1.0 INTRODUCTION

1.1 Purpose of the Study

The purpose of this study is to validate BASF Analytical Method D0506, "Method for Determination of Metconazole (BAS 555 F) and its Metabolites M11, M21, M30 and Triazol in Soil Using LC/MS/MS".

Although the method was written for calculations to be in ppm, laboratory calculations were done in ppb. The text of this report is consistent with the BASF Analytical Method D0506 (i.e. ppm). The tables and figures are kept as represented in the raw data (i.e. ppb).

2.0 SAMPLE HISTORY

As per protocol, Homogenized control soil from BASF Study No. 141527, [Oklahoma site, soil with high clay, depth 0-15 cm, (Valent study no., V-04-27027; 1u)] was provided by BASF. The control soil was received at ADPEN Laboratories on March 8, 2006. Upon receipt, the soil was stored in freezer E-16. The temperature range of freezer E-16 during the course of this study was -22 to -19°C. The control soil remained frozen during storage.

The reference substances, Metconazole (BAS 555 F), cis and trans, M11, M21, M30 and Triazol was received at ADPEN Laboratories, Inc. on March 7, 2006. Figure 1 shows structures and detailed information including lot number, purity, storage conditions, and expiration date of each compound; Appendix 2 contains copies of the COA's for the standards. The primary standards were stored in refrigerator E-9 and freezer E-9. The temperature range of refrigerator E-9 during the course of the study was 0°C to 6°C, and the temperature range of freezer E-9 during the course of the study was -20°C to -10°C. Standard solutions (concentrated, intermediate, and working) were stored at refrigerated temperature in refrigerator E-51. The temperature range of refrigerator E-51 during the course of this study was 2 to 7°C.

3.0 PROCEDURE - METHOD SYNOPSIS

BASF Analytical Method D0506 was used to determine residues of Metconazole (BAS 555 F), cis and trans, M11, M21, M30 and Triazol in soil. The following is a summary of the method:

The lowest level of fortification for all analytes was made at the method LOQ, 0.01 ppm. High-level fortifications were set at 10x LOQ (0.10 ppm).

Triazol fortification and calibration standards were prepared by serial dilution in 0.05% formic acid in DI water. Mixed fortification and calibration standards of BAS 555F (cis and trans), M11, M21 and M30 were prepared by combination and dilution in acetone, followed by serial dilution in 40:60 MeOH:0.05% formic acid in DI water.

Ten grams of sample were weighed into a 250 mL centrifuge bottle, and 100 mL of 9:1 MeOH:0.2N HCl was added to the sample. The sample was shaken and then centrifuged to separate the extract from the soil. A 10 mL aliquot of the extract was removed and transferred to a 50 mL polypropylene centrifuge tube. The remaining extract was decanted, and the soil sample was re-extracted with 100 mL of 1:1 MeOH:02.N HCl. After shaking and centrifugation, a 10 mL aliquot of the extract was removed and added to the first extract. The combined extract was then centrifuged, and labeled Extract A. For Triazol analysis, 2 mL of Extract A was transferred to a tube, and 1 mL of HPLC water and 2 mL of dichloromethane were added to the extract. The mixture was vortexed, and the DCM layer was removed. The remaining aqueous layer was evaporated to dryness using the N-Evaporator at 45 degrees C. The residue was re-dissolved in 2 mL of HPLC water, and the solution was vortexed and centrifuged. The solution was then vialed for LC/MS/MS analysis. For all other analytes, 2 mL of Extract A was transferred to a tube, and diluted with 3 mL of 40:60 MeOH:0.05% formic acid in HPLC water. The solution was then vortexed and vialed for LS/MS/MS analysis. Parameters for the LC/MS/MS analysis are detailed in Table 2.

4.0 LIMIT OF QUANTITATION AND DETECTION

The limit of quantitation (LOQ) of the method is the lowest fortification level tested (0.01 ppm) for BAS 555F (cis and trans), M11, M21, M30 and Triazol. The detector response for the standard equivalent to the LOQ level is ten times the background noise; therefore, the 0.01-ppm fortification level is an acceptable limit of quantitation. The detection limit, defined as the smallest standard injected, was 0.1 ng/mL. This standard was at least three times the background noise for all compounds.

