ABSTRACT

The Objective
The objective of the study was to independently validate the determination of Boscalid (BAS 510 F) and two of its metabolites (M510F47 (Reg.No. 107371) and M510F49 (Reg.No. 391572)) with LC-MS/MS in surface water and ground water in accordance to the guidance document SANCO/825/00, rev. 8.1, SANCO/3029/99, rev. 4 of the European Commission, and US EPA OCSPP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation.

Principle of the Method
Boscalid:
The determination of Boscalid (BAS 510 F) was accomplished in accordance to BASF Method L0127/01. Boscalid was spiked in surface water and ground water. Enrichment of the analyte was achieved by adsorption on a C_{18} SPE column. After desorption of the analyte from the SPE column with cyclohexane/ethyl acetate (1/1, v/v), the eluate was evaporated to dryness and the residues were re-dissolved in methanol/water (80/20, v/v). The residue was determined using LC-MS/MS.

M510F47:
The determination of the metabolites M510F47 (Reg.No. 107371) and M510F49 (Reg.No. 391572) was carried out in accordance to BASF Method L0127/02. Enrichment of both analytes was accomplished using solid-phase extraction.
For analysis of M510F47 the water samples were acidified to approx. pH 2, spiked with the test item and extracted using OASIS SPE cartridges. The analyte was eluted from the column with methanol. After evaporation to dryness the residues were re-dissolved in pure water. The analyte was determined using LC-MS/MS.

M510F49:
For analysis of M510F49 the water samples were spiked with the test item and extracted using C_{18} SPE column. The analyte was eluted from the column using methanol. After evaporation to dryness the residues were reconstituted in methanol/water (80/20, v/v). The analyte was determined using LC-MS/MS.

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The limit of quantification is the lowest validated fortification level for each analyte and was thus successfully established at 0.03 µg/L for all three analytes in surface water and ground water.
The limit of detection was set at 0.005 µg/L for Boscalid, 0.009 µg/L for M510F47 and M510F49 in surface water and ground water. The LOD values are equivalent to chromatographic standard concentrations of 0.025 ng/mL for Boscalid, 0.45 ng/mL for M510F47 and 0.045 ng/mL for M510F49.

Selectivity
Quantification was performed by use of highly selective LC-MS/MS detection. Two selected ion mass transitions per analyte were evaluated in order to demonstrate that the methods achieve a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the reagent blanks or control samples of each matrix, so that a high level of selectivity was demonstrated.

Linearity
Boscalid:
The linearity of the detector response was demonstrated by single determination of calibration standards at 6 concentration levels ranging from 0.025 ng/mL to 0.5 ng/mL. This range corresponds to a concentration of Boscalid from 0.005 µg/L to 0.1 µg/L in the water sample and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample.
The calibration curves obtained for both ion mass transitions and both matrices were linear with coefficients of correlation (r) greater than 0.995 and with coefficients of determination (r^2) greater than 0.990. Linear regression was performed with 1/x weighting.

M510F47:
The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at 6 concentration levels ranging from 0.35 ng/mL to 10 ng/mL. This range corresponds to a concentration of M510F47 from 0.007 µg/L to 0.2 µg/L in the water sample and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample.
The calibration curves obtained for both ion mass transitions and both matrices were linear with coefficients of correlation (r) greater than 0.995 and with coefficients of determination (r^2) greater than 0.990. Linear regression was performed with 1/x weighting.

M510F49:
The linearity of the detector response was demonstrated by single determination of calibration standards at 7 concentration levels ranging from 0.010 ng/mL to 1.0 ng/mL. This range corresponds to a concentration of M510F49 from 0.002 µg/L to 0.2 µg/L in the water sample and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a sample.
The calibration curves obtained for both ion mass transitions and both matrices were linear with coefficients of correlation (r) greater than 0.995 and with coefficients of determination (r^2) greater than 0.990. Linear regression was performed with 1/x weighting.

Matrix Effects
No significant matrix effects were found in LC-MS/MS analysis for the analytes Boscalid and M510F49. For M510F47 the matrix effects of surface water and ground water were greater or equal to 20 %, so matrix-matched standard solutions were used for quantification of this analyte.
Matrix effects were determined for each SPE column production batch.

No addition or modification to the original methods L0127/01 and L0127/02 other than the necessary optimization of instrumental parameters to set up the analytical parameters on the LC-MS/MS system was done.

No communication with the method developers or others familiar with the method was necessary to carry out the analysis.

1 INTRODUCTION

1.1 Scope of the Study

The objective of the study was to independently validate the determination of Boscalid (BAS 510 F) and two of its metabolites (M510F47 (Reg.No. 107371) and M510F49 (Reg.No. 391572)) in surface water and ground water in accordance to the guidance document SANCO/825/00, rev. 8.1, SANCO/3029/99, rev. 4 of the European Commission, and US EPA OCSPP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation.
The method L0127/01 describes the specific determination of Boscalid (BAS 510 F) in surface water and ground water and was developed at BASF SE, Agricultural Center Limburgerhof, Germany.
The method L0127/02 describes the determination of M510F47 (Reg.No. 107371) and M510F49 (Reg.No. 391572) in surface water and ground water and was also developed at BASF SE, Agricultural Center Limburgerhof, Germany.
The limit of quantification is 0.03 µg/L for each analyte in surface water and ground water. The limit of detection is 0.005 µg/L for Boscalid and 0.009 µg/L for M510F47 and M510F49.
The spiking levels were 0.03 µg/L (LOQ) and 0.3 µg/L (10xLOQ) for all analytes in surface water and ground water. All fortification levels were analysed in 5 replicates. In addition four or five untreated control samples and one reagent blank were analysed per analyte and matrix.
The analytes were determined in the final sample extracts by use of highly selective LC-MS/MS detection. Since detection by MS/MS with two characteristic mass transitions is regarded to be highly specific, no further confirmatory method is required.
Matrix effects on detection were evaluated by preparation of at least six external matrix-matched standards for each matrix and analyte and comparison with at least six solvent-matched standards for each analyte.

