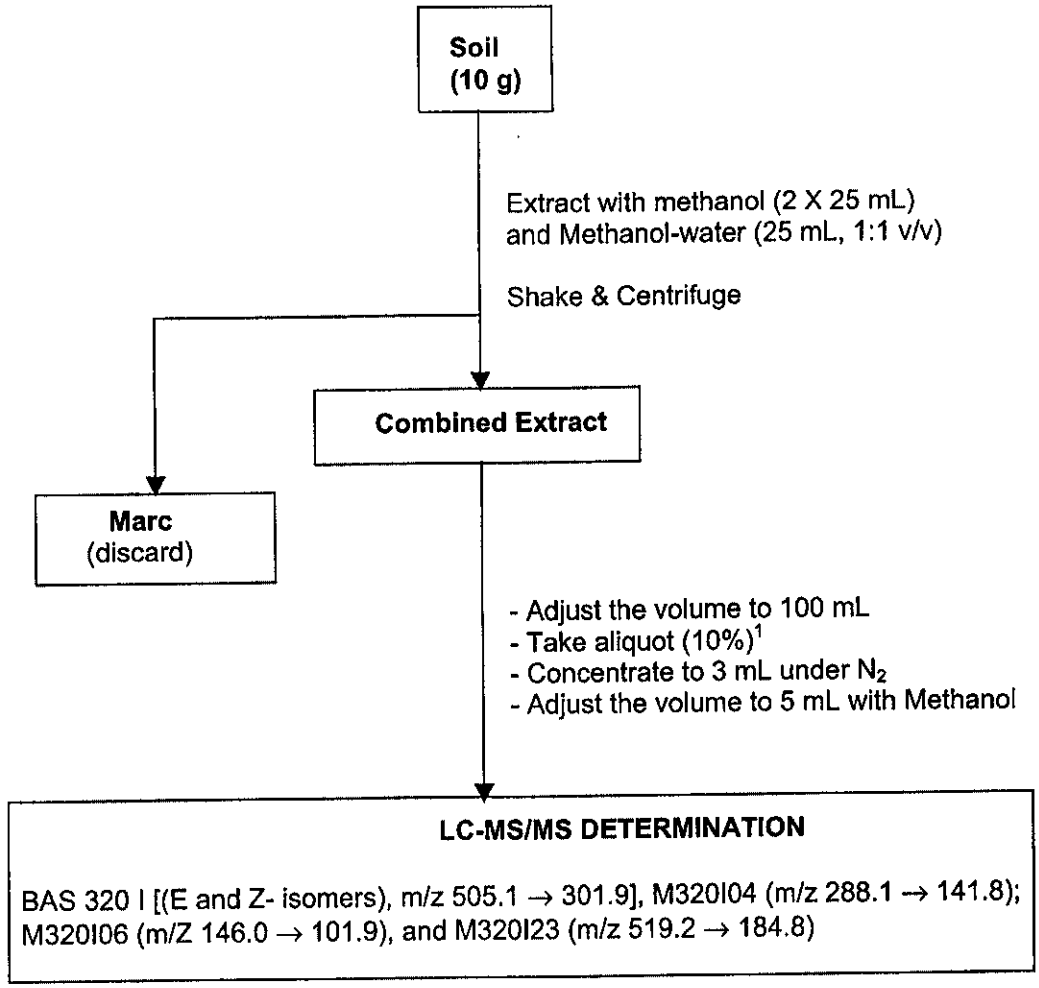
An Alternative sample preparation procedure was also used (Sections 3.2.5 and 3.3.1) in the validation. From the combined extract above, an aliquot (6%) was concentrated to dryness. The dry residue was then dissolved with methanol-water (80:20, v/v, 3 mL) for HPLC-MS/MS determination.

The LC-MS/MS quantitation was based on the following transitions as shown in the table below:

Analytes	Transitions
BAS 320 I (E and Z-isomer)	m/z 505.1→301.9
M320I04	m/z 288.1→141.8
M320I06	m/z 146.0→101.9
M320I23	m/z 519.2.1→184.8

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**.

Figure 1: Flow Diagram for Analytical Method No. D0301 in Soil [BAS 320 I (E and Z-isomer), M320I04, M320I06 and M320I23]



[1]Alternative sample Preparation: Mainly used for Sandy loam soil

- Take aliquot (6%)
- Evaporate to dryness under N_2
- Re-dissolve with Methanol-water (8:2 v/v), 3 mL

Figure 2: Typical Recovery Calculation (BASF Method D0301)

Sample Number 83435-4-C: Control Soils Fortified with 0.01 ppm of BAS 320 I and its metabolites, M320I04, M320I06 and M320I023.
Sample Number 83435-4-A: Control Soils unfortified.

