1 INTRODUCTION

1.1 Scope of the method

BAS 510 F (Boscalid) is a fungicide used in fruits and vegetables.
For registration of the compound and for monitoring purposes a residue analytical method for the BAS 510 F in groundwater and surface water with a limit of quantitation of 0.03 µg/kg is needed.

The described method L0127/01 allows the specific determination of BAS 510 F (Boscalid) with the required limit of quantitation in groundwater and surface 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 trials with spiked water samples.

The recovery trials were carried out with groundwater and surface water.

The spiking levels were 0.03 and 0.3 µg/kg for groundwater and surface water. All fortification levels were analysed in 5 replicates. In addition at least one untreated control sample was analysed per matrix and fortification level. The analyses were performed by one person, with the same equipment, in the same laboratory, within a short interval of time.

In the following, the design and results of the study are reported.

1.2 Principle of the method

Enrichment of BAS 510 F is archieved by adsorption on a C18 SPE column. After desorption of the active ingredient from the SPE column with cyclohexane/ethyl acetate, the eluate is evaporated to dryness and redissolved in water/methanol (v/v = 20/80). An aliquot of the final volume is measured using LC-MS/MS.

The method has a limit of quantitation of 0.03 µg/kg in water.

1.3 Specificity

The method allows the specific determination of BAS 510 F (Boscalid) in water.

2 MATERIALS AND METHODS

2.1 Test system water

Two different types of water were used: Groundwater (tap water of the test facility) and surface water taken from Kelmetschweiher. For more details about the groundwater and surface water see Appendix 6.1 (page 21) and Appendix 6.2 (page 22).

The groundwater was directly taken from the water pipe in building Li 445 for analysis. The surface water was taken from the polyethylene storage container.

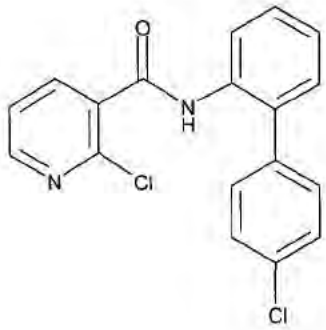
2.2 Test and reference items

2.2.1 Test items

(used for fortifications)

2.2.1.1 BAS 510 F (Boscalid)

Substance identification:	Reg. No. 300355
Common name:	Boscalid
Chemical name:	2-chloro-N-(4'-chlorobiphenyl-2-yl)nicotinamide
Structural formula:	



Empirical formula:	$C_{18}H_{12}Cl_2N_2O$
Molecular weight:	343.21
Purity:	99 %
Lot. No.:	L71-168; supplied by BASF, APR/DA, Li 721
Stability:	expected to be stable until 01.05.2014 if stored at room temperature (typically +25°C) or cooler.

2.2.2 Reference items

(used for calibration)

Same items as test items, see 2.2.1

2.3 Stability of standard solutions

Standard solutions in methanol/water are stable for at least 4 weeks when kept refrigerated (4°C) in the dark [1].

2.4 Materials and instruments

Materials, instrumentations and instrument methods were used as described in the technical procedure (see Appendix 6.5, pages 31 - 46).

2.5 Analytical procedure

The procedure described in the technical procedure was followed.

2.6 Example of calculation

Calculation of recovery of sample no. ForL0016 (tap water fortified with 0.3 µg/kg Boscalid)

Queue file: 20080ov0055 (date of analysis: November 11, 2008)
(mass transition: 343.0 -> 307.0)

Calibration curve:

Type	= linear
Peak area	= slope x concentration + intercept
Slope	= 55600.0
Intercept	= 220.0
Correlation coefficient	= 0.9995

e.g. sample no. ForL0016:

Conc. analyte [ng/mL] = (Peak area - intercept) / slope
= (7990.5 – 220.0) / 55600.0
= 0.1398 ng/mL

Data required for calculation of residues (control samples no.: ConL0003 and ConL0004)