1.2 Principle of the Method

The determination of Boscalid (BAS 510 F) was accomplished according to BASF Method L0127/01. Boscalid was spiked in surface water and ground water. Enrichment of the analyte was achieved by adsorption on a C_{18} SPE column. After desorption of the analyte from the SPE column with cyclohexane/ethyl acetate, the eluate was evaporated to dryness and the residues were re-dissolved in methanol/water (80/20, v/v). An aliquot of the final volume was measured using LC-MS/MS.

The determination of the metabolites M510F47 (Reg.No. 107371) and M510F49 (Reg.No. 391572) was carried out in accordance to BASF Method L0127/02. Enrichment of both analytes was accomplished using solid-phase extraction.
For analysis of M510F47 the water samples were acidified to approx. pH 2, spiked with the test item and extracted using OASIS HLB SPE cartridges. The analyte was eluted from the column with methanol. After evaporation to dryness the residues were re-dissolved in pure water.
For analysis of M510F49 the water samples were spiked with the test item and extracted using C_{18} SPE columns. The analyte was eluted from the column using methanol. After evaporation to dryness the residues were reconstituted in methanol/water (80/20, v/v).
The analytes were determined using LC-MS/MS.

1.3 Specificity

The method L0127/01 is highly specific for analysis of the test item Boscalid (mass transitions from the positively charged molecule ion to two typical fragment ions in MS/MS mode). The method L0127/02 is highly specific for analysis of the test items M510F47 (mass transitions from the positively charged molecule ion to two typical fragment ions in MS/MS mode) and M510F49 (mass transitions from the negatively charged molecule ion to two typical fragment ions in MS/MS mode).
The retention times of the three test items in extracts matched the retention times in solvent.
The blank values at the expected retention times of the analytes do not exceed 30 % of the LOQ, so no background interferences occurred at the retention times of Boscalid, M510F47 and M510F49.

2 MATERIALS AND METHODS

2.1 Test Systems

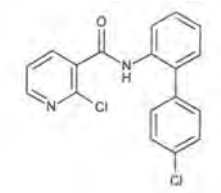
The following test systems were considered in this study of validation:

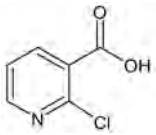
Test Matrix 1: Surface Water (Pfalz 09.10.2014) (Details are presented in the attached certificate **Figure 54**)

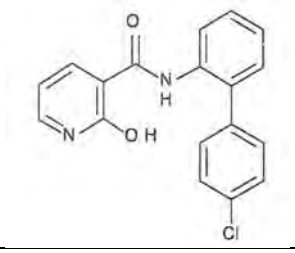
Test Matrix 2: Ground Water (Grundwasser Malsch 11.08.2015) (Details are presented in the attached certificate Figure 55)

2.2 Test and Reference Items

2.2.1 Test Items

Test Item 1			
Test item name	BAS 510 F	Batch number	L71-168
Other name(s)	Boscalid		
EAS Test item code	2011-001499	Appearance / colour	powder / white
Chemical name	2-chloro-N-(4'-chlorobiphenyl-2-yl)nicotinamide		
CAS number	188425-85-6	Purity analysed	99.0 % w/w
Chemical structure		Molecular weight	343.2 g/mol
Density	not applicable	Risk symbol(s)	N
Issue date of certificate	26 May 2015	Expiry date	01 Mar 2020
		Storage conditions	ambient (≤ +30 °C), dark, dry

Test Item 2			
Test item name	Reg.No.: 107371	Batch number	L80-188
EAS Test item code	2015-003798	Appearance / colour	solid / white
Chemical name	2-chloronicotinic acid		
CAS number	2942-59-8	Purity analysed	100 % w/w
Chemical structure		Molecular weight	157.6 g/mol
Density	not applicable	Risk symbol(s)	N
Issue date of certificate	19 May 2015	Expiry date	01 Jun 2016
		Storage conditions	ambient (≤ +25 °C), dark, dry

Test Item 3			
Test item name	Reg.No.: 391572	Batch number	L86-2
EAS Test item code	2015-003795	Appearance / colour	solid / white
Chemical name	N-(4´-chlorobiphenyl-2-yl)-2-hydroxynicotinamide		
CAS number	not available	Purity analysed	99.9 % w/w
Chemical structure		Molecular weight	324.8 g/mol
Density	not applicable	Risk symbol(s)	N
Issue date of certificate	19 May 2015	Expiry date	01 Jul 2016
		Storage conditions	ambient (≤ +25 °C), dark, dry

Specifications essential for correct identification of the test item and for use under GLP are based on the certificate of analysis as provided by the Study Sponsor/Supplier. They have not been verified by the test facility and might have not been generated under GLP, except where this is explicitly claimed on the certificate of analysis.
Copies of the certificates of analysis are included in Figure 56 to Figure 58.

2.2.2 Stability of Test and Reference Items in Standard Solution used for Calibration

It was demonstrated that the analytes Boscalid, M510F47 and M510F49 are stable in the appropriate solvent if stored in a refrigerator at 1 - 10 °C for at least 35 days.
Recovery data for each analyte/mass transition are presented in the results (section 3.1).

2.2.3 Stability of Extracts

It was demonstrated that the analytes Boscalid, M510F47 and M510F49 are stable in the extracts if stored in a refrigerator at 1 - 10 °C for at least 7 days.
Recovery data for each analyte/mass transition are presented in the results chapter (section 3.2).

