1. INTRODUCTION AND SUMMARY

1.1. Purpose of Study

The study was conducted to generate recovery data for the validation of BASF Method Number D0506: Analytical Method for the LC/MS/MS Determination of BAS 555 F and its Metabolites M11, M21, M30 and Triazol in Soil. The method was used for analysis of soils from three soil dissipation studies (Reference 1,3,4).

1.2. Summary

The validation study was conducted in three types of soils, a North Dakota (ND) loamy soil, Oklahoma (OK) loamy sand soil and a Mississippi (MS) silty loam soil. The characterization data are provided with the respective soil dissipation study (Reference 1,3,4).

The validation was performed at two levels, the LOQ of 10 ppb and 100 ppb. Five soil aliquots were fortified at each level and each set included in addition a method blank and two unfortified soil aliquots as controls. The acidic aqueous/organic extraction of the soils was diluted prior to injection to LC/MS/MS. It was demonstrated that this scheme provided acceptable recoveries.

Standard solutions of BAS 555 F and its Metabolites M11, M21, M30 and Triazol were stable on storage in solution described in the method at least for about 3 months if kept refrigerated (Reference 1).

To test the stability of analytes in sample extracts, the samples extracted from OK and ND soils were reanalyzed about 18 and 41 days, respectively after the initial analysis with storage at refrigerator. The results indicated that the analytes are stable in the sample extract.

2. MATERIALS AND METHODS

2.1. Test and Reference Substances

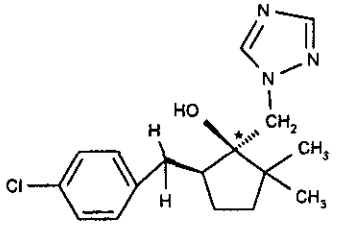
The following standard was used during method validation:

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:	

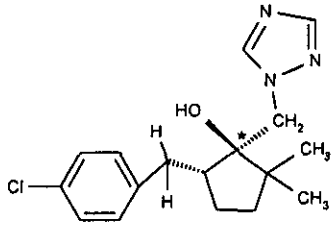
* ***Recertified in May 2006 with an expiration of 01 May 2016 (purity, 99.3%) This had no impact on the validity of the results of this study.***

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:	

* ***Recertified in May 2006 with an expiration of 01 June 2016 (purity, 99.1%) This had no impact on the validity of the results of this study.***

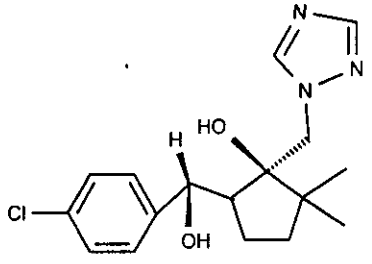


Cis-isomer

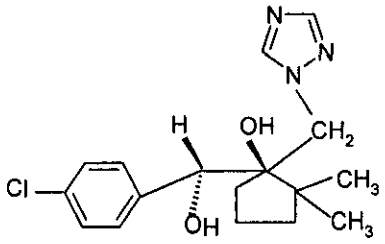


Trans-isomer

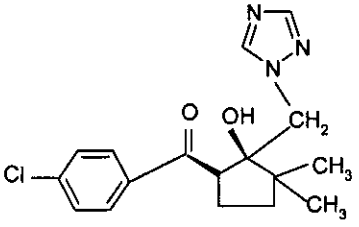
BASF Code Name:	KNF-474-M-11 (M11), R Benzylic Alcohol (CL 382390)
BASF Registry Number:	4111112
Chemical Name:	(1RS,5SR-5-[R-(4-chlorobenzyl)(hydroxy) methyl]-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular Formula:	$C_{17}H_{22}CLN_3O_2$
Molecular Weight:	335.8
Appearance:	White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



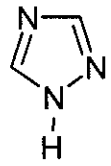
BASF Code Name:	KNF-474-M-21 (M21), R Benzyl Alcohol (CL 382391)
BASF Registry Number:	4558878
Chemical Name:	(1RS,5RS-5-[R-(4-chlorobenzyl)(hydroxy) methyl]-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol
Molecular Formula:	$C_{17}H_{22}CLN_3O_2$
Molecular Weight:	335.8
Appearance:	White 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:	



Neat standard substances were stored in the freezer (- 30 to 0 ° C) until use. BASF has retained a reserve sample of these chemicals, and has documentation at BASF Agro Research, Research Triangle Park, North Carolina, specifying the location of the synthesis and characterization information for these compounds.