5.0 CALIBRATION, CALCULATIONS AND STATISTICS

Residues of BAS 555F (cis and trans), M11, M21, M30 and Triazol were quantitated by external calibration. A calibration curve for each analyte was generated by plotting the detector's response in peak area versus nanograms of standard injected. Four calibration levels were listed in the method, but other calibration schemes may be used as indicated in the method. The analyses were conducted using six calibration levels. Although duplicate injections of each of the four calibration levels are shown in the calibration curves in the method, a single injection of the expanded calibration levels was utilized for this study. The data system derived an equation for the fit of the standard curve and this equation was used to calculate intercept and slope of the linear regression curve. Peak integration and quantitation were performed by a computer using Analyst® version 1.4.1 software. Recovery results were computed for each set of samples by Microsoft's Excel® and reported in a spreadsheet data report. Equations used for quantitation are presented in Figure 2. Statistical treatment of the data included calculation of averages and standard deviations.

TABLE 2. Typical LC-MS/MS Instrument Parameters for D0506

LC-MS/MS Parameters for BAS 555F (cis and trans), M11, M21, and M30

HPLC Instrumentation:	Hewlett Packard 1100 Series, Instrument No. 20
Detector:	PESciex API 4000 (MS/MS)
Data Acquisition System:	Analyst version 1.4.1
Column:	Luna C-18, 5um, 150x6mm (SN: 336698-8)
Mobile Phase:	A: 1% Formic Acid in Water B: 1% Formic Acid in Acetonitrile
Injection Volume:	20 uL

Step Table:

Step	Total Time (min)	Flow Rate (µL/min)	A (%)	B (%)
0	0.00	1000	90	10
1	6.50	1000	30	70
2	10.50	1000	10	90
3	10.60	1000	90	10
4	13.00	1000	90	10

MS Settings:

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	CE	CXP
BAS 555 F	320.10	70.10	400	95	53	8
M11/M21	336.10	125.10	400	70	40	7.7
M30	334.10	111.10	400	85	76	17

Curtain Gas: 10
Ionization Potential: 5500
Source Temperature: 600
Gas 1: 20
Gas 2: 50
Exit Potential: 10

LC-MS/MS Parameters for Triazol

HPLC Instrumentation:	Hewlett Packard 1100 Series, Instrument No. 20
Detector:	PESciex API 4000 (MS/MS)
Data Acquisition System:	Analyst version 1.4.1
Column:	Hypercarb, 3um, 50x4.6mm, (SN: 0361520A)
Mobile Phase:	A: 1% Formic Acid in Water B: 1% Formic Acid in Methanol
Injection Volume:	30 uL

Step Table:

Step	Total Time (min)	Flow Rate (µL/min)	A (%)	B (%)
0	0.00	800	95	5
1	2.00	800	95	5
2	2.50	800	10	90
3	5.00	800	10	90
4	5.10	800	95	5
5	7.00	800	95	5

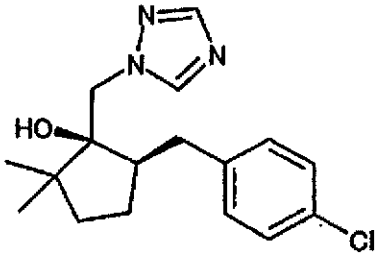
MS Settings:

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	CE	CXP
Triazol	70.10	43.20	1000	56	35	4

Curtain Gas: 10
Ionization Potential: 5000
Source Temperature: 600
Gas 1: 20
Gas 2: 20
Exit Potential: 10

10.0 FIGURES

FIGURE 1. Structure of the Test and Reference Substances

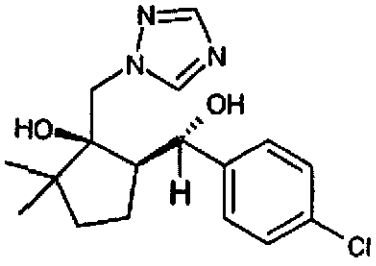


Substance Name:	BAS 555F cis
IUPAC Name:	(1RS,5RS-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular formula:	$C_{17}H_{22}ClN_3O$
Lot Number:	AC8879-136A
BASF Number:	4079468
Document Number:	115850-27-6
Purity:	98.8% (99.3% upon re-certification)
Storage Conditions:	Frozen
Expiration Date:	2/7/06 (5/1/16 upon re-certification)

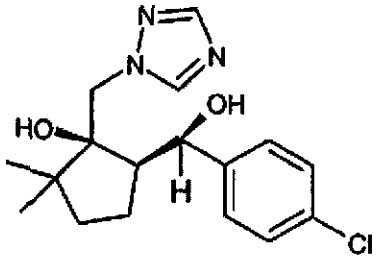
Substance Name:	BAS 555F trans
IUPAC Name:	(1RS,5SR-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular formula:	$C_{17}H_{22}ClN_3O$
Lot Number:	AC9339-122A
BASF Number:	4079654
Document Number:	115850-28-7
Purity:	98.6% (99.1% upon re-certification)
Storage Conditions:	Frozen
Expiration Date:	2/7/06 (6/1/1