2.3 Materials and Methods

2.3.1 Equipment

Equipment	Size, Description	Manufacturer
Balance	AC 120 S	Sartorius
Pipette	100 - 1000 µL	Brand
Pipette	0.5 – 5 mL	Brand
Pipette	20 - 200 µL	Brand
pH meter	Multi 340 i	WTW
Ultrasonic bath	T 900/H	Ima- Hans Schmidbauer GmbH
HPLC		Shimadzu
MS/MS	API 5500	Applied Biosystems
Evaporator	Block Heater	Stuart
Beakers	50 mL and 100 mL	VWR
Copped flask	25 mL	Duran
Glass tubes	10 mL	Pyrex
SPE columns	Bakerbond Octadecyl C_{18}; 500 mg; 3 cc	J.T. Baker
SPE columns	OASIS HLB; 500 mg; 6 cc	Waters Corporation
Pasteur pipets	150 mm	VWR
Autosampler vials	1.5 mL	VWR
Vial caps	CrimpCaps N11	Macherey-Nagel

2.3.2 Reagents

2.3.2.1 Chemicals

Chemical	Grade	Manufacturer/Supplier
Methanol	High Purity	Sigma-Aldrich
Formic acid	High Purity	Prolabo
Ultrapure water, in this method referred to as H_2O	High Purity	Merck
Cyclohexane	High Purity	Sigma-Aldrich
Ethyl acetate	High Purity	VWR, Prolabo
Hydrochloric acid	> 37 %	Sigma-Aldrich
Demineralized water	-	Elga Chorus Purelab

2.3.2.2 Solutions and Solvent Mixtures

Description	Composition
Elution solvent	cyclohexane/ethyl acetate (1/1, v/v) Add 200 mL of cyclohexane and 200 mL of ethyl acetate into a 500 mL glass bottle and mix well to ensure complete homogenous solution.
Solvent mixture 1	methanol/water (80/20, v/v) Add 800 mL of methanol and 200 mL of water into a 1 L glass bottle and mix well to ensure complete homogenous solution.
Solvent mixture 2	methanol/water (50/50, v/v) Add 15 mL of methanol and 15 mL of water into a 50 mL glass bottle, and mix well to ensure complete homogenous solution.
HPLC mobile phase A	0.1 % formic acid in pure water Add 1000 mL of water and 1 mL of concentrated formic acid into a 1 L glass bottle and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	0.1 % formic acid in methanol Add 1000 mL of methanol and 1 mL of concentrated formic acid into a 1 L glass bottle and mix well to ensure complete homogeneous solution.
6 M HCl	add 10 mL of water and 10 mL of hydrochloric acid (> 37 %) into a 50 mL glass bottle and mix well to ensure complete homogenous solution.

2.3.2.3 Standard Solutions

Stock Solutions

Stock solutions (1000 mg/L) were prepared by weighing an appropriate amount of each analyte into a flask and adding the required volume of methanol as detailed in the table below. The stock solution was allocated a unique reference number and stored at 1 - 10 °C in a brown glass vial in the dark.

Preparation of Test Item Stock Solutions

Test Item	Purity* (%)	Mass (mg)	Actual Mass** (mg)	Final Volume (mL)	Final Concentration (mg/L)	Reference of Standard Solution Produced
Boscalid	99.0	20.6	20.39	20.394	1000	S1000
M510F47	100.0	20.3	20.30	20.300	1000	S1000
M510F49	99.9	20.2	20.18	20.180	1000	S1000

* to be taken from the Certificate of Analysis (CoA)
**: Corrected for purity.

Fortification Solutions

The fortification solutions and calibration standard solutions were prepared from one stock solution in separate dilution series.
Fortification solutions (Concentration: 0.10 mg/L and 0.01 mg/L) were prepared by dilution series, prepared by using the appropriate solvent, as shown in the following tables. These solutions were also stored at 1 - 10 °C in brown glass vials in the dark.

Preparation of Fortification Solutions, Boscalid

Take Solution (mg/L)	Volume (mL)	Dilute with Methanol to a Final Volume of (mL)	Concentration (mg/L)
1000 (Stock)	1.0	10	100
100	1.0	10	10
10	1.0	10	1.0
1.0	1.0	10	0.10
0.10	1.0	10	0.01

Preparation of Fortification Solutions, M510F47

Take Solution (mg/L)	Volume (mL)	Dilute to a Final Volume of (mL)	Solvent	Concentration (mg/L)
1000 (Stock)	1.0	10	methanol	100
100	1.0	10	methanol	10
10	1.0	10	methanol/water 50/50	1.0
1.0	1.0	10	water	0.10
0.10	1.0	10	water	0.01

Preparation of Fortification Solutions, M510F49

Take Solution (mg/L)	Volume (mL)	Dilute with Methanol/Water (80/20, v/v) to a Final Volume of (mL)	Concentration (mg/L)
1000 (Stock)	1.0	10	100
100	1.0	10	10
10	1.0	10	1.0
1.0	1.0	10	0.10
0.10	1.0	10	0.01

Calibration Standard Solutions

Standard solutions for calibration of the LC-MS/MS analysis were prepared from the stock solutions of each analyte (1000 mg/L). Firstly intermediate dilutions were prepared. Afterwards, dilution series were prepared by using the appropriate solvent, as exemplified in the tables below. These solutions were also stored at 1 - 10 °C in brown glass vials in the dark.

Preparation of Standard Solutions for Calibration, Boscalid

Take solution (ng/mL)	Volume (mL)	Dilute to a Final Volume of (mL)	Solvent	Concentration (ng/mL)
Intermediate Dilutions Required				
1000000	0.50	10	MeOH	50000
50000	1.0	10	MeOH	5000
5000	0.20	10	MeOH	100
100	1.0	10	MeOH	10
10	1.0	10	MeOH/H_2O 80/20 (v/v)	1.0
Calibration Standard Solutions				
10	0.50	10	MeOH/H_2O 80/20 (v/v)	0.50
10	0.20	10	MeOH/H_2O 80/20 (v/v)	0.20
10	0.15	10	MeOH/H_2O 80/20 (v/v)	0.15
1.0	1.0	10	MeOH/H_2O 80/20 (v/v)	0.10
0.50	1.0	10	MeOH/H_2O 80/20 (v/v)	0.050
0.50	0.50	10	MeOH/H_2O 80/20 (v/v)	0.025