2.2. Extraction Solutions

Soil samples were extracted from soil by shaking two times with acidified methanol water (90:10 methanol:0.2N HCl, v/v and 50:50 methanol:0.2N HCl, v/v).

3. TEST SYSTEMS

The following three control soils were used in this study:

A control loamy soil obtained from the ND site (Valent Study V-03-26235, Event SQ3, -7 DAA, 0-30 cm)(Reference 1).

A control loamy sand soil obtained from the OK site (Valent Study V-04-27027, Event SQ3, -6 DAA, 0-30 cm)(Reference 3).

A control silty loam obtained from the MS site (Valent Study V-03-26243, Event SQ3, -19 DAA, 0-30 cm)(Reference 4).

4. SAMPLE DESCRIPTION/IDENTIFICATION

Aliquots of soil were weighed out and identified with study number-master sheet number-consecutive number as each set was analyzed. Samples were also assigned a code, " Reagent blank" for the method blank, "utc" for a unfortified sample, "utc-10 ppb" for soils fortified at 10 ppb and "utc-100 ppb" for soils fortified at 100 ppb.

5. EXPERIMENTAL DESIGN

The analytes covered in the method validation were BAS 555 F and its Metabolites M11, M21, M30 and Triazol. For each of the three soils, aliquots of 10.0 g were fortified with BAS 555 F and its Metabolites M11, M21, M30 and Triazol at two different levels: five replicates at 10 ppb, the limit of quantitation (LOQ) and five replicates at 100 ppb. An analysis set included the ten fortified samples, two unfortified control soil samples and a method blank. An analysis set included the ten fortified samples, two unfortified controls and a blank. Because the method provides options for cleanup using dichloromethane cleanup, only for one site (ND), Triazol analysis was carried out with this option.

6. METHOD OF ANALYSIS

6.1. Method Summary

A copy of the final method is attached in Appendix II. Flow charts for the procedure for extraction, cleanup and quantitation of BAS 555 F and its Metabolites M11, M21, M30 and Triazol as well as typical chromatograms for this validation study are shown in the final method (Appendix II). Residue of Metconazole (BAS 555 F) and its Metabolites M11, M21, M30 and Triazol is extracted from soil by shaking two times with acidified methanol water (90:10 methanol:0.2N HCl, v/v and 50:50 methanol:0.2N HCl, v/v). After centrifugation, aliquot of the combined extract is either diluted for determination of the Metconazole (BAS 555 F) and its metabolites M11, M21, M30 or dried and then suspended in water for Triazol determination. Residue of Metconazole (BAS 555 F) and its metabolites are determined by direct injection by liquid chromatography/mass spectrometry/ mass spectrometry (LC/MS/MS) in the positive ion mode. Residue results are calculated using a calibration curve. The limit of quantitation (LOQ) of this method is 10 ppb. Quantitation is based on peak area measurements. Using the peak area (or height) measured for BAS 555 F and its Metabolites M11, M21, M30 and Triazol, the

amount of analyte in pg can be determined from the appropriate least squares calibration curve constructed using at least duplicate injections of four levels of calibration standards. The Limit of Detection (LOD) of this method is 2 ppb.

6.2. Method Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The limit of quantitation (LOQ) is defined as the lowest fortification level successfully tested in this validation study, which is 10 ppb for each analyte. For each analyte the LOD, as defined by signal to noise, is ≤ 1/5 the lowest standard. The lowest standard is 0.1 ng/mL, equivalent to 5 ppb. The LOD at 1/5 that amount is equivalent to 1 ppb. In the validation study, an area count resulting in calculated concentration in pg > 0 was measured.

