

## 1 INTRODUCTION

### 1.1 Scope of the method

BASF method No. L0182/01 is an analytical method for the determination of the active ingredient BAS 500 F and its metabolites M500F59, M500F60 500M62, 500M76 and M500F78 in water. The described method allows the specific determination of BAS 500 F and its metabolites with a limit of quantitation (LOQ) of 0.003 µg/L for the parent compound and 0.03 µg/L for the metabolites in water.

This method was developed at BASF SE, Agricultural Center Limburgerhof, Germany.

The purpose of this study was to demonstrate the validity of the method by performing recovery experiments with spiked water samples. Spiked water samples are analysed for BAS 500 F and its metabolites M500F59, M500F60 500M62, 500M76 and M500F78. The recovery trials were carried out with ground water, surface water and tap water.

The spiking levels were 0.003 µg/L and 0.03 µg/L for BAS 500 F and 0.03 µg/L and 0.3 µg/L for the metabolites. All fortification levels were analysed in 5 replicates. In addition at least two untreated control samples have been analysed per analytical sample set. The analyses were performed by one person, with the same equipment, in the same laboratory, within a short interval of time.

### 1.2 Principle of the method

50 mL of a water sample material is acidified by adding 500 µL of formic acid and sucked a SPE column. After washing with 5 mL acidified water, the column is dried. Elute with 2 x 5 mL ethyl acetate. Combined extracts were evaporated using an N-EVap at 40°C.

As matrix effects were observed during method development, matrix matched standards were used for calibration. Determination is achieved by HPLC/MS/MS.

### 1.3 Specificity

The method allows the specific determination of BAS 500 F and its metabolites M500F59, M500F60 500M62, 500M76 and M500F78 in water using HPLC/MS-MS detection monitoring two mass transitions for each analyte.

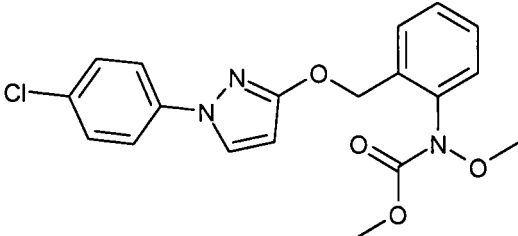
## 2 MATERIALS AND METHODS

### 2.1 Test system water

Three different types of water were used: Surface water (Kelmetschweiher), ground water (Water supply Schifferstadt) and tap water from Limburgerhof.

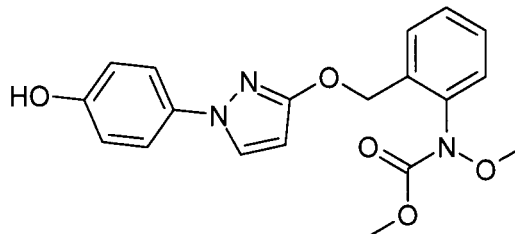
## 2.2 Test and reference items

### 2.2.1 Test Item(s)

<b>Reg.No.</b>	304428
<b>BAS Code</b>	500 F
<b>Common Name</b>	Pyraclostrobin
<b>Batch No.</b>	01815-183
<b>Test Substance Type</b>	PAI
<b>CAS-No.</b>	175013-18-0
<b>IUPAC-Name</b>	methyl N-(2-[[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxymethyl]phenyl)-(N-methoxy)carbamate
<b>Purity [%]</b>	99.9
<b>Molecular Formula</b>	C <sub>19</sub> H <sub>18</sub> ClN <sub>3</sub> O <sub>4</sub>
<b>Molecular Weight</b>	387.82
<b>Chemical Structure</b>	

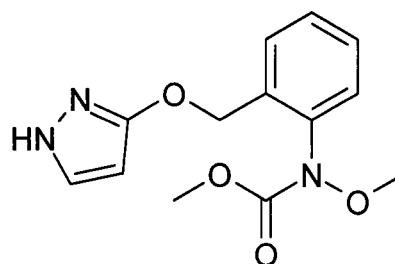
<b>Storage Advice</b>	keep in refrigerator or freezer
<b>GLP</b>	yes
<b>Expiration Date</b>	01.Aug.2019