Preparation of Standard Solutions for Calibration, M510F47

Take solution (ng/mL)	Volume (mL)	Dilute to a Final Volume of (mL)	Solvent	Concentration (ng/mL)
Intermediate Dilutions Required				
1000000	1.0	10	MeOH	100000
100000	1.0	10	MeOH	10000
10000	1.0	10	MeOH/H_2O 50/50 (v/v)	1000
1000	1.0	10	H_2O	100
Calibration Standard Solutions				
100	1.0	10	H_2O	10
100	0.50	10	H_2O	5.0
100	0.35	10	H_2O	3.5
100	0.25	10	H_2O	2.5
10	1.0	10	H_2O	1.0
5.0	1.0	10	H_2O	0.50
3.5	1.0	10	H_2O	0.35

Preparation of Standard Solutions for Calibration, M510F49

Take Solution (ng/mL)	Volume (mL)	Dilute to a Final Volume of (mL)	Solvent	Concentration (ng/mL)
Intermediate Dilutions Required				
1000000	1	10	MeOH/H_2O 80/20 (v/v)	100000
100000	1	10	MeOH/H_2O 80/20 (v/v)	10000
10000	1	10	MeOH/H_2O 80/20 (v/v)	1000
1000	1	10	MeOH/H_2O 80/20 (v/v)	100
100	1	10	MeOH/H_2O 80/20 (v/v)	10
Calibration Standard Solutions				
10	1	10	MeOH/H_2O 80/20 (v/v)	1.0
10	0.5	10	MeOH/H_2O 80/20 (v/v)	0.50
10	0.25	10	MeOH/H_2O 80/20 (v/v)	0.25
1.0	1	10	MeOH/H_2O 80/20 (v/v)	0.10
1.0	0.5	10	MeOH/H_2O 80/20 (v/v)	0.05
1.0	0.25	10	MeOH/H_2O 80/20 (v/v)	0.025
0.1	1	10	MeOH/H_2O 80/20 (v/v)	0.010

Matrix-matched Standard Solutions

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples which were then fortified with solvent standards solutions. These standards were freshly prepared for each set of analysis and used to run the linearity investigations and for quantification of residues. Matrix load was at least 90%.

Preparation of Matrix-matched Standard Solutions for Calibration, Boscalid

Take Standard Solution (ng/mL)	Volume (µL)	Volume of Matrix Blank (µL)	Concentration (ng/mL)
10	50	950	0.50
10	20	980	0.20
1.0	150	850	0.15
1.0	100	900	0.10
1.0	50	950	0.050
1.0	25	975	0.025

Preparation of Matrix-matched Standard Solutions for Calibration, M510F47

Take Standard Solution (ng/mL)	Volume (µL)	Volume of Matrix Blank (µL)	Concentration (ng/mL)
100	50	450	10
100	25	475	5.0
100	12.5	487.5	2.5
10	50	450	1.0
10	25	475	0.5
10	17.5	482.5	0.35

Preparation of Matrix-matched Standard Solutions for Calibration, M510F49

Take Standard Solution (ng/mL)	Volume (µL)	Volume of Matrix Blank (µL)	Concentration (ng/mL)
10	100	900	1.0
10	50	950	0.50
10	25	975	0.25
1.0	100	900	0.10
1.0	50	950	0.05
1.0	25	975	0.025
0.10	100	900	0.010

2.3.2.4 Stability of Standard Solutions

Stability of selected standard solutions was assessed for at least 35 days at 1 - 10 °C in the dark (storage in refrigerator).

Reference Item	Concentration [ng/mL]	Solvent	Storage Conditions	Time Interval [days]
Boscalid (BAS 510 F)	0.50	MeOH/H_2O 80/20 (v/v)	1 - 10 °C	35
	0.20	MeOH/H_2O 80/20 (v/v)	1 - 10 °C	35
	0.10	MeOH/H_2O 80/20 (v/v)	1 - 10 °C	35
M510F47	10	H_2O	1 - 10 °C	35
	5.0	H_2O	1 - 10 °C	35
	1.0	H_2O	1 - 10 °C	35
M510F49	1.0	MeOH/H_2O 80/20 (v/v)	1 - 10 °C	35
	0.10	MeOH/H_2O 80/20 (v/v)	1 - 10 °C	35

2.3.3 Set-up of the Analytical Run

Each sequence started and ended with an injection of a calibration standard. For each analyte, at least six calibration levels were injected.
At the start of the analytical sequence, the detector response was monitored over the calibration range of interest by constructing a calibration function of peak area versus concentration.
Injections of sample extracts were interspersed with injections of quality control standards after max. 5 samples to verify the detector response.

2.4 Analytical Procedure

2.4.1 Sample Preparation

The water samples were not filtered, but sufficiently homogenized before analysis. Particulates were allowed to settle before taking aliquots for analysis.
An aliquot of water sample (10 mL for the analysis of Boscalid and M510F49 and 50 mL for the analysis of M510F47) was added to a glass beaker. In case of M510F47 the water sample was acidified to pH < 2 by addition of 200 µL 6 M HCl prior to fortification.

2.4.2 Fortification

Control (untreated) specimens of the water samples were fortified with the fortification solutions prior to extraction as described in the following table:

Sample Type	Sample Aliquot	Concentration of Spiking Solution [ng/mL]	Volume of Spiking Solution [mL]	Level of Fortification [µg/kg]
Boscalid				
Reagent Blank	-	-	-	0.00
Control	10	-	-	0.00
Fortification (LOQ)	10	10	30	0.03
Fortification (10xLOQ)	10	100	30	0.3
M510F47				
Reagent Blank	-	-	-	0.00
Control	50	-	-	0.00
Fortification (LOQ)	50	10	150	0.03
Fortification (10xLOQ)	50	100	150	0.3
M510F49				
Reagent Blank	-	-	-	0.00
Control	10	-	-	0.00
Fortification (LOQ)	10	10	30	0.03
Fortification (10xLOQ)	10	100	30	0.3

2.4.3 Extraction of Sample Material

Boscalid:

The SPE column (C_{18} Bakerbond) was first pre-conditioned by addition of 2 x 2.5 mL cyclohexane/ethyl acetate (1/1, v/v), 2 x 2.5 mL methanol and 2 x 2.5 mL pure water. Afterwards the conditioned columns were filled with 2 mL pure water. Then the fortified water sample (10 mL) was allowed to flow through the SPE column by applying an appropriate vacuum resulting in a slow flow of approximately 3-4 mL/min. The SPE column was then rinsed with 2 mL pure water, which was also used to rinse the glass beaker in which the fortified sample was prepared. All eluates up to this point were discarded. The column was then dried under vacuum for approximately 60 min at room temperature. The dried SPE-column was prewashed with 2.5 mL cyclohexane. The cyclohexane wash was discarded. The analyte was then eluted with 2 x 2.5 mL cyclohexane/ethyl acetate (1/1, v/v). The eluate was collected in a glass tube and evaporated to dryness, using a stream of nitrogen at 40 °C. The dry residue was re-dissolved in methanol/water (80/20, v/v). The volume depended on the amount of residue, which was expected. For the control samples and the fortified samples at LOQ it was 2 mL, for the fortified samples at 10xLOQ it was 20 mL. To ensure complete dissolution, ultrasonic treatment was used for a few seconds.

M510F47:

The SPE column (OASIS HLB) was first pre-conditioned by addition of 2 x 6 mL methanol followed by 2 x 6 mL pure water. Then the acidified and fortified water sample (50 mL) was allowed to flow through the SPE column without any vacuum applied to have an approximate flow of 1 drop per second. The SPE column was then rinsed with 4.5 mL pure water, which was also used to rinse the glass beaker in which the fortified sample was prepared. All eluates up to this point were discarded. The column was then dried under vacuum to remove most of the residual water to facilitate later concentration of the eluate. This was done for 5 min at room temperature. The analyte was then eluted with 2 x 4.5 mL methanol with no vacuum applied for elution and at the end vacuum applied to remove residual methanol. The eluate was collected in a glass tube and evaporated to dryness, using a stream of nitrogen at 40 °C. The dry residue was reconstituted in 1 mL pure water. To ensure complete dissolution, ultrasonic treatment was used for a few seconds.

M510F49:

The SPE column (C_{18} Bakerbond) was first pre-conditioned by addition of 2 x 2.5 mL methanol followed by 2 x 2.5 mL pure water. Then the fortified water sample (10 mL) was allowed to flow through the SPE column without any vacuum applied to have an approximate flow of 1 drop per second. The SPE column was then rinsed with 2 mL pure water, which was also used to rinse the glass beaker in which the fortified sample was prepared. All eluates up to this point were discarded. The column was then dried under vacuum to remove most of the residual water to facilitate later concentration of the eluate. This was done for 2-3 min at room temperature. The analyte was then eluted with 2 x 2.5 mL methanol with no vacuum applied for elution and at the end vacuum applied to remove residual methanol. The eluate was collected in a glass tube and evaporated to dryness, using a stream of nitrogen at 40 °C. The dry residue was reconstituted in 2 mL methanol/water (80/20, v/v). To ensure complete dissolution, ultrasonic treatment was used for a few seconds.

2.4.4 Preparation for Measurement

In case of Boscalid the extracts were directly analysed by LC-MS/MS.
In case of M510F47 the extracts of reagent blank, control samples and LOQ samples were directly analysed. The 10xLOQ fortification samples were diluted (factor 10) with matrix blank prior LC-MS/MS analysis.
In case of M510F49 the extracts of reagent blank, control samples and LOQ samples were directly analysed. The 10xLOQ fortification samples were diluted (factor 10) with methanol/water (80/20, v/v) prior LC-MS/MS analysis.
The extracts or dilutions were analysed by LC-MS/MS as described in section 2.5.1.

2.4.5 Influence of Matrix Effects on Analysis

In order to test the influence of the matrix effects on the analysis, the response of each analyte in both matrices (surface and ground water) as compared to standards in solvent was studied. Therefore, calibration standard solutions were compared against their respective calibration standards prepared in blank matrix extracts (matrix-matched standards, see section 2.3.2.3).
The influence of matrix effects on the analysis is presented in the results (section 3.4).

2.5 Instrumental Analysis

2.5.1 Instrumentation and Conditions

Boscalid:

	Parameter		
Chromatographic System			
Analytical Column	Thermo Betasil C_{18}, 100 mm x 2.1 mm, 5 µm (No. 70105-102130)		
Column Temperature	30 °C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	water/formic acid (1000/1, v/v) methanol/formic acid (1000/1, v/v)		
Flow Rate	0.6 mL/min		
Gradient (including wash and equilibration)	Time (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
Mass Spectrometric Conditions			
MS System	API 5500™ LC/MS/MS System (Sciex)		
Ionisation Type	Electrospray (ESI, TurboIon Spray)		
Polarity	Positive Ion Mode		
Scan Type	MS/MS, Multiple Reaction Monitoring (MRM)		
Capillary Voltage (IS)	5000 V		
Curtain Gas (CUR)	30 (arbitrary units)		
Collision Gas (CAD)	6 (arbitrary units)		
Entrance Potential (EP)	10 V		
Ionspray Turbo Heater (TEM)	500 °C		
Gas Flow 1 (GS1)	60 (arbitrary units)		
Gas Flow 2 (GS2)	60 (arbitrary units)		
Declustering Potential (DP)	125 V		
Analyte	**Transitions**	**Collision Energy**	**Cell Exit Potential (CXP)**
Boscalid (BAS 510 F)	342.9 → 271 m/z* 342.9 → 307 m/z	45 V 29 V	26 V 28 V
Expected Retention Time	approx. 2.8 min		

* Proposed as quantification transition.