6.3. Stability of Extracts

To test the stability of analytes in sample extracts, the samples extracted from OK and ND soils were reanalyzed about 18 and 41 days, respectively after the initial analysis with storage at refrigerator. The results indicated that the analytes are stable in the sample extract.

6.4 Method Accountability

Soil samples were extracted from soil by shaking two times with acidified methanol water (90:10 methanol:0.2N HCl, v/v and 50:50 methanol:0.2N HCl, v/v). Aqueous/methanol extraction scheme was used in the aerobic soil metabolism study (Reference 5); thus no method accountability study was needed.

Figure 1 Sample Calculations

Calculation of results is based on peak area measurements. Using the peak area measured for BAS 555 F (data set 060209ap.rdb) the amount of analyte in pg can be determined from the appropriate least squares calibration curve. In the validation study, no adjustment was made for soil moisture. Calculate the residue concentration in ppb for soil using the equation:

$$C_S = \frac{W_A \times V_F \times DF}{W_S \times V_I \times A_F \times 1}$$

W_A = Amount of analyte calculated from calibration curve (pg)

V_F = Final Volume after all dilutions (mL)

W_S = Sample weight extracted (g)

V_I = Injection volume (µL)

A_F = (aliquot used for dilution) / (total extract volume)

1 = Number remaining after all unit conversions

DF= Dilution Factor

To calculate W_A, the amount (in pg) of analyte BAS 555 H/trans from the calibration curve, a linear least squares curve was constructed (Master Sheet 138074-01).

File Type	Sample Name	Injection Vol.	Area 1st Inj.	Area 2nd Inj	Area 3rd Inj
Standard	0.1 ng/mL	10µL	758.17	744.29	728.11
Standard	0.3 ng/mL	10 µL	2008.3	2206.7	2207.9
Standard	1 ng/mL	10 µL	6885.4	8435.4	7528.8
Standard	3 ng/mL	10 µL	22108	25568	22786

Intercept, b	-162
Slope, m	787
Correlation Coeff.	0.9959

The area for sample 138074-01-04 fortified at 10 ppb was 1528.3. Using equation y = mx + b, solving for x:

$$x = \frac{(y - b)}{m} = \frac{1528.3 - (-162)}{787} = 2.15 \text{ pg } (= W_A)$$

Sample wt. = 10.0 g (=W_S), Final Vol. = 5.0 mL (= V_F), V_I = Injection volume (10 µL)
Aliquot factor = 2 mL/ 200 mL=0.01 (= A_F)

$$\text{Residue (ppb, wet)} = \frac{V_f \times A_a\,(pg) \times DF}{W \times A_f \times V_{inj}\,(\mu L)} \times \frac{1\ ng}{1000\ pg} \times \frac{1000\ \mu L}{1\ mL}$$

$$= \frac{5\ mL \times 2.15\ pg \times 1}{10.0\ g \times 0.01 \times 10.0\ \mu L}$$

$$= 10.75\ ppb$$

Recovery:

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

$$\text{\% Recovery} = \frac{(10.75-0) \times 100}{10} = 108\ \%$$

Sample ppb (dry weight basis):

Moisture correction was not done in the validation study.

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

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
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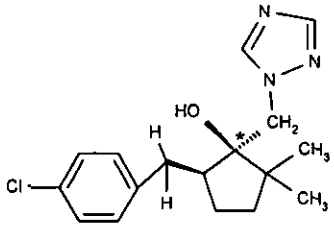
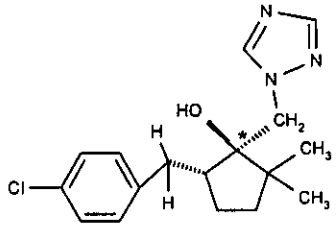
Lot No./Batch-No.:	AC8879-136A
Purity:	98.8%
Structural Formula:	

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:	