6 upon re-certification)

FIGURE 1. Structure of the Test and Reference Substances (Continued)

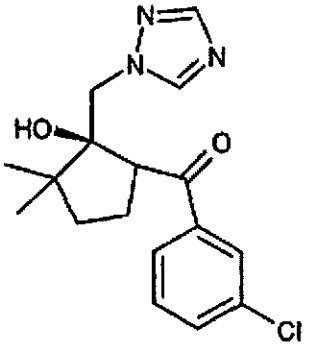


Substance Name:	KNF-474-M-11, R Benzylic Alcohol (M11)
IUPAC Name:	(1RS,5SR-5-[R-(4-chlorobenzyl)(hydroxyl) methyl]-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular formula:	$C_{17}H_{22}ClN_3O_2$
Lot Number:	AS2106a
BASF Number:	4111112
Document Number:	N/A
Purity:	98.5%
Storage Conditions:	Frozen
Expiration Date:	7/12/06

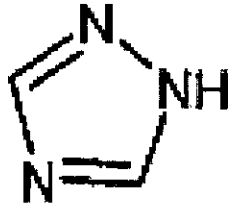


Substance Name:	KNF-474-M-21, R Benzyl Alcohol (M21)
IUPAC Name:	(1RS,5RS-5-[R-(4-chlorobenzyl)(hydroxyl) methyl]-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular formula:	$C_{17}H_{22}ClN_3O_2$
Lot Number:	AS2110a
BASF Number:	4558878
Document Number:	N/A
Purity:	98.3%
Storage Conditions:	Frozen
Expiration Date:	7/13/06

FIGURE 1. Structure of the Test and Reference Substances (Continued)



Substance Name:	KNF-474-M-30, R Benzyl Ketone (M30)
IUPAC Name:	Methanone, 4-chlorophenyl-[2-hydroxy-3,3-dimethyl-2-(1H-1,2,4-triazol-1-ylmethyl) cyclopentyl]-, cis-(±)-(9CI)
Molecular formula:	$C_{17}H_{20}ClN_3O_2$
Lot Number:	AS2111a
BASF Number:	4110625
Document Number:	N/A
Purity:	98.4%
Storage Conditions:	Frozen
Expiration Date:	7/13/06



Substance Name:	BF 480-16 (Triazol)
IUPAC Name:	1,2,4-(1H)-Triazole
Molecular formula:	$C_2H_3N_3$
Lot Number:	AC10194-134
BASF Number:	87084
Document Number:	87084
Purity:	99.0%
Storage Conditions:	Frozen
Expiration Date:	3/1/12

FIGURE 2. Calculations for the Quantitation of D0506

Residue results are calculated by comparison to the standard curves obtained from a *linear regression analysis of the data found by the data system. The equation for the fit* of the standard curve was used to calculate intercept and slope of the linear regression curve. The intercept and the slope were used in the equation used for quantitation. Excel is used to calculate the ppb and percent recovery and to present the data in a report format. The following equations were taken from the method and were used for quantitation:

Residue in ppm (ppm Found) = $\frac{\text{pg found per injection}}{\text{mg injected}} \times \frac{\text{ng}}{1000\ \text{pg}}$

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

pg found per injection = Amount of Analyte calculated from calibration curve

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

mg injected = $\frac{\text{Sample weight extracted}}{\text{Final extraction volume}} \times (\mu\text{L injected}) \times (\text{aliquot factor})$

Aliquot factor = $\frac{\text{Aliquot taken}}{\text{Final volume}}$

As an example, calculations to obtain BAS 555F (trans) percent recovery in soil, values using sample 2K6-SOIL-F1 from work order 2K5-903-SOIL-ILV-1 are shown below:

a) ppb* = $\frac{0.00375}{0.00040}$ = 9.375 ppb

b) Percent recovery** = $\frac{(9.375 - 0.000)}{10} \times 100$ = 93.8%

* Residue detected values converted to ppm (x1000) for report text.

** Raw values used for Statistical Evaluation.
Recovery percent values rounded as whole number (i.e. 99%) in the text of the report.

1 INTRODUCTION

Metconazole is a fungicide utilized in crops. The current technical procedure allows the determination of BAS 555 F and its metabolites (M11, M21, M30 and Triazol) residues by quantitation of the each analyte at the required limit of quantitation (LOQ) of 10 ppb.

2 MATERIALS

Standard substances are stored in a refrigerator at around 4 °C until use. Information on the characterization of these substances is available from BASF Aktiengesellschaft, Agricultural Center, and Limburgerhof, Germany and Valent USA Corporation, Dublin, CA.