Sample no.:	ConL0003 and ConL0004	ForL0016
Sample weight:	10 g	10 g
Fortification:	0 µg/kg = untreated	0.3 µg/kg Boscalid
Final volume (V_{end}):	2.0 mL	20 mL
Peak area:	1037.3[1)]	7990.5
Conc. of analyte (C_B):	0.01470	0.1398

[1)]Mean area of two control samples in the same worklist

Equation:

$$R\,[\mu g/kg] = \frac{V_{end} \times C_B}{S_M}$$

R	=	Residue in the water sample [µg/kg]
V_{end}	=	End volume of the extract after all dilution steps [mL]
C_B	=	Conc. of analyte in the inj. vol. as read from the calibration curve [ng/mL]
S_M	=	Weight of the water sample extracted [g]

$$R\,(untreated\ sample) = \frac{2.0 \times 0.01470}{10.0} = 0.0029\ \mu g/kg$$

$$R\,(fortified\ sample) = \frac{20 \times 0.1398}{10.0} = 0.28\ \mu g/kg$$

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

$$= \frac{0.28}{0.30} \times 100 = 93.3$$

Since the blank value (untreated samples) is less than the limit of detection (0.005 µg/kg), the corrected recovery is not calculated.

3.3 Summary of method and findings

Method

Type of method:	LC-MS/MS
Test systems:	Groundwater and surface water
Analyte detected:	BAS 510 F (Boscalid)
Extraction:	Solid phase extraction
Determined as:	Individual compound
Confirmatory technique:	Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. The quantification is based on two mass transitions for BAS 510 F (Boscalid). Recovery data are given for both mass transitions (Tables 1 – 4).
Time required:	The analysis of one series of samples (= 15 unknown samples, 2 fortified samples for recovery experiments, 1 blank sample, and 2 quality control samples) requires 1.5 working days (12 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

Findings

Limit of detection:	The limit of detection for BAS 510 F is 1.25 pg. It is here defined as the absolute amount of analyte injected into the LC-MS/MS instrument using the lowest standard of the calibration curve. For a water sample this would equal to a concentration of 0.005 µg/kg (17% of LOQ).
Limit of quantitation:	0.03 µg/kg for water
Specificity:	Specific to BAS 510 F (Boscalid).
Levels of fortification:	0.03 and 0.3 µg/kg

Appendix 6.4 continued: Technical procedure (Non-GLP)

1 Introduction

1.1 Scope of the Method

Determination of BAS 510 F in water is currently archieved by method 411/0 with quantification by GC-MS. The present method L0127/1 uses LC-MS/MS, which is also accepted as confirmatory technique.
For registration of the compound and for monitoring purposes a residue analytical method for the active ingredient BAS 510 F in water with a limit of quantitation of 0.03 µg/kg is needed.
The described method L0127/1 allows the determination of BAS 510 F with the required limit of quantitation in surface and groundwater.
This method was developed at BASF SE, Agricultural Center Limburgerhof, Germany.

1.2 Principle of the Method

Enrichment of BAS 510 F is archieved by adsorption on a C18 SPE column. After desorption of the active ingredient from the SPE column with cyclohexane/ethyl acetate, the eluate is evaporated to dryness and redissolved in water/methanol (v/v = 20/80). An aliquot of the final volume is measured using LC-MS/MS. Compared to method 411/0, the water volume is reduced by a factor of 100, even archieving a lower LOQ.

The method has a limit of quantitation of 0.03 µg/kg in water.

1.3 Specificity

BAS 510 F is identified and quantified as individual compound.

1.4 Safety

(1) Normal laboratory precautions are sufficient for safe handling of BAS 510 F.
(2) Methanol, ethyl acetate, and cyclohexane are flammable and should not be used near heat, sparks or open flames. Methanol is toxic. Cyclohexane is harmful and severe eye irritant. Ethyl acetate is irritant. Formic acid is corrosive and irritating.
(3) All solvents should be used only in well ventilated laboratories.
(4) Protective glasses and clothing should be worn during all laboratory procedures.
(5) Disposal of samples and standards must be done in compliance with on-site safety policies and procedures.

