

1.0 INTRODUCTION

1.1 Scope of the Method

BASF Method L0182/02 was developed to determine residues of BAS 500 F (Pyraclostrobin) and its metabolites BF 500-5 (Reg. No. 298327), BF 500-12 (Reg. No. 412053), BF 500-11 (Reg. No. 411847), BF 500-13 (Reg. No. 412785), BF 500-14 (Reg. No. 413038) and BF 500-15 (Reg. No. 377613) in drinking and surface water by BASF Agricultural Center, Limburgerhof, Germany and independently validated by JRF America, Audubon, PA.

The independent lab validation was conducted using two fortification levels for drinking and surface water. The fortification levels were 0.003 and 0.03 µg/L for BAS 500 F and 0.03 and 0.3 µg/L for the metabolites. For each fortification level and matrix, five replicates were analyzed. Additionally, two replicates of unfortified samples were examined.

1.2 Principle of the Method

The residues of BAS 500 F and its metabolites BF 500-5, BF 500-12, BF 500-11, BF 500-13, BF 500-14, and BF 500-15 were extracted from water (50 mL) by acidification with formic acid followed by solid phase extraction on a C18 SPE column. Residues were then eluted with ethyl acetate twice. The extracts were concentrated to dryness and reconstituted in acetonitrile/water (20:80, v/v). The final determination was conducted using LC/MS/MS in positive ion mode.

In drinking and surface water, the method has a limit of quantitation of 0.003 µg/L for BAS 500 F and 0.03 µg/L for each metabolite. The method LOD was set at 0.0006 µg/L for BAS 500 F and 0.006 µg/L for each metabolite (20% of LOQ).

1.3 Specificity

BAS 500 F and its metabolites BF 500-5, BF 500-12, BF 500-11, BF 500-13, BF 500-14, and BF 500-15 were identified and quantified as individual analytes.

2.0 MATERIALS AND METHODS

2.1 Test Systems

The following test systems were considered for this study:

Test System 1: Surface Water, Jordan Lake, Cary, NC

Test System 2: Drinking (Well) Water, BASF Research Triangle Park, NC (130516-01)

Control drinking and surface water matrices were provided by BASF Crop Protection and sent to JRF America via Fed-Ex. The samples were logged in at JRF America and given unique laboratory identification numbers. The samples were stored refrigerated.

The description and characterization of the water used is given in the respective attached reports (Appendix B).

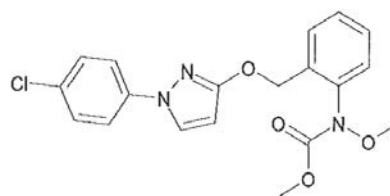
2.2 Test and Reference Substances

Reference substances BAS 500 F and its metabolites BF 500-5, BF 500-12, BF 500-11, BF 500-13, BF 500-14, and BF 500-15 were used for fortifications and LC/MS/MS calibration standards.

BAS 500 F (Pyraclostrobin)

BASF Registry Number:	304428
Molecular Formula:	C ₁₉ H ₁₈ ClN ₃ O ₄
Molecular Weight:	387.8 g/mol
IUPAC Name:	Methyl N-(2-[[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxymethyl]phenyl)-N-methoxy)carbamate
CAS Number:	175013-18-0
Batch No.:	01815-183
Purity:	99.9%
Storage Advice:	Keep in refrigerator or freezer
Expiration Date:	August 01, 2019

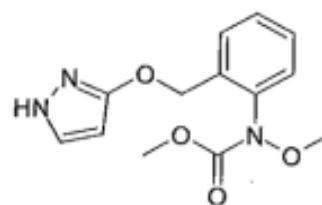
Chemical Structure:



BF 500-11 (Pyraclostrobin metabolite)

BASF Registry Number:	411847
Molecular Formula:	C ₁₃ H ₁₅ N ₃ O ₄
Molecular Weight:	277.3
IUPAC Name:	Methyl N-methoxyN-{2-[(1H-pyrazol-3-yl)oxymethyl]phenyl}carbamate
CAS Number:	175013-17-9
Batch No.:	01183-172
Purity:	98.9%
Storage Advice:	Keep at room temperature or cooler
Expiration Date:	May 1, 2020

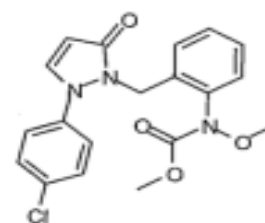
Chemical Structure:



BF 500-14 (Pyraclostrobin metabolite)

BASF Registry Number:	413038
Molecular Formula:	C ₁₉ H ₁₈ ClN ₃ O ₄
Molecular Weight:	387.8
IUPAC Name:	Methyl N-{2-[2-(4-chlorophenyl)-5-oxo-2,5-dihydro pyrazol-1-ylmethyl]-phenyl} N-methoxy carbamate
CAS Number:	na
Batch No.:	L83-122
Purity:	99.0%
Storage Advice:	Keep at room temperature or cooler
Expiration Date:	April 1, 2014

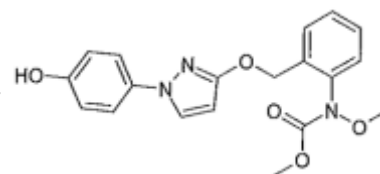
Chemical Structure:



BF 500-12 (Pyraclostrobin metabolite)

BASF Registry Number: 412053
Molecular Formula: $C_{19}H_{19}N_3O_5$
Molecular Weight: 369.4
IUPAC Name: Methyl N-(2-[[1-(4-hydroxyphenyl)-1H-pyrazol-3-yl]oxymethyl]phenyl) N-methoxy carbamate
CAS Number: na
Batch No.: 01586-56
Purity: 98.2%
Storage Advice: Keep at room temperature or cooler
Expiration Date: May 1, 2020

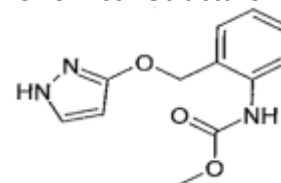
Chemical Structure:



BF 500-13 (Pyraclostrobin metabolite)

BASF Registry Number: 412785
Molecular Formula: $C_{12}H_{13}N_3O_3$
Molecular Weight: 247.3
IUPAC Name: Methyl N-[2-[(1H-pyrazol-3-yl)oxymethyl]phenyl] carbamate
CAS Number: na
Batch No.: 01586-60
Purity: 99.5%
Storage Advice: Keep at room temperature or cooler
Expiration Date: May 1, 2020

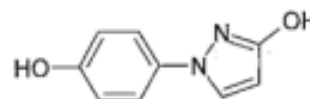
Chemical Structure:



BF 500-15 (Pyraclostrobin metabolite)

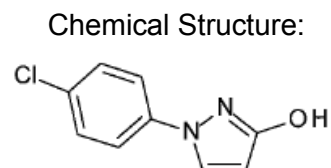
BASF Registry Number: 377613
Molecular Formula: $C_9H_8N_2O_2$
Molecular Weight: 176.2
IUPAC Name: 1-(4-hydroxyphenyl)-1H-pyrazol-3-ol
CAS Number: na
Batch No.: 01586-94
Purity: 97.2%
Storage Advice: Keep at room temperature or cooler
Expiration Date: February 01, 2018

Chemical Structure:



BF 500-5 (Pyraclostrobin metabolite)

BASF Registry Number: 298327
 Molecular Formula: C₉H₇ClN₂O
 Molecular Weight: 194.6
 IUPAC Name: 1-(4-chlorophenyl)-1H-pyrazol-3-ol
 CAS Number: na
 Batch No.: L84-174
 Purity: 99.6%
 Storage Advice: Keep at room temperature or cooler
 Expiration Date: April 01, 2020



2.3 Materials and Methods

Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Analytical Balance	AT 200	Mettler	----
Flasks, Volumetric	Various sizes	Various	----
Cylinder, graduated	Various sizes	Various	----
Pipettes	Various volumes	Eppendorf	----
Bottle, glass	250 mL, clear	QEC	2117-0008
Bottle, HDPE	250 mL	QEC	6213-B008
Disposable SPE columns	C18, 6 mL, 500 mg	Agilent	12102052
SPE box	Visiprep DL5, 12 valve	Supelco	5-7044
Vacuum Pump	Laboport	KNF Neuberger	----
Plastic Centrifuge Tubes	15 mL	VWR	525-0400
Evaporator	N-Evap 112	Organomation	----
Mixer	Vortex Genie 2	Scientific Industries	----
Ultrasonic bath	Fisher FS30	Fisher Scientific	----
HPLC vials	2 mL	Agilent Technologies	5182-0716
HPLC vial caps	PTFE/red silicone septa	Agilent Technologies	5182-0717
Liquid Chromatographic System	Waters Acquity UPLC	Waters	----
Mass Spectrometer	AB Sciex API 4000 QTrap	AB Sciex	----
HPLC Column	Atlantis T3 3µm, 3.0×150 mm	Waters	186003723