2.1 List of Abbreviation

HPLC	High Performance Liquid Chromatography
LOQ	Limit of Quantitation
LOD	Limit of Detection
DCM	Dichloromethane
MEOH	Methanol
FA	Formic Acid
MS	Mass Spectrometry

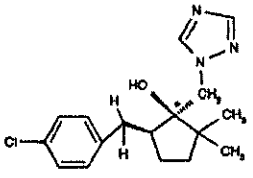
2.2 Fortification/Reference Substances

Analytical Standards are available from BASF Aktiengesellschaft, Agricultural Center, Limburgerhof, Germany and Valent USA Corporation, Dublin, CA.

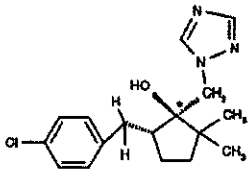
The following standard was used during method validation:

BASF Code Name:	BAS 555 F cis (CL 354,801)
BASF Registry Number:	4079468
Chemical Name:	(1RS,5RS-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular Formula:	$C_{17}H_{22}ClN_3O$
Molecular Weight:	319.80
Appearance:	White powder
Expiration date:	February 2006
Lot No./Batch-No.:	AC8879-136A
Purity:	98.8%
Structural Formula:	

BASF Code Name:	BAS 555 F trans (CL 354,802)
BASF Registry Number:	4079654
Chemical Name:	(1RS,5SR-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular Formula:	$C_{17}H_{22}CLN_3O$
Molecular Weight:	319.80
Appearance:	White powder
Expiration date:	February 2006
Lot No./Batch-No.:	AC9339-122A
Purity:	98.6%
Structural Formula:	

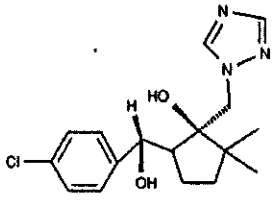


Cis-isomer



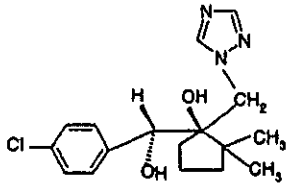
Trans-isomer

BASF Code Name:	KNF-474-M-11 (M11), R Benzylic Alcohol (CL 382390)
BASF Registry Number:	4111112
Chemical Name:	(1RS,5SR-5-[R-(4-chlorobenzyl)(hydroxy) methyl]-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular Formula:	$C_{17}H_{22}CLN_3O_2$
Molecular Weight:	335.8
Appearance:	White powder
Expiration date:	July 12, 2006
Lot No./Batch-No.:	AS2106a
Purity:	98.5%
Structural Formula:	

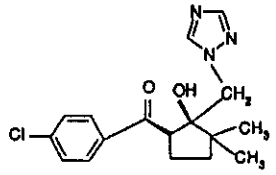


BASF Code Name:	KNF-474-M-21 (M21), R Benzyl Alcohol (CL 382391)
BASF Registry Number:	4558878
Chemical Name:	(1RS,5RS-5-[R-(4-chlorobenzyl)(hydroxy) methyl]-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol

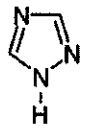
Molecular Formula:	$C_{17}H_{22}CLN_3O_2$
Molecular Weight:	335.8
Appearance:	White powder
Expiration date:	July 13, 2006
Lot No./Batch-No.:	AS2110a
Purity:	98.3%
Structural Formula:	



BASF Code Name:	KNF-474-M-30 (M30), R Benzyl Ketone (CL 382389)
BASF Registry Number:	4110625
Chemical Name:	Methanone, 4-chlorophenyl)-[2-hydroxy-3,3-dimethyl-2-(1H-1,2,4-triazol-1-ylmethyl) cyclopentyl]-,cis-(±)-(9CI) (CA INDEX)
Molecular Formula:	$C_{17}H_{20}CLN_3O_2$
Molecular Weight:	333.8
Appearance:	White powder
Expiration date:	July 13, 2006
Lot No./Batch-No.:	AS2111a
Purity:	98.4%
Structural Formula:	



BASF Code Name:	BF 480-16 (Triazol)(CL 196719)
BASF Registry Number:	87084
Chemical Name:	1,2,4-(1H)-Triazole
Molecular Formula:	$C_2H_3N_3$
Molecular Weight:	69.1
Appearance:	White powder
Expiration date:	March 01, 2012
Lot No./Batch-No.:	AC10194-134
Purity:	99%
Structural Formula:	