Calculations are shown only for BAS 320 I. Following equations were used to calculate procedural recoveries (%):

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

Residue (ppm) = (ng value calculated from calibration curve)/mg sample injected

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

Calibration curve: ng = (Peak Area – intercept)/slope

$$\textbf{mg injected} = \frac{\text{Sample weight (10 g) extracted}}{\text{Final extraction volume (100 mL) [Section 3.2.4]}} \times \mu\text{L injected} \times \text{Aliquot factor (F1} \times \text{F2)}$$

$$\textbf{First Aliquot factor (F1)} = \frac{\text{Aliquot taken [Section 3.2.5]}}{\text{Final volume [Section 3.3.1]}} = \frac{10\text{ mL}}{5\text{ mL}} = 2.0$$

(Samples with residue at the limit of quantitation, 0.01 ppm)

Second Aliquot factor (F2) = 1, 0.1 and 0.01 for 0.01, 0.1 and 1.0 ppm fortification samples, respectively

Slope (m): 13700 Intercept (b): 22600

$$\text{ng (BAS 320 I) calculated from curve} = \frac{\text{Peak Area - (-22600)}}{13700} = \frac{282000 - (22600)}{13700}$$

= 18.9 pg = 0.0189 ng

$$\text{mg sample injected} = \frac{10.0\text{ (g)} \times 10\text{ (µL)} \times 2.0\text{ (F1)} \times 1.0\text{ (F2)}}{100\text{ (mL)}} = 2.0\text{ mg}$$

Sample Residue (ppm) = (0.0189)/2 = 0.000945 ppm

Sample Residue (ppm) for unfortified control = 0 ppm

$$\text{Percent Recovery} = \frac{(0.000945\text{ ppm} - 0\text{ ppm})}{0.01\text{ ppm}} \times 100 = 95\%$$

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.

1. Introduction

1.1 Scope of the method

BAS 320 I is a new insecticide that will be used for cotton, potato and other crops in the US and Europe. For registration of the insecticide 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 is developed. The method D0301 allows the determination of BAS 320 I and its metabolites with the required limit of quantitation in soil.

2. Materials

Standard substances are stored in a freezer (<-5^{0}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

Description of Test and Reference substances used in the validation study is provided in pages 11 through 13.

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	
3.2.1-3.2.3	Centrifuge	Refrigerated Centrifuge Model CS-6KR	Beckmann	
3.1, 3.2	Centrifuge Tubes (Teflon®)	50 mL	VWR	21009-477
3.2.1-3.2.3	Centrifuge Adapter	for 50 mL tubes	VWR	
Various	Cylinder, Graduated	Various sizes	Various	
3.2.1-3.2.3	Filter paper	15.0 cm, Whatman 4	VWR	1004070
Various	Flask, Erlen Meyer, 24/40	1000 mL	Various	
Various	Flask, Volumetric	100, 50, 25 ,10 and 5 mL	Various	
3.2.1-3.2.3	Funnel, long stem; glass	top i.d (65 mm), stem o.d.(8.0 mm) and stem length (65 mm)	Various	
3.3	Gelman PTFE acrodisc	0.45 um, 13 mm and 22 mm	Gelman Science	4543 & 4219
Various	Hot Plate, Magnetic Stirring		Various	
Various	Pipet, Volumetric	0.5, 1-10, 25 mL	Various	
Various	Pasteur Pipet, disposable	Various size	VWR	
3.2.5	Nitrogen-Evaporator	Model 112	Organomation Assoc.	11250
3.2.1-3.2.3	Laboratory Shaker	Model HS501-D	Janke and Kunkel	