Reagents

2.3.1.1 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Formic acid	ACS	EMD	FX0440-5
Formic acid	LC/MS	Fluka	94318-250 mL
Acetonitrile	OmniSolv	EMD	AX0156-1
Ethyl acetate	OmniSolv	EMD	EX0241-1
Water	HPLC	Honeywell	AH365-4

2.3.1.2 Solutions and Solvent Mixtures

Description	Code	Composition
Solvent system 1	SS1	0.1% Formic acid in water Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Solvent system 2	SS2	Acetonitrile / Millipore H ₂ O = 2 + 8(v + v) Add 200 mL of a acetonitrile and 800 mL of water into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution
HPLC mobile phase A	LC1	0.1% Formic acid in water Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	0.1% Formic acid in acetonitrile Add 1000 mL of acetonitrile and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

2.3.1.3 Standard Solutions

Stock Solutions

Stock Solution Preparation

A 1 mg/mL stock solution was prepared for each individual analyte by weighing an appropriate amount of each analyte into a 10 mL volumetric flask and diluting to volume in acetonitrile.

Analyte ID	Purity	Weight (mg) ^a	Final Volume (mL)	Concentration (mg/mL)
BAS 500 F	99.9%	14.7	10	1.47
BF 500-5	99.6%	10.1	10	1.01
BF 500-12	98.2%	11.3	10	1.13
BF 500-11	98.9%	10.1	10	1.01
BF 500-13	99.5%	10.1	10	1.01
BF 500-14	99.0%	10.3	10	1.03
BF 500-15	97.2%	12.2	10	1.22

^a Corrected for purity

Intermediate Stock Dilution Solutions Preparation

Intermediate stock dilutions of individual analytes were prepared by dilution of stock solutions with acetonitrile/water (20:80, v/v) in 20 mL volumetric flasks.

Analyte	Concentration (µg/mL)	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (µg/mL)
BAS 500 F	1470	0.137	20	10
BF 500-5	1010	1.98	20	100
BF 500-12	1130	1.77	20	100
BF 500-11	1010	1.98	20	100
BF 500-13	1010	1.98	20	100
BF 500-14	1030	1.94	20	100
BF 500-15	1220	1.64	20	100

Stock Solutions (continued)

Mixed Analyte Solution Preparation

A mixed analyte solution was prepared by dilution of 2.5 mL of the intermediate stock dilution solutions with acetonitrile/water (20:80, v/v) in a 25 mL volumetric flask.

Analyte	Concentration (µg/mL)	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (µg/mL)
BAS 500 F	10	2.5	25	1.0
BF 500-5	100	2.5		10
BF 500-12	100	2.5		10
BF 500-11	100	2.5		10
BF 500-13	100	2.5		10
BF 500-14	100	2.5		10
BF 500-15	100	2.5		10

Fortification Solutions

Fortification Solutions Preparation

Fortification solutions were prepared by serial dilution of the mixed analyte solution with acetonitrile/water (20:80, v/v) in a 25 mL volumetric flask.

Analyte	Concentration (µg/mL) ^a	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (µg/mL) ^a
Mixed	1.0/10.0	2.5	25	0.1/1.0
Mixed	0.1/1.0	2.5	25	0.01/0.1
Mixed	0.01/0.1 ^d	2.5	25	0.001/0.01

^a Pyraclostrobin (BAS 500 F)/metabolites

Calibration Standard Solutions

Preparation of Standard Solutions for Calibration

Standard solutions for calibration were prepared by dilution of the fortification solutions with acetonitrile/water (20:80, v/v) in 25 mL volumetric flasks.