2.3 Equipment – Suggested Sizes/Suppliers, Manufactures

Method Step	Equipment	Size, Description	Manufacturer / Supplier	Catalog Number [1)]
Various	Spatula	Various	VWR	
Various	Volumetric Flasks	10, 50, 100-mL	VWR	
Various	Pipettes	0.5, 1, 2, 5, 10-mL	VWR	
Various	MicroMan Pipettes	10 µL-1000 µL	Rainin	M-25, M-50, M-250, M-1000
Various	General laboratory supplies	Various	Various	
Various	Disposable Pasteur Pipets	5 3/4 inch and 9 inch	VWR	14673-010 53283-915
2.5	Analytical Balance	Mettler AE-240 DeltaRange®, Weighing range 0-160 g	Mettler	
3.1	Bulk Floor Chopper	Homoloid Machine, Model J.	Fitzpatrick, Co.	
3.2	Syringe	100 µL	Hamilton	
3.2	Top-loading balance	Mettler PM 4800 DeltaRange®, Weighing range 0-3100 g	Mettler	
3.2	Wide-Mouth Centrifuge Bottle	250-mL	Nalgen/VWR	2189-0008
3.2	Shaker–reciprocal	Model KS501	IKA Labortechnik	
3.2	Centrifuge	RC5C With GSA rotor	Sorval Instrument	
3.2	Centrifuge	Allegra 6 R	Beckman	
3.2	Disposable Pasteur Pipets	5 3/4 inch and 9 inch	VWR	14673-010 53283-915
3.2	Liquid Scintillation vial	Borosilicate Glass	VWR	74511-20
3.2	Vortex	Vortex-Genie2	VWR	
3.2	Muti-Tube Vortexer	VX-2500	VWR	58816-116
3.2	Nitrogen Evaporator with water bath	N-Evap 112	Organomotion Associate, Inc,	
3.2	Test Tube	16 x 100 mm	Corning Incorp.	VWR
3.2	HPLC Cap	Individual Screw-thread, 9 mm	Sun-Sri	502235
3.2	HPLC Vials	1.8 mL, 12 x 32 mm	Agilent	5182-0716

Method Step	Equipment	Size, Description	Manufacturer / Supplier	Catalog Number [1)]
3.3	LC/MS/MS	API-4000 and API-4000QTrap	P/E Sciex	
3.3	HPLC Column	Luna C18(2), 150 mm x 4.6 mm, 5 µ	Phenomenex	00F-4252-EO
3.3	HPLC Column	Thermo Hydrocarb, 50 mm x 4.6 mm, 3 µ	Thermo Electron Corp.	0264538E
3.3	LC System, Auto Sampler and Pumps	P/E Series 200	Perkins Elmer	
4.2	Aluminum dish	57 mm	VWR	25433-008
8	Sample Concentrator	Tecne, DRI-Block Model DB.3	Techne Inc.	

[1)] NOTE: Equivalent equipment from other suppliers proven to be equivalent may be substituted.

2.4 Reagents and Chemical -- Suggested Sources

2.4.1 Chemicals

Chemical	Grade	Manufacturer	Catalog No.
Water	HPLC	B & J	365-4
Hydrochloric acid	GR 38%	E.M. Science	HX0603P-5
Acetone	HPLC	B & J	010-4
Formic Acid (FA)	98%	E.M. Science	EM-11670-1
Methanol	HPLC	B & J	230-4
Dichoromethane	HPLC	B & J	300-4

NOTE: Equivalent reagents and chemicals from other suppliers may be substituted if proven to be equivalent.

2.4.2 Solvent Mixtures

Standard preparation and sample solution (40%methanol: 60% of 0.05%FA in Water, v/v) Step 2.5 e.g. Mix 400 mL methanol and 600 mL of 0.05%FA in HPLC water
0.2 N HCl step 3.2 e.g. Take 16.7 mL of 12 N HCl to 1 liter volumetric flask and bring to volume with HPLC water and mix.
Extraction Solution (90 methanol: 10, 0.2 N HCl, v/v) step 3.2 e.g. Mix 900 mL Methanol and 100 mL of 0.2 N HCl.
Extraction Solution (50 methanol: 50, 0.2 N HCl, v/v) step 3.2 e.g. Mix 500 mL Methanol and 500 mL of 0.2 N HCl
0.05% formic acid in HPLC water (0.05 % formic acid: 99.95% mL HPLC water, v/v) step 3.2 e.g. Add 0.5 mL formic acid to 999.5 mL HPLC water

2.5 Standard Solutions

2.5.1 Standard Solution Storage and Stability

Standard solutions are stored in 4 oz. Amber bottles and kept refrigerated. Stock and fortification and calibration solutions of analytes prepared in acetone and aqueous organic have been shown to be stable for a period of at least 3 months in refrigerator (see Reference 1).