2. Materials (Continued)

Method Step	Equipment	Size, Description	Manufacturer/ Supplier	Catalog Number
Various	Spatula		Various	
Various	Stopper, Teflon®	24/40	Various	
3.3	Syringes, plastic, disposable	1 mL	Various	
Various	Ultrasonic Bath	Model FS 7652H	Fisher Scientific	
3.3	Vials, HPLC Screw caps	9 mm; 2.0 mL	Sun Brokers International	502-130 502-235
Various	Vials, Collection, PTFE screw cap	1 oz	VWR	GLC-01008
Various	Vials, Amber Borosilicate	8 and 40 mL	VWR	224984 and 15900-018
Various	Votex mixer	Genie 2	Fisher Scientific Co	12-812
3.5	LC-MS	API 4000 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
Ammonium acetate	97%	E.M. Science	AX1220-32
Acetic Acid, glacial	99.7%	E.M. Science	AX0074-6
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: Methanol-water, 50:50, v/v Add 500 mL of methanol and 500 mL of water into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution. **Note:** Significant amount of heat may occur while mixing.	3.2.4
Solution II: Methanol-water, 80:20, v/v Add 800 mL of methanol and 200 mL of water into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution. **Note:** Significant amount of heat may occur while mixing.	3.3.1, 3.3.2 and 2.4.2.3
LC-MS Mobile Phase A: Water with 4 mM ammonium acetate and 0.1% acetic acid: Add 308 mg of ammonium acetate and 1 mL of acetic acid into a 1L volumetric flask. Dilute to the mark with water and mix well to ensure complete homogeneous solution.	3.5
LC-MS Mobile Phase B: Water with 4 mM ammonium acetate and 0.1% acetic acid: Add 308 mg of ammonium acetate and 1 mL of acetic acid into a 1L volumetric flask. Dilute to the mark with methanol and mix well to ensure complete homogeneous solution.	3.5

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 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.

2.4.2 Standard Solutions

2.4.2.1 Stock solutions of (1 mg/mL): BAS 320 I (E and Z-isomer), M320I04, M320I06 and M320I23

Prepare a 1.0 mg/mL stock solution individualy by weighing an appropriate amount of each analyte into a volumetric flask. Dissolve with methanol and dilute to mark. For example, to prepare a 25 mL stock solution of BAS 320 I (E-isomer), place 25.0 mg of BAS 320 I (E-isomer) into a 25 mL volumetric flask. Dissolve and dilute to mark with methanol. Sonicate and vortex to ensure a complete homogeneous solution.

2. Materials (Continued)

2.4.2.2 Mix Standards for Fortifications

Prepare a 10 µg/mL mixed standard solution for fortification by combining 1.0 mL of stock solutions of each analyte (2.4.2.1) into a 100 mL volumetric flask. Dilute to mark with methanol and vortex to ensure a complete homogeneous solution. Prepare serial dilution's of this combined solution as needed. Suggested concentrations of mixed standards for fortifications are 10, 1.0, 0.1 µg/mL, in methanol.

2.4.2.3 Injection Standard Solutions for LC-MS/MS Analysis (Calibration Standards): 20,10, 5.0, 2.5, and 1.0 pg/µL in Solution II.

Prepare a 20.0 pg/µL mixed injection standard solution by transferring an appropriate amount of the 1.0 µg/mL of mixed fortification solution (2.4.2.2) with a volumetric pipet into a volumetric flask and dilute to the mark with **Solution II**. Prepare serial dilutions of this solution as needed. Suggested concentrations of mixed standards are 10, 5.0, 2.5 and 1.0 pg/µL, in **Solution II.**

NOTE:
- Use amber bottles with Teflon®-lined screw caps as storage containers for standard solutions. **Avoid exposure of direct/indirect sunlight with these solutions. BAS 320 I (E-isomer) converts rapidly to Z-isomer in the sunlight.** It is also recommended to turn off the hood light, if possible, while working with these 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 blender or mill. Homogenized soil samples are stored frozen (<-5°C) before analysis. Weigh a 10 g or to the nearest tenth of a gram aliquot of the soil sample into a 50 mL Teflon® centrifuge bottle.

3.2 Fortification and Extraction

3.2.1 For the fortification samples, add an appropriate volume of mixed standard solution of BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 to the respective control sample by volumetric pipet. For example, for a 0.01 ppm fortification sample, pipet 1 mL of the 0.1 µg/mL mixed standard fortification solution (2.4.2.2) onto 10 g of control sample.

3. ANALYTICAL PROCEDURES (Continued)

3.2.2 Add 25 mL **methanol** into the centrifuge bottle containing the soil and vortex to mix the solvent to the soil. Place the centrifuge bottle horizontally in the shaker and shake at 300 RPM for 30 minutes. Centrifuge at 3000 rpm for 5 minutes at about 0-5°C. Attach a funnel fitted with Whatman filter paper into a 100 mL volumetric flask, transfer the supernatant by decantation through the funnel and collect. Rinse the filter paper with 5-10 mL of **methanol** (use a disposable pipet or wash bottle) and collect.