Reg.No. 412053  
BAS Code 500M59  
Batch No. 01586-56  
Test Substance Type ME  
IUPAC-Name methyl N-(2-([1-(4-hydroxyphenyl)-1H-pyrazol-3-yl]oxymethyl)phenyl) N-methoxy carbamate  
Purity [%] 99.2  
Molecular Formula  $C_{19}H_{19}N_3O_5$   
Molecular Weight 369.38  
Chemical Structure



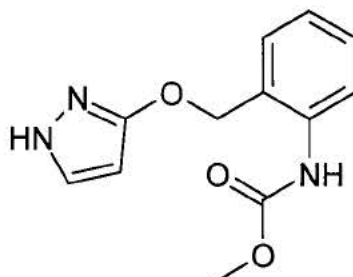
Storage Advice keep at room temperature (typically +25°C) or cooler  
GLP yes  
Expiration Date 01.May.2020

Reg.No. 411847  
BAS Code 500M60  
Batch No. 01183-172  
Test Substance Type ME  
CAS-No. 175013-17-9  
IUPAC-Name methyl N-methoxy N-{2-[(1H-pyrazol-3-yl)oxymethyl]phenyl}carbamate  
Purity [%] 98.9  
Molecular Formula  $C_{13}H_{15}N_3O_4$   
Molecular Weight 277.28  
Chemical Structure



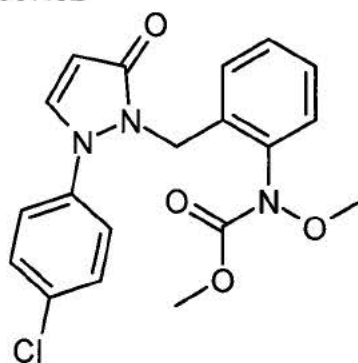
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GLP yes  
Expiration Date 01.May.2020

Reg.No. 412785  
BAS Code 500M62  
Batch No. 01586-60  
Test Substance Type ME  
IUPAC-Name methyl N-[2-(1H-pyrazol-3-yloxymethyl)phenyl]carbamate  
Purity [%] 99.5  
Molecular Formula  $C_{12}H_{13}N_3O_3$   
Molecular Weight 247.25  
Chemical Structure

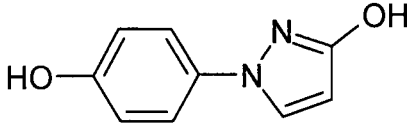


Storage Advice keep at room temperature (typically +25°C) or cooler  
GLP yes  
Expiration Date 01.May.2020

Reg.No. 413038  
BAS Code 500M76  
Batch No. 01882-198  
Test Substance Type ME  
IUPAC-Name methyl N-{2-[2-(4-chlorophenyl)-5-oxo-2,5-dihydro-pyrazol-1-ylmethyl]-phenyl} N-methoxy carbamate  
Purity [%] 97.0  
Molecular Formula  $C_{19}H_{18}ClN_3O_4$   
Molecular Weight 387.82  
Chemical Structure



Storage Advice keep at room temperature (typically +25°C) or cooler  
GLP yes  
Expiration Date 01.May.2012

Reg.No.	377613
BAS Code	500M78
Batch No.	01586-94
Test Substance Type	ME
IUPAC-Name	1-(4-hydroxyphenyl)-1H-pyrazol-3-ol
Purity [%]	97.2
Molecular Formula	C <sub>9</sub> H <sub>8</sub> N <sub>2</sub> O <sub>2</sub>
Molecular Weight	176.18
Chemical Structure	

Storage Advice	keep at room temperature (typically +25°C) or cooler
GLP	yes
Expiration Date	01.May.2012

### 2.2.2 Reference Items

Same as test items, see 2.2.1

### 2.3 Stability of standard solutions

The stability of standard solutions of BAS 500 F, 500M59, 500M60, 500M62, 500M76 and 500M78 was investigated. Standard solutions were prepared in acetonitril/water 20+80 (v+v) and stored in a refrigerator. They were analysed against freshly prepared calibration standards after one week and 16 days.

### 2.4 Materials and instruments

The procedure described in the technical procedure (see Appendix, Chapter 7) was followed with no modifications. As HPLC-MS/MS conditions are only exemplified in the technical procedure, the actual conditions are given below.