NOTE: Suggested standard concentrations are listed below. A different concentration scheme may be used and additional standards may be prepared as needed.

2.5.2 Preparation of Standard Solutions

cis-BAS 555 F Stock Solution, **1.0 mg/mL**

Prepare a **1 mg/mL** *cis*-BAS 555 F stock solution by weighing an appropriate amount of *cis*-BAS 555 F into a volumetric flask. Dissolve with acetone and dilute to mark. Record the concentration of *cis*-BAS 555 F in this solution after correcting for purity.

For example, to prepare a 10 mL stock solution, place 10.0 mg of *cis*-BAS 555 F into a 10 mL volumetric flask. Dissolve and dilute to mark with acetone.

trans-BAS 555 F Stock Solution, **1.0 mg/mL**

Prepare a **1 mg/mL** *trans*-BAS 555 F stock solution by weighing an appropriate amount of *trans*-BAS 555 F into a volumetric flask. Dissolve with acetone and dilute to mark. Record the concentration of *trans*-BAS 555 F in this solution after correcting for purity.

For example, to prepare a 10 mL stock solution, place 10.0 mg of *trans*-BAS 555 F into a 10 mL volumetric flask. Dissolve and dilute to mark with acetone.

KNF-474-M-21 (M21) Stock Solution, **1.0 mg/mL**

Prepare a **1 mg/mL** M21 stock solution by weighing an appropriate amount of M21 into a volumetric flask. Dissolve with acetone and dilute to mark. Record the concentration of M21 in this solution after correcting for purity.

For example, to prepare a 10 mL stock solution, place 10 mg of M21 into a 10 mL volumetric flask. Dissolve and dilute to mark with acetone.

KNF-474-M-11 (M11) Stock Solution, **1.0 mg/mL**

Prepare a **1 mg/mL** M11 stock solution by weighing an appropriate amount of M11 into a volumetric flask. Dissolve with acetone and dilute to mark. Record the concentration of M11 in this solution after correcting for purity.

For example, to prepare a 10 mL stock solution, place 10 mg of M11 into a 10 mL volumetric flask. Dissolve and dilute to mark with acetone.

KNF-474-M-30 (M30) Stock Solution, **1.0 mg/mL**

Prepare a **1 mg/mL** M30 stock solution by weighing an appropriate amount of M30 into a volumetric flask. Dissolve with acetone and dilute to mark. Record the concentration of M30 in this solution after correcting for purity.

For example, to prepare a 10 mL stock solution, place 10 mg of M30 into a 10 mL volumetric flask. Dissolve and dilute to mark with acetone.

BF 480-16 (Triazol) Stock Solution, **1.0 mg/mL**

Prepare a **1 mg/mL** Triazol stock solution by weighing an appropriate amount of Triazol into a volumetric flask. Dissolve with acetonitrile and dilute to mark. Record the concentration of Triazol in this solution after correcting for purity.

For example, to prepare a 10 mL stock solution, place 10 mg of Triazol into a 10 mL volumetric flask. Using vortex, dissolve and dilute to mark with acetonitrile.

Mixed Fortification Standards of *cis/trans*-BAS 555 F, M11, M21 and M30

Prepare a **10 µg/mL** mixed standard of *cis/trans*-BAS 555 F, M11, M21 and M30 solution by transferring 1mL of each of 1 mg/mL individual Stock solution into a 100 mL volumetric flask. Dilute to mark with acetone

Prepare a serial dilution with acetone to make **1 µg/mL** Mixed Fortification Standards.

Prepare another serial dilution with 40% methanol/60% of 0.05%FA in HPLC water to make a **0.1 µg/mL** Mixed pre-calibration Standard.

Fortification Standards of Triazol

Prepare a **10 µg/mL** standard Triazol solution by transferring 1mL of 1 mg/mL Triazol Stock solution into a 100 mL volumetric flask. Dilute to mark with 0.05% FA/HPLC water.

Prepare a serial dilution with 0.05% FA/HPLC water to make **1 µg/mL** Fortification Standard.

Standards for calibration of *cis/trans*-BAS 555 F, M11, M21 and M30

Prepare a **1 and 3 ng/mL** calibration standard solution by transferring 1 and 3 mL each of the 0.1 µg/mL fortification solution into a 100 mL volumetric flask. Dilute to mark with 40% methanol/60% of 0.05%FA in HPLC water. Prepare serial dilutions of these standards as needed. Suggested concentrations of standards in 40% methanol/60% of 0.05%FA in HPLC water for LC/MS/MS analyses are: **0.3 and 0.1 ng/mL**. Other concentration schemes may be used, if required.