3.2.3 Add 25 mL aliquot of **methanol** on to the soil marc, and vortex to loosen the soil and to mix. Mix well to obtain a homogeneous suspension. Repeat the extraction step above (3.2.2) for 30 minutes. After centrifugation, transfer the supernatant into the above 100 mL volumetric flask by decantation through the funnel and collect. Rinse the filter paper with 5-10 mL of **methanol** (use a disposable pipet or wash bottle) and collect.

3.2.4 Add 25 mL aliquot of **Solution I** on to the soil marc, and vortex to loosen the soil and to mix. Mix well to obtain a homogeneous suspension. Repeat the extraction step above (3.2.2) for 30 minutes. After centrifugation, transfer the supernatant into the above 100 mL volumetric flask by decantation through the funnel. and collect. Rinse the filter paper with 5-10 mL of **methanol** (use a disposable pipet or wash bottle) and collect. Bring the volumes of the combined extracts to 100 mL with methanol. **Mix well and to obtain a homogeneous extract**.

NOTE:

- In case of sandy soil, centrifugation may be conducted at about 20 °C
- Centrifugation must be continued until the solid residue forms a compact pellet. If the soil extract is heavy (dark non-homogeneous appearance), centrifuge at 4000 rpm with a small number of samples (2-4 samples) to obtain an adequate centrifugation.
- In case of heavy clay or loamy soil, tap the bottom of the centrifuge tube repeatedly on a solid surface and vortex or use a flat head spatula to break the soil marc.
- Extreme caution should be taken while mixing the samples after volume adjustment. It is recommended initially to hand mix the extract in the volumetric flask with occasional venting by opening the stopper followed by the mixing with Vortex mixer. Vigorous mixing in stages will avoid sample loss due to pressure build-up. Also, not enough mixing will cause low recoveries.

3.2.5 Vortex the extract in the volumetric flask and transfer a 10 mL (10%) aliquot of the extract (3.2.4) into a 1 oz. vial (VWR Catalog number GLC-01008). Carefully evaporate the extract to about 2-3 mL using a nitrogen evaporator at about 0°C under positive flow of nitrogen. Remove the samples immediately after evaporation.

(Alternative) Vortex the extract in the volumetric flask and transfer a 6 mL (6%) aliquot of the extract (3.2.4) into a 1 oz. vial (VWR Catalog number GLC-01008). Carefully evaporate the extract to dryness using a nitrogen evaporator at about 30°C under positive flow of nitrogen. Remove the samples immediately after evaporation.

3. ANALYTICAL PROCEDURES (Continued)

NOTE:

- Alternative procedure mentioned in Section 3.2.5 could only be used, if necessary for sandy loam soil or German 2.2 soil.
- To determine how much 2-3 mL of solution represents in a 1 oz. vial during N_2 evaporation, it is suggested that the analyst add 3 mL of water into an 1 oz. vial (40 mL) prior to conducting step 3.2.5 and compare. This will give the analyst a "picture" of how much 3 mL of solution is and prevent over evaporation. Do not allow the samples to go to dryness. This causes low recovery. If the sample goes to dryness, do not proceed to the next step. Start over with a new extract aliquot.
- Aliquot amount may vary for different types of LC-MS instrument used for the analysis to achieve adequate sensitivity of all analytes at the limit of quantitation (0.01 ppm)
- Do not allow samples to remain dry longer than necessary. Proceed immediately to the next step. The evaporation step usually takes 35-45 minutes.
- It is recommended to clean the needles of the N-evaporator prior to each use. Use the following procedure:

a. Sonicate the needles in 50 % methanol and water
b. Rinse with methanol
c. Dry under slow stream of nitrogen

3.3 Sample Preparation for LC-MS/MS Analysis

3.3.1 For LC-MS/MS determination, transfer the samples from Section 3.2.5 into a 5 mL volumetric flask using a disposable pipet. Rinse and vortex the vial with methanol twice with about 1 mL of methanol and add to the volumetric flask. Adjust the volume with methanol. Swirl, sonicate and vortex to ensure a complete homogeneous solution.