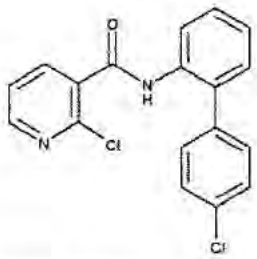
Appendix 6.4 continued: Technical procedure (Non-GLP)

2 TEST AND REFERENCE ITEMS

2.1 Test Items

2.1.1 BAS 510 F ai

Substance identification: Reg. No. 300355
Common name: Boscalid
Chemical name: 2-chloro-N-(4'-chlorobiphenyl-2-yl)nicotinamide
Structural formula:



Empirical formula: $C_{18}H_{12}Cl_2N_2O$

Molecular weight: 343.21
Purity: 99 %
Lot. No.: L71-168; supplied by BASF, APR/DA, Li 721
Stability: expected to be stable until 01.05.2014 if stored at room temperature (typically +25°C) or cooler.

2.2 Reference Items

Compounds described in chapter 2.1 were used.

2.3 Stability of Calibration Solutions and Residues in Water

Standard solutions are kept refrigerated at 4°C. An earlier study demonstrated storage stability of BAS 510 F water/methanol standard solutions for at least 30 days [1].

Appendix 6.4 continued: Technical procedure (Non-GLP)

3 MATERIALS AND METHODS

Note: Materials, chemicals, and equipment specified below were used for method development. They are specified as examples only and may be substituted with supplies of similar specifications. If the use of supplies other than those stated is intended, applicability to this method must be confirmed prior to method validation and/or routine analysis.

3.1 Equipment for Extraction and Sample Clean-up

Equipment	Size, Description	Manufacturer/ Supplier	Catalog Number
Balance	Analytical, PM4800 Delta Range	Mettler (Germany)	
Electronic pipet	HandyStep electronic	Brand	705000
PD-Tips for HandyStep electronic	12.5mL 50 mL	Brand	702378 702382
Gilson pipets	M1000 M250 M50	Gilson	
Pistons for Gilson pipets	CP1000 CP250 CP50	Gilson	
Processing Station	VacMaster Sample	International Sorbent Technology	
Evaporator	TurboVapLV Evaporator	Zymark	
Column drier	see attachment 1	made in-house	
SPE columns Bakerbond C18	500mg/3mL	J.T.Baker	7020-03
Beaker	glass; 50 mL		
Pasteur pipets	L =155 mm; up to 3ml	Fortuna (Germany)	2600111
Culture tubes (with screwing tops)	10 mL		
Autosampler vials	2 mL		
Vial caps	Teflon®-lined snap-caps		
HPLC-MS/MS	PE Sciex API 4000	PE Sciex Instruments	HPLC-MS/MS

Appendix 6.4 continued: Technical procedure (Non-GLP)

3.2 Reagents

Note: Equivalent chemicals from other suppliers may be substituted but all chemicals used must be at least of "analytical grade" or must meet equivalent specifications.

3.2.1 Chemicals

Chemical	Grade	Manufacturer/ Supplier	Catalog Number
Ethyl acetate	p.a	Riedel-de Haen (Germany)	33211
Cyclohexane	SupraSolv	Merck, Germany	1.02817
CH_3OH (methanol)	SupraSolv	Merck, Germany	1.06011
Ultra pure water, in this method referred to as pure water (H_2O)	High Purity	prepared with Millipore apparatus Milli-Q plus 185 (in-house system)	Millipore (France)

3.2.2 Solutions and solvent mixtures

Cyclohexane/ethyl acetate (v/v = 1/1)
Methanol/water (v/v = 80/20)

HPLC eluents:
mobile phase A: H_2O/HCOOH (v/v = 1000/1)
mobile phase B: CH_3OH/HCOOH (v/v = 1000/1)

3.2.3 Solutions for fortification purposes

Stock solution for fortifications
Prepare a 1 mg/mL stock solution from BAS 510 F in methanol by weighing the appropriate amount into a volumetric flask.