		Parameter		
<b>Chromatographic System</b>		Agilent HP 1100 with CTC Autosampler		
Analytical-column		Atlantis T3, 150 x 3 mm, 3 µm particle size		
Pre-column:		No		
Column Temperature		40°C		
Injection Volume		50µL		
Mobile Phase A		Water / formic acid, 1000/1, v/v		
Mobile Phase B		ACN/ formic acid, 1000/1, v/v		
Flow Rate		500µl/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B	
	0.0	85	15	
	1	85	15	
	7	10	90	
	13	10	90	
	13.10	85	15	
	17	85	15	
<b>Detection System</b>		PE Sciex API 4000 Mass Spectrometer		
Ionisation		Electrospray (ESI)		
<b>Analyte</b>	<b>Transitions</b>	<b>Polarity</b>	<b>Expected Retention Time</b>	
304428 (BAS 500F)	388→163 388→194*	positive	approx. 9.21 min.	
412053 (500M59)	370→278 370→194*	positive	approx. 7.61 min.	
411847 (500M60)	278→194* 278→149	positive	approx. 6.63 min.	
412785 (500M62)	248→132* 248→216	positive	approx. 6.58 min.	
413038 (500M76)	388→300 388→241*	positive	approx. 7.06 min.	
377613 (500M78)	177→132 177→135*	positive	approx. 5.12 min.	

\* proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time

## 2.5 Example of calculation

Calculation of recovery of sample no. ForL0036 (surface water, fortified with 0.03 µg/L BAS 500 F, mass transition 388->194, worklist 2011pg70015).

Calibration curve:   Type           linear  
                          Slope         1110000  
                          Intercept    1260

For sample no ForL0036:

Concentration of analyte [ng/mL]   = (Peak area - intercept)/slope  
  = (27518.6 - 1260)/1110000  
  = 0.0237

Equation:

$$R[\mu\text{g/L}] = \frac{V_{\text{end}} \times C}{S_m \times A_I}$$

R       = Residue in water sample

$V_{\text{end}}$  = End volume of the extract after all dilution steps [mL]  
 $C$  = Concentration of analyte in the inj. Vol. as read from calibration curve [ng/mL]  
 $S_m$  = Volume of the water sample extracted [mL]  
 $AI$  = Aliquotation factor of water extract taken for analysis

$$R \text{ (fortified sample)} = \frac{60 \times 0.0237}{50 \times 1} = 0.02844$$

$$\% \text{ Recovery} = \frac{R \text{ (found, fortified)} - R \text{ (found, untreated)}}{R \text{ (fortified)}} \times 100$$

$$\% \text{ Recovery} = \frac{0.02844 - 0^*}{0.03} \times 100 = 94.8$$

\*Residue in control sample was zero

Due to interferences between analyte and matrix leading to variable response, matrix matched standard were used throughout this study.

Confirmatory technique: Due to the high selectivity and specificity of HPLC/MS/MS an additional confirmatory technique is not necessary, if two mass transitions for the respective analyte were validated. The validation was performed with two mass transitions for each analyte. All recovery data were within the acceptable range.

Time required for analysis: The analysis of one series of samples (10 unknown samples, 2 fortified samples for recovery determination and a blank sample requires one working day (8 hours). This time includes all experimental work, calculation of results and documentation according to GLP.

## 1 INTRODUCTION

BAS 500 F (pyraclostrobin) is a fungicide used against in many crops. The analytical method L0182/01 succeeds method 455 developed earlier (see Reference 1). A new version became necessary to meet current guideline requirements. The new method offers the possibility to determine residues of BAS 500 F (pyraclostrobin) and its metabolites Reg No. 412053 (500M59), Reg No. 411847 (500M60), Reg No. 412785 (500M62), Reg No. 413038 (500M76), and Reg No. 377613 (500M78) in ground-, surface- and drinking water by LC-MS/MS. Method L0182/01 was successfully tested during method development in ground-surface- and tap (drinking) water

This method was developed at BASF SE, Limburgerhof, Germany.

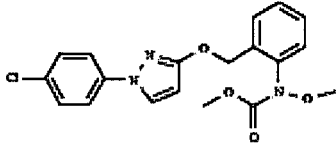
## 2 MATERIALS

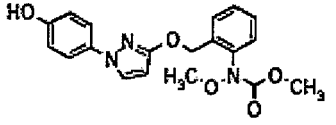
### 2.1 Safety

The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. No eating, drinking, smoking or tobacco use at the place of work. Hands and/or face should be washed before breaks and at the end of the shift. Details are given in the Materials Safety Data Sheets (MSDS) of the individual substances. All procedures involving organic solvents should be performed in a well-ventilated hood. Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

### 2.2 Test and Reference Items

Test and reference items should be stored according to the information provided in the certificate of analysis.