(Alternative) For LC-MS/MS determination, add 3 mL of **Solution II** to the dry residue (Alternative Section 3.2.5). Swirl, sonicate and vortex to ensure a complete homogeneous solution. Typically the following procedures are used to prepare the samples for analysis:

3.3.2 **For control and 0.01 ppm fortifications**, filter the sample solution (3.3.1) through a syringe filter (a 0.45 micron Gelman membrane filter fitted to 1.0 mL disposable plastic syringe) and collect the filtrate (about 1-2 mL) into an injection vial. [Note: Use 22 mm syringe filter for extract with heavy clay].

For 0.1 ppm fortifications, take 1 mL of the sample solution (3.3.1) and dilute to 10 mL with **Solution II** or directly add 30 mL of **Solution II** to the dry residue (Section 3.2.5).

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

3.5. Instrumentation

Suggested LC-MS/MS Operating condition:

Instrument:	PE Sciex API 4000 Biomolecular Mass Analyzer
Inlet [HPLC System]:	PE Series 200 Micro Pump system with Series 200 Autosampler
Data System:	Analyst 1.1
Column:	Metachem Inertsil ODS-3, 5µ, 150 X 2.0 mm, [P/N 0396]
Injection:	Typically 10 µL
Mobile Phase: [Gradient]	A = water with 4 mM ammonium acetate and 0.1 % acetic acid B = methanol with 4 mM ammonium acetate and 0.1 % acetic acid **Time (min.)** **Composition** 0.0 70% A + 30% B 2.0 15% A + 85% B 5.0 15% A + 85% B 6.0 0% A + 100% B 7.5 0% A + 100% B 8.0 70% A + 30% B 11.0 70% A + 30% B Run every 11 minutes
Flow Rate:	400 µL/minute

	BAS 320 I (E-Isomer)	**BAS 320 I (Z-Isomer)**	**M320I04**	**M320I06**	**M320I23**
Expected Retention Times	6.56 minutes	5.44 minutes	3.80 minutes	3.06 minutes	4.09 minutes
Transitions:	505.1 → 301.9	505.1 → 301.9	288.1 → 141.8	146.0 → 101.9	519.2 → 184.8
Ionization Mode:	Negative ion for all analytes; Turbospray (425°C)				

3. ANALYTICAL PROCEDURES (Continued)

NOTE:

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 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.

3.6 Calibration Procedures

Calculation of results is based on peak area measurements using a calibration curve.

The calibration curve is obtained by direct injection of 10 µL of the mixed BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 standards for LC-MS/MS in the range of 1.0 pg/µL to 20.0 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, 25, 50, 100 and 200 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 505.1 → 301.9 for BAS 320 I (E and Z- isomers), and 288.1 → 141.8, 146.0 → 101.9, and 519.2 → 184.8 for M320I04, M320I06 and M320I23, respectively.

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.

3.7 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 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23. The limit of detection has not been determined, but set at 20 % of the limit of quantitation [e.g. at the LOQ (0.01 ppm), the LOD is 0.002 ppm]. 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

An example calculation is provided in **Figure 1.**

5. Time Requirement for Analysis

The time required for a set of 13 samples (10 fortified, 2 controls and one reagent blank) is approximately 6 person-hours, provided that no special problems arise, such as matrix interference.

6. Confirmatory Techniques

The method allows for the determination of BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 using LC-MS/MS which is a highly selective and self confirmatory detection technique. Therefore, no additional confirmatory technique is required.

7. Potential Problems

Avoid exposure of direct/indirect sunlight with the solutions containing BAS 320 I (E-isomer) due to rapid conversions to Z-isomer in the sunlight.

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.

Recovery of metabolite M320I04 will be reduced significantly if extract is concentrated to dryness (alternative sample preparation procedure) in most of the soil types.

Peak enhancement could be a potential problem form matrix build-up in the mass spectrometer. It is highly recommended to perform instrument check routinely during LC-MS/MS analysis for standard peak enhancement or suppression. During method development, it was observed that the response of the BAS 320 I could be enhanced or suppressed due to a heavy load of matrix (soil residue) in the LC-MS/MS analysis. As a result increased/decreased sensitivity (high/low signal) of the target analytes and chromatograms with choppy base lines were produced. These problems could be observed by an instrument check sample prior or during the actual sample analysis. The instrument check sample is basically prepared by adding known amount of standard to the control matrix at the limit of quantitation, (this case 0.01 ppm level). Once the problem is observed, it was absolutely required to clean the LC-MS thoroughly. 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.