Diluted standard solutions for fortifications
Prepare a standard solution containing 10 ng/mL by appropriately diluting the corresponding stock solution with methanol. Suggested concentrations of standard solutions are 10 ng/mL (for 0.03 µg/kg spiking) and 100 ng/mL (for 0.3 µg/kg spiking).

3.2.4 Standard solutions for calibration

Starting from the 10.0 ng/mL solution described under 3.2.3 working solutions are prepared by dilution with methanol/water (v/v = 80/20) as needed.
Suggested concentrations of standards for calibration are 0.025, 0.05, 0.1, 0.15, 0.2, and 0.5 ng/mL. If required, other concentration schemes, and different or additional standard concentrations may be used.

Appendix 6.4 continued: Technical procedure (Non-GLP)

4 Analytical Procedure

4.1 Sample Storage

Samples are not filtered in order to include analytes sorbed to floating particles and to avoid losses due to filter sorption. Until analysis, water samples are stored in clean amber glass bottles in a refrigerator at ca. +4 °C or in plastic bottles ca. -20°C, respectively.

4.2 Sample Preparation and Fortification

10 mL of water sample is added to a 50 mL glass beaker. For fortfications, spiking solutions with analyte concentrations of 10 and 100 ng/mL are added to 10 mL of control water samples. The correlation between the concentration of the spiking solution and the resulting final analyte concentration in the sample is shown below:

Blank Sample Volume [mL]	Concentration of Spiking Solution	Volume of Spiking Solution [µL]	Level of Fortification
10			0.00 µg/kg*
10	10 ng/mL	30	0.03 µg/kg **
10	100 ng/mL	30	0.3 µg/kg

* control sample
** proposed limit of quantification

4.3 Extraction of Sample Material

4.3.1 SPE column conditioning

Mount C18 SPE columns onto the Baker extraction system and rinse the columns with 2 x 2.5 mL cyclohexane/ethyl acetate (v/v = 1/1), with 2 x 2.5 mL methanol and with 2 x 2.5 mL pure water. If necessary, apply an appropriate vacuum. Make sure that columns do not run dry. The washing solutions are discarded and the conditioned columns are filled with 2 mL pure water.

4.3.2 Water extraction

10 mL of water sample is transferred to the C18 SPE column of the water extraction apparatus by pipetting aliquots of similar volume directly onto the column. To assure quantitative transfer, the beaker is then rinsed with 2 mL of pure water and this water is transferred onto the column. Aliquots are sucked through the column by applying an appropriate vacuum resulting in a slow flow (approximately 3-4 mL/min).

4.3.3 SPE-column drying

Columns from 4.3.2 are mounted onto the column drier (attachment 1) and dried with a stream of N_2 at a temperature of 30 °C for 45 min.

4.3.4 SPE-column elution

Dried SPE columns are mounted onto the Baker extraction system and prewashed with 2.5 mL cyclohexane. The cyclohexane wash is discarded. Then the sample collector rack is provided with 10 mL glass tubes and the analyte BAS 510 F is eluted from the columns with 2 x 2.5 mL cyclohexane/ethyl acetate (v/v = 1/1). The collected eluates are evaporated to

Appendix 6.4 continued: Technical procedure (Non-GLP)

dryness in the evaporator at 40 °C water bath temperature. The residues are dissolved in methanol/water (v/ v = 80/20) solution. The volume depends on the amount of residue, which is expected. For the limit of quantification of the method (0.03 µg/L) it is 2 mL (V_{End}).

4.4 Quantitation

From the final volume V_{End} an aliquot of 50 µL is injected into the LC-MS/MS instrument for quantitation.

The LC system is coupled to a triple quadrupole mass spectrometer operated in MS/MS mode. The instrument is equipped with an ESI interface.

Note: It is advisable to verify the retention time and the sensitivity of the analyte on the chromatography system prior to each analytical series. For this, appropriate standard solutions can be injected into the chromatography system to verify peak retention time, resolution, and sensitivity of the reference substance and show the stability of the system. The retention time depends strongly on type and dimensions of the chromatography system.
The equipment and the conditions listed were used for the test of the method in water. They may be substituted, however, by comparable ones, if the applicability was proven before.