BAS-Code	500 F	
Common Name	Pyraclostrobin	
IUPAC Name	methyl N-(2-[[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxymethyl]phenyl)-(N-methoxy)carbamate	
BASF Reg. No.	304428	
CAS-No.	175013-18-0	
Molecular Formula	C <sub>19</sub> H <sub>18</sub> ClN <sub>3</sub> O <sub>4</sub>	
Molecular Weight	387.8	

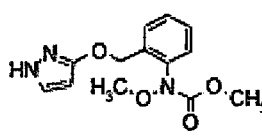
BAS-Code	500M59 (BF 500-12)	
Common Name	-	
IUPAC Name	methyl N-(2-[[1-(4-hydroxyphenyl)-1H-pyrazol-3-yl]oxymethyl]phenyl)-(N-methoxy)carbamate	
BASF Reg. No.	412053	
CAS-No.	-	
Molecular Formula	C <sub>20</sub> H <sub>19</sub> N <sub>3</sub> O <sub>5</sub>	
Molecular Weight	369.4	

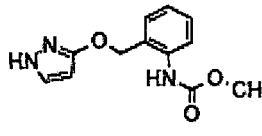


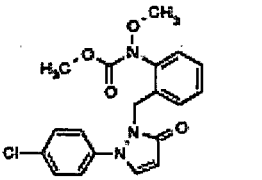
Method L0182/01

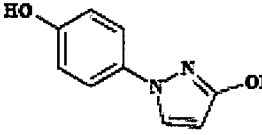
Technical Procedure

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BAS-Code	500M60 (BF 500-11)	
Common Name	-	
IUPAC Name	methyl N-methoxy N-(2-((1H-pyrazol-3-yl)oxymethyl)phenyl)carbamate	
BASF Reg. No.	411847	
CAS-No.	175013-17-9	
Molecular Formula	C <sub>13</sub> H <sub>15</sub> N <sub>3</sub> O <sub>4</sub>	
Molecular Weight	277.3	

BAS-Code	500M62 (BF 500-13)	
Common Name	-	
IUPAC Name	methyl N-(2-((1H-pyrazol-3-yl)oxymethyl)phenyl)carbamate	
BASF Reg. No.	412785	
CAS-No.	-	
Molecular Formula	C <sub>12</sub> H <sub>13</sub> N <sub>3</sub> O <sub>3</sub>	
Molecular Weight	247.3	

BAS-Code	500M76 (BF 500-14)	
Common Name	-	
IUPAC Name	methyl N-(2-(2-(4-chlorophenyl)-5-oxo-2,5-dihydro-pyrazol-1-ylmethyl)phenyl) N-methoxy carbamate	
BASF Reg. No.	413038	
CAS-No.	-	
Molecular Formula	C <sub>18</sub> H <sub>18</sub> ClN <sub>3</sub> O <sub>4</sub>	
Molecular Weight	387.8	

BAS-Code	500M78 (BF 500-15)	
Common Name	-	
IUPAC Name	1-(4-hydroxyphenyl)-1H-pyrazol-3-ol	
BASF Reg. No.	377613	
CAS-No.	-	
Molecular Formula	C <sub>8</sub> H <sub>9</sub> N <sub>2</sub> O <sub>2</sub>	
Molecular Weight	176.2	

## 2.3 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Analytical balance	AT 261	Mettler, Giessen Germany	
Balance	PM4800	Mettler,	
Pipette	Various volumes	Gässon Medical Electronics S.A., F 95400 Villier-le-Bel, France	
Handy step electronic dispenser		Brand	705000
Disposable SPE columns, C18	6ml, 500mg	VARIAN	1210205
SPE box	VacMaster, 20 valves	IST	
Glass bottle	150 ml		
Pasteur plastic pipettes	up to 3 ml		
TurboVap LV, EVAPORATOR		Zymark	
HPLC vials	32* 11.6 mm		
Teflon-lined plastic caps for HPLC-vials			
Ultrasonic (Ultraschallbad)	TRANSSONIC 460	Elma	
Volumetric cylinder	1000 mL, 250 ml		

**Note:** The equipment and instrumentation listed above may be substituted by that of similar specifications. The applicability is confirmed if the recoveries of the fortification experiments are in the expected concentration range.