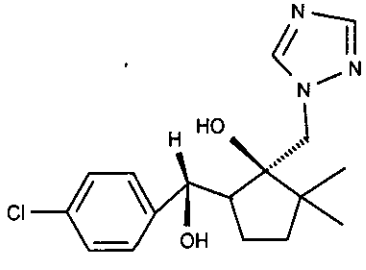


Cis-isomer



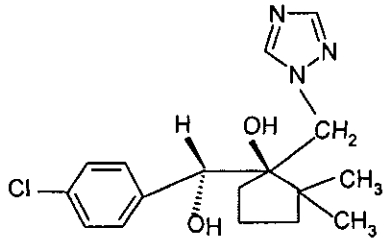
Trans-isomer

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:	

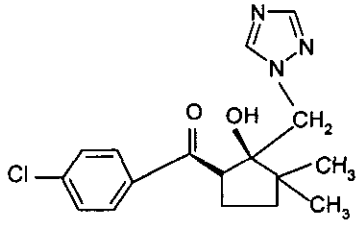


BASF Code Name:	KNF-474-M-21 (M21), R Benzyl Alcohol (CL 382391)
BASF Registry Number:	4558878

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:	

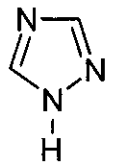


BASF Code Name:	KNF-474-M-30 (M30), R Benzyl Ketone (CL 382389)
BASF Registry Number:	4110625
Chemical Name:	Methanone, 4-chlorophenyl)-[2-hydroxy-3,3-dimethyl-2-(1H-1,2,4-triazol-1-ylmethyl) cyclopentyl]-,cis-(±)-(9CI) (CA INDEX)
Molecular Formula:	$C_{17}H_{20}CLN_3O_2$
Molecular Weight:	333.8
Appearance:	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,	

Method Step	Equipment	Size, Description	Manufacturer / Supplier	Catalog Number [1]
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
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 (<-5°C) 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 1µg/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% 0f 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. For *cis/trans*-BAS 555 F, M11, M21 and M30

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:

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

5. TIME REQUIREMENT FOR ANALYSIS

The laboratory time required for a set of 35 samples, including 2 recoveries and 1 control is approximately 5 person-hours. This does not include LC/MS/MS analysis and calculation times, and also does not account for any special problems that may arise, such as matrix interference.

6. CONFIRMATORY TECHNIQUES

The method allows for the determination of BAS 555 F and its metabolites by LC/MS/MS, which is a highly selective detection technique, however other transition ions may be used if needed.

7. SAFETY AND HEALTH CONSIDERATIONS

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

8. POTENTIAL PROBLEMS

A) In the case of suppression or enhancement or high background in LC/MS/MS detection, the following may be examined for only *cis/trans*-BAS 555 F, M11, M21 and M30:

Transfer 1 mL of the extract (step 3.2.f) into a 100 mm x10 mm test tube and add 1 mL of water. Partition with 2 x 4 mL DCM using a vortex for approximately 30 seconds in a moderate speed. Collect DCM fraction (lower layer) each time into a 20 mL liquid scintillation vial using a Pasteur pipette. Dry the combined DCM fraction completely using a sample concentrator under nitrogen and heated block (40°-60°C). Add exactly 2 mL of methanol cap the vial and vortex for approximately 30 second. Dilute the sample further with 3 mL of 0.05% FA in HPLC water. Inject 10 µL into LC/MS/MS.

B) In the case of suppression or enhancement or high background in LC/MS/MS detection, the following may be examined for only Triazol:

Transfer 2 mL of the extract (step 3.2.f) into a 100 mm x10 mm test tube and add 1 mL of water. Partition one time with 2 mL DCM using a vortex for approximately 30 seconds in a moderate speed. After complete phase separation, discard DCM fraction (lower layer) using a Pasteur pipette. Dry the aqueous fraction completely using a sample concentrator under nitrogen and water bath (40-50° C). Add 2 mL HPLC water into test tube 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.

REFERENCE:

1. John W. Stearns. "Determination of Metconazole and its Metabolites M11/M21 & M30 in Soil", April 2005, Method RM-41S-2, Valent USA Corporation, Dublin, CA.

FLOW CHART FOR THE ANALYTICAL METHOD

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