4.4.1 Chromatographic conditions

LC system:	Agilent 1100 LC Binary Pump		
Autosampler:	CTC PAL		
Injection volume:	50 µL		
LC column:	Betasil C18, 100 x 2.1 mm 5 µm		
Column temperature:	25 °C		
Mobile phase:	Solvent A – water/formic acid, (v/v = 1000/1)		
	Solvent B – methanol/formic acid, (v/v = 1000/1)		
Gradient:	Time	Composition	
	(min)	(% A)	(%B)
	0	66	34
	2.0	26	74
	4.0	10	90
	6.0	10	90
	6.1	66	34
	9.0	66	34
Flow rate:	0.6 mL/min		
Retention time:	BAS 510 F :	approx. 3.3 min	
Run time:	approx. 9.0 min		

Appendix 6.4 continued: Technical procedure (Non-GLP)

4.4.2 Mass spectrometric conditions

Mass spectrometer:	AB Sciex API 4000 triple stage quadrupole	
Interface:	ESI	
Ion mode:	BAS 510 F:	(+) MRM

Ion source temperature: 500 °C

Transitions:	BAS510 F	343.0 -> 271.0 and 343.0 -> 307.0

4.4.3 Calibration procedures

Calibration curves are generated by plotting peak area versus the concentration of the analytes measured by direct injection of reference standards containing known amounts of BAS 510 F. The linear least squares working curve in the form y = bx + c is used for the construction of the calibration curve.
A typical curve could cover a range from 0.025 to 0.5 ng/mL. In a given analytical series, the same injection volume is used for all samples and standards.
In a measuring series standards and samples are injected alternately to show the stability of the detection response during the whole series.
For each series, the set should begin and end with standard injections. Each standard level should be injected at least in duplicate.

4.4.4 Determination of instrumental recovery with QCSs

Within each analytical series at least two additional quality control samples are analyzed to check for potential matrix effects.
For this purpose, a control water sample is extracted as described in chapter 4.3.1 - 4.3.4. Yet, the residual matrix after evaporation of the eluate is dissolved in 2 mL of standard solution containing 0.15 ng/mL of BAS 510 F.
The concentration is determined from the calibration curve and related to the nominal concentration of 0.15 ng/mL.

5 Calculation of Residues

5.1 Principle

Calculation of results is based on calibration curves recorded within each analytical series. Peak area is plotted versus the concentration of analyte. The residue of BAS 510 F is calculated from its calibration curve and the equation is shown in section 5.2.

Appendix 6.4 continued: Technical procedure (Non-GLP)

5.2 Equation

The residue (R) in the water sample in µg/kg is calculated as shown in the following equation:

$$R = \frac{V_{End} \times C_B}{S_M}$$

R = Residue in the water sample [µg/kg]
V_{End} = End volume of the extract after all dilution steps [mL]
C_B = Conc. of analyte in the injection volume as read from the calibration curve [ng/mL]
S_M = Mass of water sample extracted [g]. 1 mL of water is treated as 1 g of water, since differences are negligible compared to analytical errors.

If residue data are to be corrected for loss of analyte during sample extraction and clean-up procedures the residue [R] has to be corrected with the results of the procedural recoveries as shown in the following equation:

$R_{RC} = R \times R_{FE}$

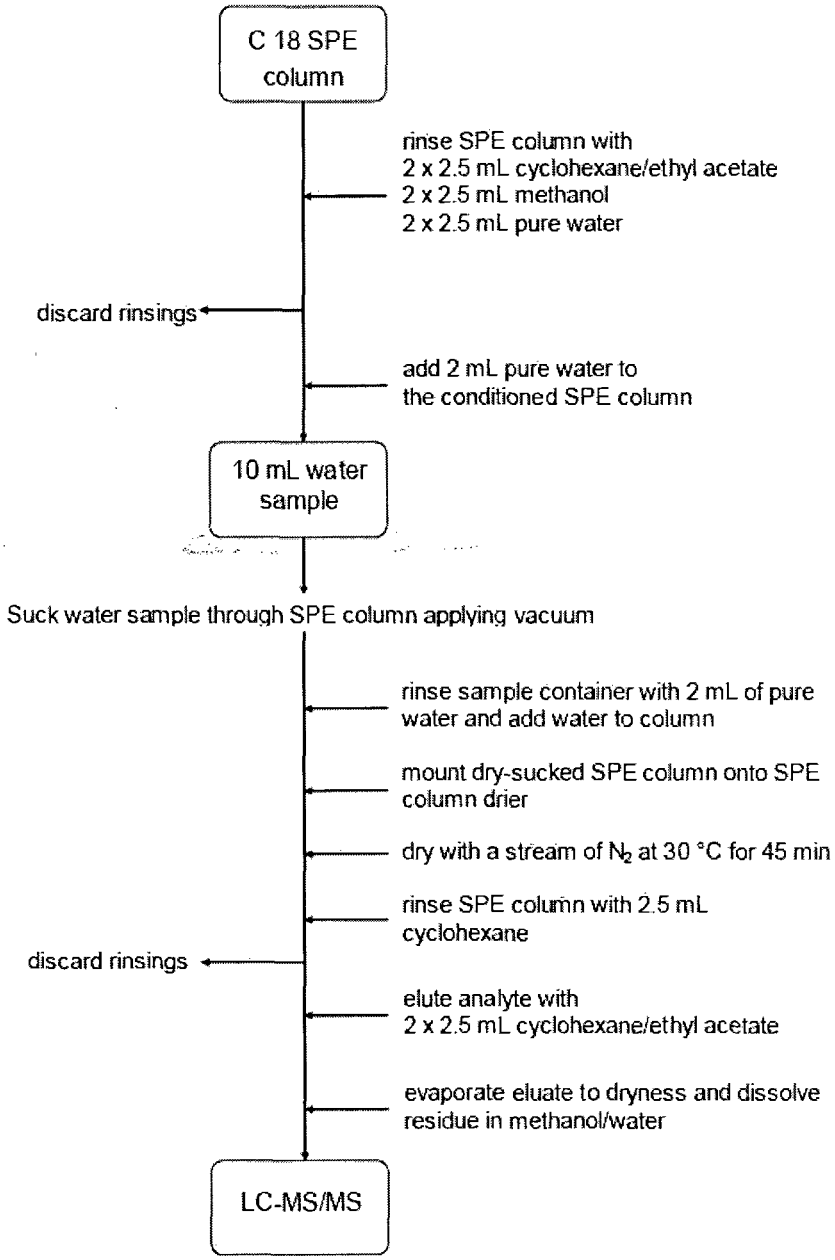
R_{RC} = Residue concentration of the analyte in the sample corrected with the procedural recovery of the analyte in fortification experiments [µg/kg sample material]
R_{FE} = Procedural recovery of the analyte as determined from fortification experiments performed in parallel to the sample analysis

$$= \frac{100\ \%\ (\text{level of fortification})}{\%\ \text{recovery}}$$

Note: *For routine analysis requirements, residue data should not be corrected for procedural recoveries. Results of fortification experiments should be listed individually.*

Appendix 6.4 continued: Technical procedure (Non-GLP)

6 Flow Chart of Method L0127/1



1 Parts of the report to be changed or appended:

1.1 Appendices

Present	No existing information on the influence of matrix load on the analysis based on quality control samples and detailed measurement tables.
New	Additional information are provided to demonstrate the influence of matrix load on the analysis using quality control samples. Detailed appendix tables show further measurement results.
Reason for alteration	Additional information are required for re-registration purposes according to SANCO/825/00 rev 8.1 (16/11/2010) and SANCO/3029/99 rev. 4 (11/07/2000).

1.2 Study Director

Present	Dr. Holger Penning
New	Dr. Tanya Ertunc
Reason for alteration	The former study director has moved to another field of responsibility within BASF.