## 2.4 Reagents

### 2.4.1 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Formic acid 98-100%	Pro analysi	Bernd Kraft GmbH	05314.3010
Acetonitrile	LiChrosolv	Merck KGaA, Darmstadt	1.00030
Ethyl acetate	LiChrosolv	Merck KGaA, Darmstadt	1.00868
Water, e.g. Baker <sup>®</sup> or Millipore <sup>®</sup>	Gradient Grade	J.T. Baker / Millipore/Waters	

**Note:** Equivalent reagents and chemicals from other suppliers may be used instead.

**2.4.2 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 <sub>2</sub> O = 2 + 8(v + v) Add 200 mL of methanol 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 methanol 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.

**Note:** If necessary, the solutions may also be prepared in different volumes as long as the proportions are not modified.

### 2.4.3 Standard Solutions

#### Stock Solutions

Prepare a 1 mg/mL stock solution individually for each analyte by weighing an appropriate amount into a flask and add the required volume of acetonitrile.

For example, to prepare 10 mL of 1.0 mg/mL stock solution, separately weigh 10 mg of BAS 500 F, (Reg No. 304428), 500M59 (Reg No. 412053), 500M60 (Reg No. 411847), 500M62 (Reg No. 412785), 500M76 (Reg No. 413038) and 500M78 (Reg No. 377613) into a 10 mL volumetric flask and dissolve in 10 mL of acetonitrile). Ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Independence of standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved for example by using one of the following approaches:

- Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
- Fortification and calibration standard solutions should be prepared from one stock solution in separate dilution series.

For subsequent preparations of solutions, freshly prepared solutions can be compared directly to previous standard solutions.

A correction for purity is done if the purity of the reference compounds is  $\leq 95\%$ . If the purity is  $> 95\%$  correction is optional.

Due to large differences in sensitivity, the concentration of pyraclostrobin in standard and fortification solutions is 10 times lower than the concentration of the metabolites. This also leads to lower LOQ and LOD for pyraclostrobin.

#### Preparation of mixed analyte solution from stock solutions

The following codes are used for the description of the solutions prepared:  
D = Dilution step (only used as intermediate dilution step)

F = Fortification solution

S = Solution used as analytical standard

Take 0.1 mL of the pyraclostrobin stock solution and 1.0 mL of each metabolite stock solution and dilute to 10 mL with SS2 separately. The concentration of the analytes in the resulting solutions is 10  $\mu\text{g/mL}$  for pyraclostrobin and 100  $\mu\text{g/mL}$  for the metabolites. To prepare a mixed analyte solution (D1), mix 1 mL from each solution and dilute to 10 mL with SS2.

#### Fortification Solutions

Prepare mixed standard solutions for fortification from stock in a flask. Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution.

#### Preparation of mixed Fortification solutions

Take solution	Volume (mL)	To prepare solution	Dilute with SS 2 to a final volume of (mL)	Concentration of pyraclostrobin	Concentration of metabolites
D1	-	-	-	1 $\mu\text{g/mL}$	10 $\mu\text{g/mL}$
D1	1	D2	10	0.1 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$
D2	1	F1	10	0.01 $\mu\text{g/mL}$	0.1 $\mu\text{g/mL}$
F1	1	F2	10	1 ng/mL	10 ng/mL

**Note:** A different concentration scheme may be used, if other fortification levels are needed for the analysis.

If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

### Calibration Standard Solutions

#### Preparation of matrix solution for the preparation of matrix matched standards

To prepare matrix to dilute standards, take a control water sample and follow step 3.4 of this method. Dilute the residue from step 4 with 3 mL SS2 (instead of 6 mL for the sample) to account for the dilution of matrix during mixing with standard solutions S3 to S8. If necessary, process several control water samples as described in 3.4.