Standards for calibration of Triazol

Prepare a **50 ng/mL** stock calibration standard solution by transferring 5 mL of the **1** µg/mL fortification solution into a 100 mL volumetric flask. Dilute to mark with 0.05% FA/HPLC water. Prepare serial dilutions of this standard. Suggested concentrations of standards in HPLC water for

LC/MS/MS analyses are: **5, 1, 0.5 and 0.25 ng/mL**. Other concentration schemes may be used, if required.

3 ANALYTICAL PROCEDURES

(Flow diagram included at the end of the this technical procedure)

3.1 Sample Preparation

Soil cores collected in the field and micromilled utilizing a Wretch Ultra Centrifugal Mill aided by liquid nitrogen cooling are stored frozen (<-5ºC) before analysis. .

Keep all samples frozen until ready for analysis. Allow the frozen soil samples to thaw completely in an air-tight container just prior to extraction.

3.2 Fortification and Sample Extraction

(See section 8 for Potential Problems)

a) Weigh 10 g (± 0.1g) of soil sample into a 250 mL bottle. For the fortification samples, add accurately an appropriate volume of standard solution to the respective control sample by syringe or volumetric pipette. For example, for a 10 ppb fortification sample, take accurately 0.1 mL of the 1 μg/mL standard solution onto a control sample and for 100 ppb fortification sample, take 0.1 mL of 10 μg/mL standard solution onto a control sample.

Note: If the sample amount is limited, the sample size and extraction solvent can be decreased to the half.

b) Use graduated cylinder to add 100 mL of the first extraction solution (90% Methanol:10% of 0.2 N HCl) into the sample bottle, seal with a polyethylene-lined screw cap. Shake at moderate speed for 60 minutes on the reciprocating shaker.

c) Centrifuge the sample for 10 minutes at approximately 5,000 rpm.

d) Transfer quantatively aliquot of the extract (e.g 10 mL) into a 50 mL centrifuge tube. Discard the entire remaining solution (care must be taken to not disturb the soil and remove all the remaining solution)(suggestion: use a 10 mL disposable pipette). Using a graduated cylinder, add 100 mL of second extraction solution (50% Methanol:50% of 0.2 N HCl) into the same sample bottle, seal with a polyethylene-lined screw cap. Shake at moderate speed for 20 minutes on the reciprocating shaker.

e) Centrifuge the sample for 10 minutes at approximately 5,000 rpm.

f) Mix quantatively equal volume of the second extraction solution (e.g 10 mL) into the same 50 mL centrifuge bottle containing the first extraction solution, cap and centrifuge at approximately 2000 rpm for 10 min.

g) Preparation of samples for LC/MS/MS

1. <u>For *cis/trans*-BAS 555 F, M11, M21 and M30</u>

Transfer 2 mL aliquot from step 3.2.f into 20 mL liquid scintillation vial. Add 3 mL of 40% methanol in 60% of 0.05% FA in HPLC water and vortex. Dilute the

sample further with 40% methanol in 60% of 0.05% FA, if required. Inject 10 µL into LC/MS/MS.

2. For Triazol

Transfer 2 mL of the extract from step 3.2.f into a test tube. Dry completely under nitrogen (gentle) and water bath (40-50° C). Add 2 mL HPLC water and cap it. Using a multi-tube vortexer (VX-2500), vortex extensively by for 4 min. Centrifuge at approximately 2000 rpm for 10 min. Dilute the sample further with HPLC water, if required. Inject 20 µL into LC/MS/MS.

3.3 LC/MS/MS Instrumentation and Conditions

3.3.1 For *cis/trans*-BAS 555 F, M11, M21 and M30

Instrument:	PE Sciex API 4000 Q Trap Mass Spectrometer			
Inlet [HPLC System]	PE 200 Micro Pump System + Perkin Elmer 200 Series Auto Sampler			
Column:	Luna C18 (2), 150 mm x 4.6 mm, 5 µm			
Injection:	10 µL			
Mobile Phase Gradient	Solution A : Water, 0.1% Formic Acid Solution B : Acetonitrile, 0.1% Formic Acid			
	Time (min.)	%Solution A	%Solution B	Switch Valve
	0.0	90	10	
	6.5	30	70	
	10.5	10	90	
	10.6	90	10	
	13.0	90	10	
Flow Rate:	1000 µL/minute			
Expected Retention Time	BAS 555 F ~9.80 cis ~ 9.43 trans	M11 ~ 8.27 M21 ~ 9.15	M30 ~ 9.04	
Ionization Mode:	Turbo Ion Spray – Positive MRM Mode			