Analyte	Concentration (µg/mL) ^a	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (ng/mL) ^a
Mixed	0.01/0.1	2.5	25	1.0/10.0
		0.625	25	0.25/2.5
		0.5	25	0.2/2.0
		0.375	25	0.15/1.5
	0.001/0.01	2.5	25	0.1/1.0
		1.25	25	0.05/0.5
		0.5	25	0.02/0.2
		0.25	25	0.01/0.1
		0.125	25	0.005/0.05

^a Pyraclostrobin (BAS 500 F)/metabolites

Preparation of Matrix-Matched Standard Solutions for Calibration

Matrix matched standards were prepared by dilution of the standard solutions for calibration with a control sample of matrix carried through the procedure. The control sample of matrix was prepared using a final volume of 6 mL (as noted in section 3.6 of the method) instead of 3 mL (as noted in section 2.4.3 of the method) acetonitrile/water (20:80, v/v). A comparison of matrix matched standards was made for respective reference standards prepared from control matrix brought to a final volume in 3 mL and 6 mL. No significant difference in signal response was noted for multiple reference standard concentrations. See Appendix E for data details addressing the deviation.

Analyte	Concentration (ng/mL)	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (ng/mL) ^a
Mixed	2.0	0.750	1.5	0.1/1.0
	1.5	0.750	1.5	0.075/0.75
	1.0	0.750	1.5	0.05/0.5
	0.5	0.750	1.5	0.025/0.25
	0.2	0.750	1.5	0.010/0.1
	0.1	0.750	1.5	0.005/0.05

^a Pyraclostrobin (BAS 500 F)/metabolites

3.0 Analytical Procedure

3.1 Weighing and Fortification

Twelve samples were prepared for each matrix (surface and drinking water): Two control samples, five samples treated at the limit of quantitation (LOQ) and five treated at 10 times the LOQ. For each, 50 mL of water was measured into a 250 mL HDPE bottle and spiked with the appropriate fortification solution.

The following scheme was used:

Sample Type	Sample Volume	Concentration of Spiking Solution [$\mu\text{g/mL}$] ^a	Volume of Spiking Solution [μL]	Level of Fortification [$\mu\text{g/L}$] ^a
Control	50 mL	-	-	0.00
Fortification (LOQ)	50 mL	1.0/10.0	150	0.003/0.03
Fortification (10× LOQ)	50 mL	10.0/100	150	0.03/0.3

^a Pyraclostrobin (BAS 500 F)/metabolites

3.2 Extraction of Sample Material

Residues of Pyraclostrobin and its metabolites were recovered from water samples by acidification with formic acid followed by solid phase extraction on a C18 SPE column. Residues were then eluted with ethyl acetate twice.

3.3 Preparation for Measurement

Each extract was brought to dryness using a nitrogen evaporator. The residue was reconstituted with 6.0 mL acetonitrile/water (20:80, v/v).

For Control and LOQ levels, samples were ready for injection. The 10×LOQ samples were diluted 10 times with acetonitrile/water (20:80, v/v). The final concentration of each sample, as prepared, is presented below:

Sample Type	Pyraclostrobin Concentration (ng/mL)	Metabolite Concentration (ng/mL)
Control	0.0	0.0
Fortification (LOQ)	0.025	0.25
Fortification (10× LOQ)	0.025	0.25

4.0 Instrumentation and Conditions

UPLC/MS/MS Conditions for the Analysis of BAS 500 and Metabolites BF 500-5, BF 500-12, BF 500-11, BF 500-13, BF 500-14, and BF 500-15

Waters Acquity UPLC Conditions			
Analytical Column	Waters Atlantis T3 3.0x150 mm 3 μ m, S/N 013533280140 16		
Column Temperature	40 °C		
Flow Rate	0.5 mL/min		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	Initial	85	15
	1.0	85	15
	7.0	10	90
	13.0	10	90
	13.1	85	15
	17.0	85	15
Mobile Phase A:	0.1 % Formic Acid in Water		
Mobile Phase B:	0.1 % Formic Acid in Acetonitrile		
Injection Volume (μ L):	50		

MS/MS Conditions	
Interface	AB SCIEX 4000 Qtrap® with Turbo Ion Spray®
Polarity	Positive
Curtain gas (CUR)	30.00
Temperature (TEM)	550.00 °C
Ion transfer voltage (IS)	4500.00
Collision gas setting (CAD)	9.00
GS1	50.00
GS2	20.00
Entrance potential (EP)	10.00
Scan type	MRM

The primary (quantitative) and secondary (confirmatory) transition ions monitored are presented below:

Analyte	Transitions (m/z)		polarity	Approximate Retention Time (min.)
	Primary	Secondary		
BA 500 F	389 \rightarrow 195	389 \rightarrow 164	Positive	6.62
BF 500-5	196 \rightarrow 154	196 \rightarrow 118		6.62
BF 500-12	370 \rightarrow 194	370 \rightarrow 278		7.25
BF 500-11	278 \rightarrow 194	278 \rightarrow 149		6.13
BF 500-13	248 \rightarrow 132	248 \rightarrow 164		6.10
BF 500-14	389 \rightarrow 242	389 \rightarrow 301		6.60
BF 500-15	177 \rightarrow 135	177 \rightarrow 132		4.55

4.1 Calibration Procedures

Calculation of results is based on peak area measurements using a calibration curve. The calibration curve is obtained by direct injection of the calibration standards containing known amounts of Pyraclostrobin and its metabolites by injecting standard solutions at appropriate concentrations for each analyte. Calibration standard concentrations for Pyraclostrobin and its metabolites ranged from 0.005 to 0.10 ng/mL and 0.05 to 1.0 ng/mL, respectively. Typical calibration curves are presented for Pyraclostrobin and its metabolites in Figures 11.1 through 11.13. Representative chromatograms are presented in Figures 11.14 through 11.143.

4.2 Calculation of Residues and Recoveries

Calculations of results were based on peak area measurements. Analyst® 1.5.1 software created the standard curve based on linear regression analysis using 1/x weighting. The recoveries of the analytes were calculated relative to the linearity curve generated with each set.

$$\text{I. Concentration (ng/mL)} = \frac{\text{Response} - \text{Intercept}}{\text{Slope}} = C_A$$

The recoveries of spiked compounds are calculated according to equation II:

$$\text{II. Recovery \%} = \frac{\text{Conc. of Fortified Sample (ng / mL)} - \text{Conc. of Control (ng / mL)}}{\text{Conc. of Sample as Prepared}} \times 100$$

Example Calculation: BAS 500 F (389 → 195), Surface Water Trial 1, 10xLOQ #1 fortified at 0.03 ppm, analytical set: Surface_Water_Trial 1_03132014.wiff

The following values were used in this calculation:

Response of fortified sample	8787.21
Response of control sample	0.000
Slope:	309000
Intercept:	589

$$\text{I. Concentration (ng/mL)} = \frac{8787.21 - 589}{309000} = 0.0265 \text{ ng/mL}$$

$$\text{II. Recovery \%} = \frac{0.0265 \text{ ng / mL}}{0.025 \text{ ng / mL}} = 106\%$$

Note: All concentration results calculated by Analyst® software. Percent recovery calculations were performed using Microsoft® Excel. No calculations were made with rounded numbers. Results were rounded for reporting purposes only. Hand calculated results may differ as a result of rounding.

5.2 Summary of Method

Type of method: LC/MS/MS

Test systems: Surface (Lake) Water
Drinking (Well) Water

Analytes and selected mass transitions (*m/z*):

<u>Analyte</u>	<u>Primary</u>	<u>Secondary</u>
BAS 500 F	389 → 195	389 → 164
BF 500-5	196 → 154	196 → 118
BF 500-12	370 → 194	370 → 278
BF 500-11	278 → 194	278 → 149
BF 500-13	248 → 132	248 → 164
BF 500-14	389 → 242	389 → 301
BF 500-15	177 → 135	177 → 132

Analytical procedure: The residues of BAS 500 F (Pyraclostrobin) and its metabolites BF 500-5, BF 500-12, BF 500-11, BF 500-13, BF 500-14, and BF 500-15 were extracted from water (50 mL) by acidification with formic acid followed by solid phase extraction on a C18 SPE column. Residues were then eluted with ethyl acetate twice. The extracts were concentrated to dryness and reconstituted in acetonitrile/water (20:80, v/v). The final determination was conducted using LC/MS/MS in positive ion mode.

Confirmatory technique: For every compound the quantitation is possible at two different transitions. Due to the high selectivity and specificity of LC/MS/MS an additional confirmatory technique was not necessary.

Limit of detection (LOD): 0.0006 µg/L for Pyraclostrobin and 0.006 µg/L for all metabolites.

Limit of quantification (LOQ): 0.003 µg/L for Pyraclostrobin and 0.03 µg/L for the metabolites, corresponding to concentrations of 0.025 and 0.25 ng/mL in the final extract respectively.

Levels of fortification: 0.003 and 0.03 µg/L for Pyraclostrobin and 0.03 and 0.3 µg/L for the metabolites

Time required: The analysis of 13 samples requires about 8 hours of work including the calculation of the results and reporting of all raw data under GLP.