#### Preparation of standard solutions for calibration

Take solution	Volume (µl)	To prepare	Dilute with SS2 to a final volume of (mL)	Concentration of pyraclostrobin (ng/mL)	Concentration of metabolites (ng/mL)
F1	1000	S1	10	1.0	10
F1	250	S2	10	0.25	2.5
F1	200	S3	10	0.20	2.0
F1	150	S4	10	0.15	1.5
F2	1000	S5	10	0.10	1.0
F2	500	S6	10	0.05	0.5
F2	200	S7	10	0.02	0.2
F2	100	S8	10	0.01	0.1

#### Matrix-matched standards

Take solution	Volume (µL)	Dilute with matrix to a final volume of (mL)	Concentration of pyraclostrobin (ng/mL)	Concentration of metabolites (ng/mL)
S3	500	1	0.10	1.0
S4	500	1	0.075	0.75
S5	500	1	0.05	0.5
S6	500	1	0.025	0.25
S7	500	1	0.010	0.10
S8	500	1	0.005	0.05

\* in case matrix-matched standards (= instrument recovery samples) are needed for successful analysis, calibration standard solution are prepared in matrix solution, i.e., final volume of a control sample carried through the analytical procedure. Matrix-matched standards should be prepared in a way that the matrix load is at least 90% of the matrix load in the unknown samples. In addition the matrix load should be the same in all calibration standard solutions.

**Note:** A different concentration scheme may be used and additional standards may be prepared as needed.

If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

**Additional Information:**

- Use amber bottles with Teflon-lined screw caps as storage containers for all standard solutions.

**2.4.4 Stability of Standard Solutions**

The stability of stock solutions of the metabolites 500M59, 500M60, 500M62, 500M76 and 500M78 in acetonitrile at a concentration of 10µg/mL was investigated over a period of 120 days (Reference 2). All metabolites proved to be stable for 120 days. The corresponding information for the parent compound pyraclostrobin can be found in Reference 3.

**3 ANALYTICAL PROCEDURE****3.1 Sample Preparation**

Water samples can be considered as homogenous, shaking is sufficient to assure that the aliquot taken for residue analysis is representative for the whole sample.

**3.2 Sample Storage**

Until analysis, water samples are stored in glass bottles in a refrigerator at 4°C.

Storage stability in water at 4°C for BAS 500 F (pyraclostrobin) was investigated over a period of two years (Reference 4). Under the mentioned conditions, residues of pyraclostrobin can be considered to be stable for one year.

Storage stability in water at 4°C for metabolites 500M59 (Reg No. 412053), 500M60 (Reg No. 411847), 500M62 (Reg No. 412785), 500M76 Reg No. (413038) and 500M78 Reg No. (377613) in water was investigated for 24 months (Reference 5). Metabolites 500M60, 500M62 and 500M76 can be assumed to be also stable for at least one year, while 500M59 and 500M78 should be analysed within 30 days.

**3.3 Aliquotation and Fortification**

For treated samples and control samples, fill 50 mL water sample into a 150 mL glass flask.

For fortified samples, fill at this stage 50 mL of control water into a 150 mL glass flask and add fortification solutions on the matrix.

The following scheme may be used:

Sample Type	Sample Volume	Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	50 mL	-	-	0.00 µg/L
Fortification (LOQ)	50 mL	F2	150 µL	0.003/0.03 µg/L*
Fortification (10xLOQ)	50 mL	F1	150 µL	0.03/ 0.3 µg/L
Treated	50 mL	-	-	-

\* limit of quantification

**Note:** Volume of spiking solution added to generate the fortified sample should not exceed 10% of sample weight or volume.

### 3.4 Extraction of Sample Material

Preparation of extraction column: To condition the extraction C18-SPE column, wash column with 5 mL ethyl acetate followed by 5 mL of acidified water (SS1). Do not allow to run dry.

Extraction: Fill 50 mL of sample material into a glass flask, and acidify by adding 500  $\mu$ L of formic acid (see chapter 2.4.2). Suck the water slowly through the SPE column and wash with 5 mL SS1. Apply full vacuum for 30 seconds to dry the column. Elute twice with 2 x 5 mL ethyl acetate. Combine extracts and evaporate solvent using an N-EVap at 40°C or similar instrument.

### 3.5 Sample Clean-up

No further sample clean-up is required.