M510F47:

	Parameter		
Chromatographic System			
Analytical Column	Waters XSelect HSS T3, 100 mm x 2.1 mm, 2.5 µm (No. 186006151)		
Column Temperature	30 °C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	water/formic acid (1000/1, v/v) methanol/formic acid (1000/1, v/v)		
Flow Rate	0.3 mL/min		
Gradient (including wash and equilibration)	Time (min)	% A	% B
	0	90	10
	0.5	90	10
	1.0	70	30
	4.1	40	60
	4.2	0.1	99.9
	6.0	0.1	99.9
	6.1	90	10
	7.0	90	10
Mass Spectrometric Conditions			
MS System	API 5500™ LC/MS/MS System (Sciex)		
Ionisation Type	Electrospray (ESI, TurboIon Spray)		
Polarity	Positive Ion Mode		
Scan Type	MS/MS, Multiple Reaction Monitoring (MRM)		
Capillary Voltage (IS)	5000 V		
Curtain Gas (CUR)	30 (arbitrary units)		
Collision Gas (CAD)	6 (arbitrary units)		
Entrance Potential (EP)	10 V		
Ionspray Turbo Heater (TEM)	500 °C		
Gas Flow 1 (GS1)	60 (arbitrary units)		
Gas Flow 2 (GS2)	60 (arbitrary units)		
Declustering Potential (DP)	56 V		
Analyte	**Transitions**	**Collision Energy**	**Cell Exit Potential (CXP)**
M510F47	157.9 → 122 m/z* 157.9 → 94 m/z	23 V 27 V	14 V 10 V
Expected Retention Time	approx. 2.9 min		

* Proposed as quantification transition.

M510F49:

	Parameter		
Chromatographic System			
Analytical Column	Thermo Betasil C_{18}, 100 mm x 2.1 mm, 5 µm (No. 70105-102130)		
Column Temperature	30 °C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	water/formic acid (1000/1, v/v) methanol/formic acid (1000/1, v/v)		
Flow Rate	0.6 mL/min		
Gradient (including wash and equilibration)	Time (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
Mass Spectrometric Conditions			
MS System	API 5500™ LC/MS/MS System (Sciex)		
Ionisation Type	Electrospray (ESI, TurboIon Spray)		
Polarity	Negative Ion Mode		
Scan Type	MS/MS, Multiple Reaction Monitoring (MRM)		
Capillary Voltage (IS)	-4500 V		
Curtain Gas (CUR)	30 (arbitrary units)		
Collision Gas (CAD)	6 (arbitrary units)		
Entrance Potential (EP)	-10 V		
Ionspray Turbo Heater (TEM)	500 °C		
Gas Flow 1 (GS1)	60 (arbitrary units)		
Gas Flow 2 (GS2)	60 (arbitrary units)		
Declustering Potential (DP)	-125 V		
Analyte	**Transitions**	**Collision Energy**	**Cell Exit Potential (CXP)**
M510F49	322.9 → 202 m/z* 322.9 → 94 m/z	-36 V -34 V	-19 V -9 V
Expected Retention Time	approx. 3.1 min		

* Proposed as quantification transition.

2.5.2 Calibration Procedures

Calculation of results was based on peak area measurements using a calibration function. At least six calibration levels were injected per each analyte. The calibration function was obtained by direct injection of the analyte standards for LC-MS/MS. Linear regression was performed with 1/x weighting.
In all runs, the same injection volume was used for samples and standards.
Injections of sample extracts were interspersed with injections of quality control standards after max. 5 samples to verify the detector response.

2.5.3 Calculation of Residues and Recoveries

The method required that the sample volume was 10 mL (Boscalid and M510F49) and 50 mL (M510F47) for fortification samples.
The residue of analyte was calculated as shown by the following equations:

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

II. Residues in the Sample Matrix [µg/L] $R \quad \frac{V_{end} \times C_A \times DF}{V_{sample}}$

V_{end}	=	Final volume of the extract [mL]
C_A	=	Concentration of analyte obtained from the calibration curve [ng/mL]
DF	=	Dilution Factor
V_{sample}	=	Volume of the sample [mL]

Remark: The response is the peak area determined after integration; intercept and slope are the factors of the linear regression function.

Recovery is the percentage of the fortified amount (µg), which is recovered through the method. The recoveries of spiked compounds are calculated according to equation **III**:

III. Recovery [%] $\text{Rec} \quad \frac{\text{Residue in fortified sample} \times 100}{\text{Amount of analyte fortified}}$

Example 1:
Boscalid (343 → 271 m/z) in Matrix Surface Water, fortified at 0.03 µg/L (SET01)
The following values were used in this calculation:

Analysis Package	*SET01*
Peak area of fortified sample (ID 1026)	*18044*
Slope	*108000*
Intercept	*2390*
Sample Aliquot (A)	*10 mL*
Final Volume (V_{end})	*2 mL*
Dilution Factor DF	*1.0*

Concentration of fortified sample (C_A) $= \frac{18044 - 2390}{108000} = 0.145 ng/mL$

Residue (fortified sample) $\frac{2\ mL \times 0.145\ ng/mL \times 1.0}{10\ mL}$ 0.0290 µg/L

Recovery [%] $\frac{0.0290\ (\mu g/L)}{0.03\ (\mu g/L)} \times 100$ 97 %

Example 2:
M510F47 (158 → 122 m/z) in Matrix Surface Water, fortified at 0.03 µg/L
(SET05: Matrix-matched standards were used for quantification)
The following values were used in this calculation:

Analysis Package	*SET05*
Peak area of fortified sample (ID 5025)	*80412*
Slope	*51500*
Intercept	*2530*
Sample Aliquot (A)	*50 mL*
Final Volume (V_{end})	*1 mL*
Dilution Factor DF	*1*

Concentration of fortified sample (C_A) $= \frac{80412 - 2530}{51500} = 1.51 ng/mL$

Residue (fortified sample) $\frac{1\ mL \times 1.51\ ng/mL \times 1}{50\ mL}$ 0.0302 µg/L

Recovery [%] $\frac{0.0302\ (\mu g/L)}{0.03\ (\mu g/L)} \times 100$ 101%

Example 3:
M510F49 (323 → 202 m/z) in Matrix Surface Water, fortified at 0.03 µg/L (SET04)
The following values were used in this calculation:

Analysis Package	*SET04*
Peak area of fortified sample (ID 4026)	*45615*
Slope	*333000*
Intercept	*3070*
Sample Aliquot (A)	*10 mL*
Final Volume (V_{end})	*2 mL*
Dilution Factor DF	*1.0*

Concentration of fortified sample (C_A) $= \frac{45615 - 3070}{333000} = 0.128 ng/mL$

Residue (fortified sample) $\frac{2\ \text{mL} \times 0.128\ \text{ng/mL} \times 1.0}{10\ \text{mL}}$ 0.0256 µg/L

Recovery [%] $\frac{0.0256\ (\mu g/L)}{0.03\ (\mu g/L)} \times 100$ 85 %

3 RESULTS

3.1 Storage Stability of Working Solutions

The working solutions prepared in solvent were stored at 1 - 10 °C for 35 days in the dark, which was sufficient to cover the length of time they were used in this study. After this time stock solutions (1000 mg/L) of each analyte and selected standard solutions were prepared freshly to check the stability of the analytes in the appropriate solvent.

For each analyte and solvent, at least three different concentrations were checked if possible. Therefore selected working solutions were diluted to the highest standard calibration level and compared to a freshly prepared solution of this concentration by triplicate injection of each. Only one ion mass transition was evaluated.

Furthermore two or three calibration standards per each analyte were compared to a freshly prepared solution of the same concentration by triplicate injection of each.

3.5 Summary of Method

Type of method: LC-MS/MS

Test systems: Surface Water (Pfalz 09.10.2014) (Details are presented in the attached certificate **Figure 54**)

Ground Water (Grundwasser Malsch 11.08.2015) (Details are presented in the attached certificate Figure 55)

Analytes and selected mass transitions:

Boscalid	343 → 271 m/z
	343 → 307 m/z
M510F47	158 → 122 m/z
	158 → 94 m/z
M510F49	323 → 202 m/z
	323 → 94 m/z

Analytical procedure: Boscalid and two of its metabolites (M510F47 and M510F49) were spiked separately in surface water and ground water at two fortification levels (0.03 µg/L and 0.3 µg/L). The analytes were extracted using SPE and determined using LC-MS/MS.

Confirmatory technique: Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique was not necessary.

The quantification was based on the monitoring of two mass transitions for each analyte. Recovery data was reported for each mass transition and matrix considered.

Limit of detection (LOD): Boscalid: 0.005 µg/L, corresponding to a concentration of 0.025 ng/mL in the extract.

M510F47: 0.009 µg/L, corresponding to a concentration of 0.45 ng/mL in the extract.

M510F49: 0.009 µg/L, corresponding to a concentration of 0.045 ng/mL in the extract.

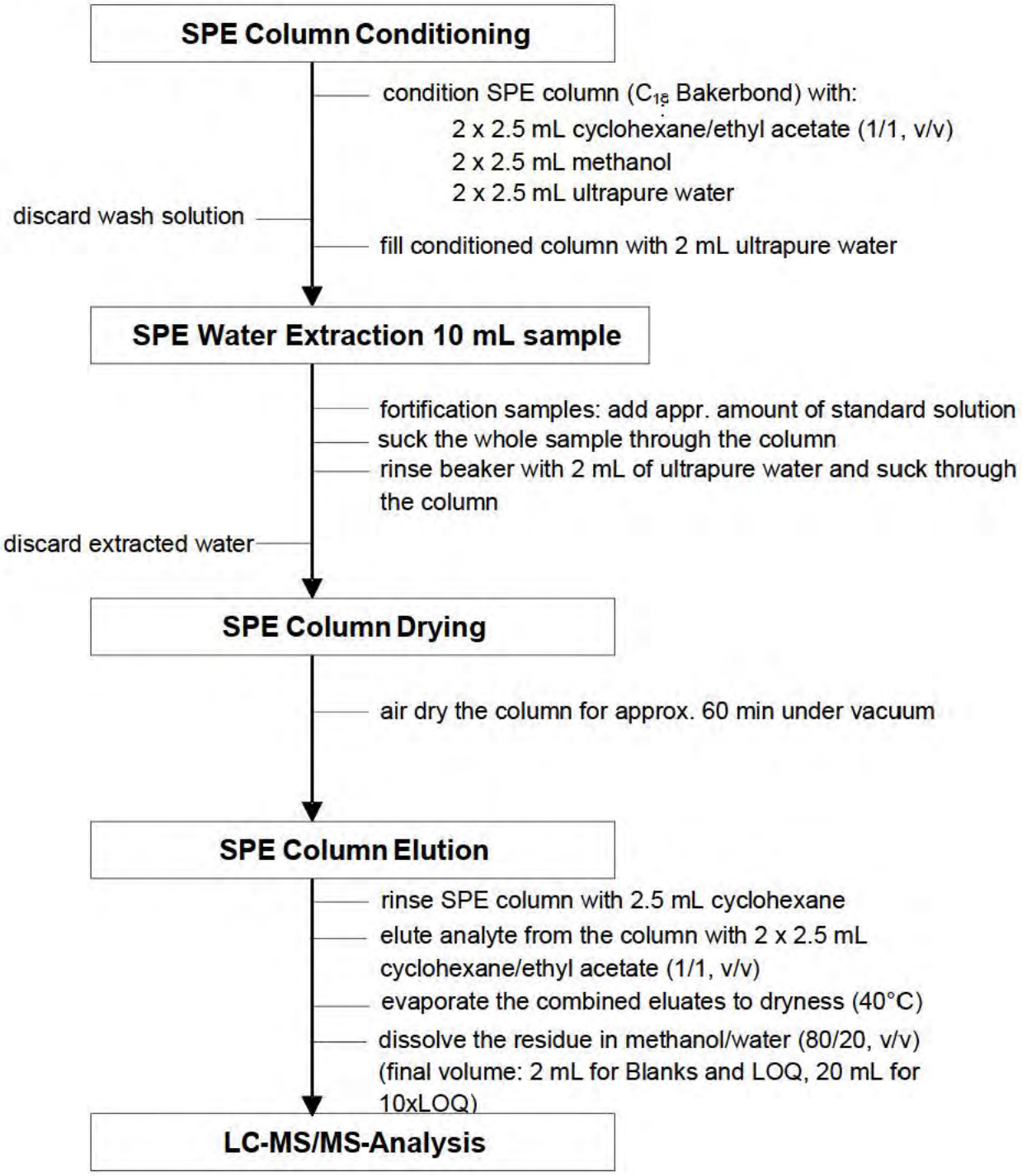
Limit of quantification (LOQ):	0.03 µg/L (lowest fortification level) for all three analytes, corresponding to a concentration in the extract of 0.15 ng/mL for Boscalid, 1.5 ng/mL for M510F47 and 0.15 ng/mL for M510F49.
Levels of fortification:	0.03 µg/L and 0.3 µg/L (LOQ and 10xLOQ)
Matrix effects:	Matrix effects on the detection of Boscalid and M510F49 were found to be insignificant (<20 %). Significant matrix effects were observed for M510F47 (mean >20 % for surface water and about 20 % for ground water), so matrix-matched standards were used for quantification of M510F47 in both matrices.
Time required:	A set of e.g. 15 samples, including preparation of matrix-matched standards for calibration can be prepared by a skilled lab technician with one working day. LC-MS/MS analysis is usually performed automatically over night, evaluation of LC-MS/MS results and subsequent calculation is done within few hours. Thus within 24 hours about 15 samples can be processed and evaluated.