Transitions:	BAS 555 F	M11, M21	M30	
Primary	320.1/70.1	336.1/125.1	334.1/111.1	
Secondary	320.1/125	336.1/109.2	334.1/139	

3.3.2 For Triazol

Instrument:	PE Sciex API 4000 Mass Spectrometer			
Inlet [HPLC System]	PE 200 Micro Pump System + Perkin Elmer 200 Series Auto Sampler			
Column:	Thermo Hypercarb 50 mm x 4.6 mm, 3 µm			
Injection:	20 µL			
Mobile Phase Gradient	Solution A : Water, 1% Formic Acid Solution B : Methanol, 1% Formic Acid			
	Time (min.)	%Solution A	%Solution B	
	0.0	95	5	
	2.0	95	5	
	2.5	10	90	
	5.0	10	90	
	5.1	95	5	
	7.0	95	5	
Flow Rate:	800 µL/minute			
Expected Retention Time	Triazol ~ 1.60			
Ionization Mode:	Turbo Ion Spray – Positive MRM Mode			
Transitions: Primary	Triazol 70.1/43.2			

NOTE:

1	The equipment listed was used for method development and validation. Other equivalent hardware may be used if proven to be equivalent.
2	The recommended chromatographic systems were found to be optimal for the types of instruments utilized for the method validation. Different chromatographic systems might be necessary to be developed for a different type of instrument or matrix.

3.4 Calibration Procedures

Calculation of results is based on peak area measurements (or peak height) using a calibration curve. The standard curve is obtained by direct injection of the BAS 555 F and its metabolites into LC/MS/MS in the range of 0.1 ng/mL to 5 ng/mL. In a given injection run, the same volume injection is used for all samples and standards. The calibration curves are obtained by plotting peak area or height, monitoring transitions m/z 320.1/70.1 for BAS 555 F (cis/trans), m/z 336.1/125.1 for M11/M21,m/z 334.1/111.1 for M30 and m/z 70.1/43.2 for Triazol. Other transition ions may be used if needed. The linear least squares working curve in the form $y = bx + c$ is used for the construction of the calibration curve. Each injection set should begin and end with standard injections, and each standard level should be injected at least in duplicate. The correlation co-efficient (r^2) must be ≥ 0.98

3.5 Limit of Quantitation and Limit of Detection

The limit of quantitation is defined as the lowest fortification level successfully tested. The limit of quantitation (LOQ) of the method is 10 ppb. The estimated limit of detection (LOD) is 2 ppb.

4. CALCULATION OF RESULTS

4.1 Principal

Calculation of results is based on peak area (or height) measurements. The residue of BAS 555 F and its metabolites is calculated from the calibration curve and the equations shown in Section 4.2.

4.2 Calculation of Residues

The residues of the analytes in the sample ng/g (ppb) are then calculated with the following formula.

$$\text{Residue (ppb) ng/g} = \frac{V_f \times A_A\,(pg) \times DF}{W \times A_F \times V_{inj}\,(\mu L)} \times \frac{1\ ng}{1000\ pg} \times \frac{1000\ \mu L}{1\ mL}$$

V_f	=	Final Volume (5 mL for BAS 555 F, M11, M21 and M30: 2 mL for Triazol)
A_A	=	Amount of analyte from calibration curve (pg)
W	=	Sample weight extracted (10 g)
A_F	=	Aliquot factor (2 mL/200 mL=0.01)
V_{inj}	=	Injection volume (10-20 µL)
1000	=	Conversion factor for pg to ng
1000	=	Conversion factor for µL to mL
DF	=	Dilution Factor

Moisture correction is not needed for the method validation study, however the residue results for soil analysis from field studies may need to be reported on a 'dry weight' basis. The procedure is suggested as follow:

Weigh 5 g of wet soil accurately into a weighed aluminum dish or other container and place into an oven (approximately 100° C) for at least 16 hours (overnight). Remove the container from the oven quickly and allow it to cool down to room temperature in a desiccators and then obtain the dry sample weight (g).

% Moisture=[wet sample wt (g)-dry sample wt (g)] x 100/5 (g)

Residue in ppb (dry sample wt.) = Residue in ppb in wet sample/[100-% moisture)/100].

4.3 Calculation of Recoveries

The recoveries of spiked analytes are calculated with the following formula:

$$\text{Recovery \%} = \frac{\text{Residue in fortified sample (ppb)} - \text{Residue in control (ppb)}}{\text{Amount analyte fortified (ppb)}} \times 100$$

FLOW CHART FOR THE ANALYTICAL METHOD

(*cis/trans*-BAS 555 F, M11, M21, M30 and Triazol)