### 3.6 Preparation for Measurement

Dilute the residue from step 4 with 6 mL of SS2 and, if higher dilution is required, proceed with SS2 (for fortifications at LOQ and control samples, residues are dissolved in a volume of 6 mL). If further dilution is required, use mixture of SS2 and control matrix extract 1 + 1

### 3.7 Influence of matrix effects on analysis

During method development matrix effects were observed leading to variable recoveries. It was shown that more stable recoveries could be obtained, when matrix matched standards were used. Therefore, samples should be analyzed using matrix matched calibration standard prepared from control matrix solution (see 2.4.3).

### 3.8 Stability of Extracts and Final Volumes

The stability of extracts and final volume if stored in the refrigerator for 7 days will be demonstrated in the validation study. Procedural recoveries can be used to prove the stability even over a longer time interval, if necessary.

## 4 QUANTIFICATION AND CALCULATION

### 4.1 Set-up of the analytical run

A sequence for measurement generally consists of:

- o Calibration standards
- o Control samples
- o Procedural recovery samples
- o Unknown samples

Reagent Blanks or blanks can also be injected if necessary. Each injection set should begin and end with an injection of a calibration standard. Standards should be interspersed with samples. Each calibration standard should be at least injected twice. At least 5 calibration levels need to be injected.

## 4.2 Instrumental analysis

### 4.2.1 Instrumentation and Conditions

		Parameter		
Chromatographic System		Agilent HP 1100 with CTC Autosampler		
Analytical column		Atlantis T3, 150 x 3 mm, 3 µm particle size		
Pre-column:		No		
Column Temperature		40°C		
Injection Volume		50 µL		
Mobile Phase A		Water / formic acid, 1000/1, v/v		
Mobile Phase B		ACN/ formic acid, 1000/1, v/v		
Flow Rate		500 µl/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B	
	0.0	85	15	
	1	85	15	
	7	10	90	
	13	10	90	
	13.10	85	15	
	17	85	15	
Detection System		PE Sciex API 4000 Mass Spectrometer		
Ionisation		Electrospray (ESI)		
Analyte	Transitions	Polarity	Expected Retention Time	
304428 (BAS 500F)	388→163 388→194*	positive	approx. 9.21 min.	
412053 (500M59)	370→278 370→194*	positive	approx. 7.61 min.	
411847 (500M60)	278→194* 278→149	positive	approx. 6.63 min.	
412785 (500M62)	248→132* 248→216	positive	approx. 6.58 min.	
413038 (500M76)	388→300 388→241*	positive	approx. 7.06 min.	
377613 (500M78)	177→132 177→135*	positive	approx. 5.12 min.	

\* proposed as quantification transition. Any of these transitions could be used for quantification in case interference is observed at the same retention time

**Note:** Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of the fortification experiments are in the acceptable range.  
In general a divert valve is used to reduce the matrix load on the detection system.  
Instrument conditions, e.g. injection volumes, columns, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the fortification experiments are in the acceptable range.  
Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the used instrument.



#### 4.2.2 Calibration procedures

Calculation of results is based on peak area measurements using a calibration curve. At least 5 calibration levels need to be injected (e.g., required for enforcement). The calibration curve is obtained by direct injection of mix standards for LC-MS/MS in the range of 1 ng/mL to 0.05 ng/mL for the metabolites and 0.1 ng/mL to 0.005 ng/mL for pyraclostrobin. In a given injection run, the same injection volume is used for all samples and standards.

#### 4.2.3 Calculation of Residues and Recoveries

Calculation of results is based on peak area measurements. For the procedural recoveries, the sample volume will be considered 50 mL in the final calculation of residues [ $\mu\text{g/L}$ ]. The method requires that the sample volume to be  $50 \pm 0.1$  mL for fortification samples. The recovery is the percentage of the fortified amount ( $\mu\text{g}$  or  $\text{ng}$ ), which is recovered through the method and the weights cancels out, as shown in the equation below, during the final calculation step.

The residues of BAS 500 F in  $\text{mg/kg}$  are calculated as shown in equations I and II:

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

$$\text{II. Residue [mg/kg]} = \frac{V_{\text{end}} \times C_A}{G \times A_f \times 1000}$$

$V_{\text{end}}$	=	Final volume of the extract after all dilution steps [mL]
$C_A$	=	Concentration of analyte as read from the calibration curve [ng/mL]
$G$	=	Weight of the sample extracted [g]
$A_f$	=	Aliquotation factor
1000	=	Factor remaining after all unit conversions

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

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

## 5 FLOWCHART