4 RECOMMENDATION FROM THE ILV

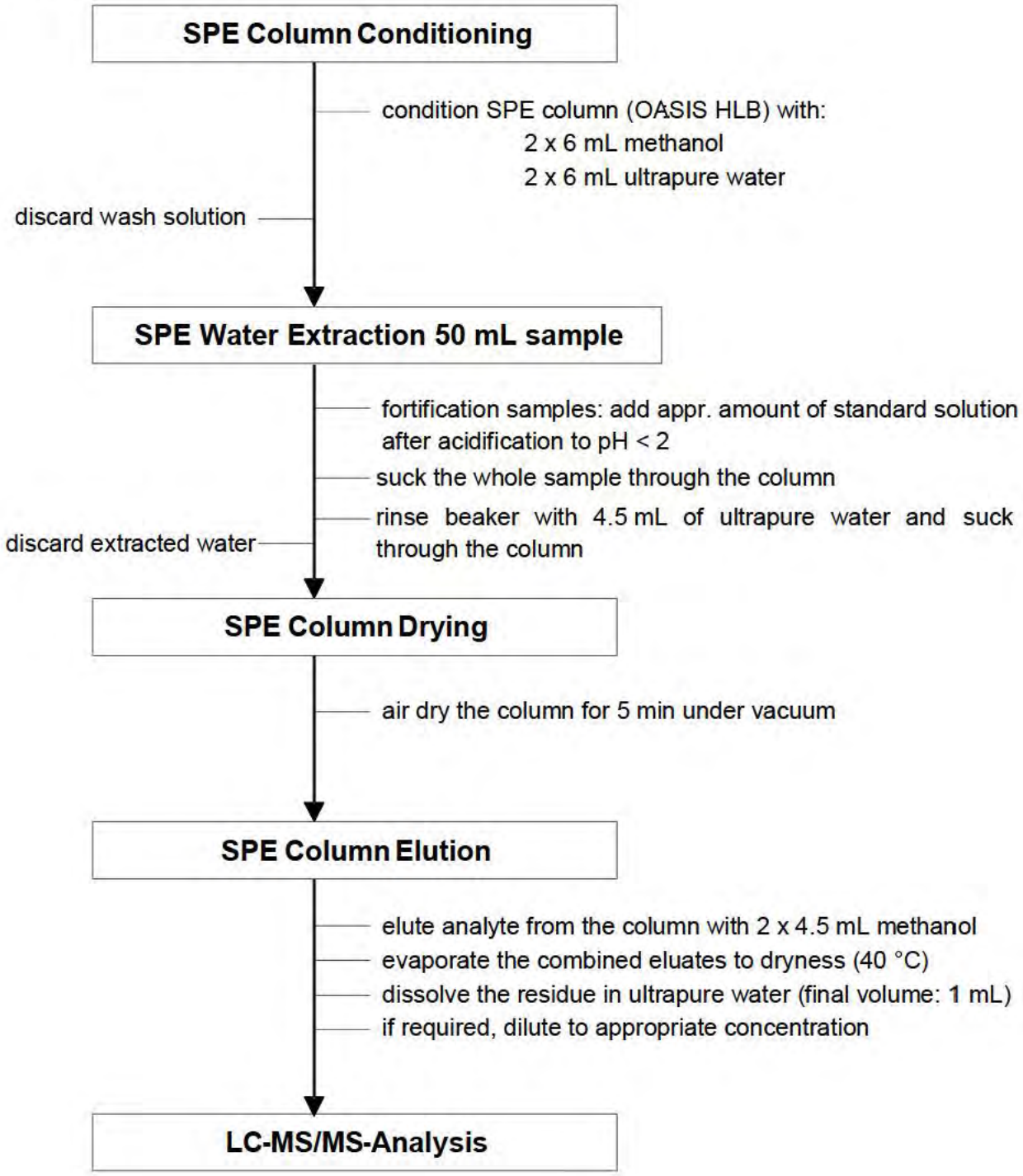
No addition or modification to the original methods L0127/01 and L0127/02 other than the necessary optimization of instrumental parameters to set up the analytical parameters on the LC-MS/MS system was done.
Matrix effects were determined for each SPE column production batch.
No communication for technical support/advice with the method developers or others familiar with the method for establishing the method and conducting the analysis was necessary to carry out the analysis.
The matrix effect of each production batch of the SPE-cartridges should be assessed.

Appendix 7.3: Additional Information on the Method

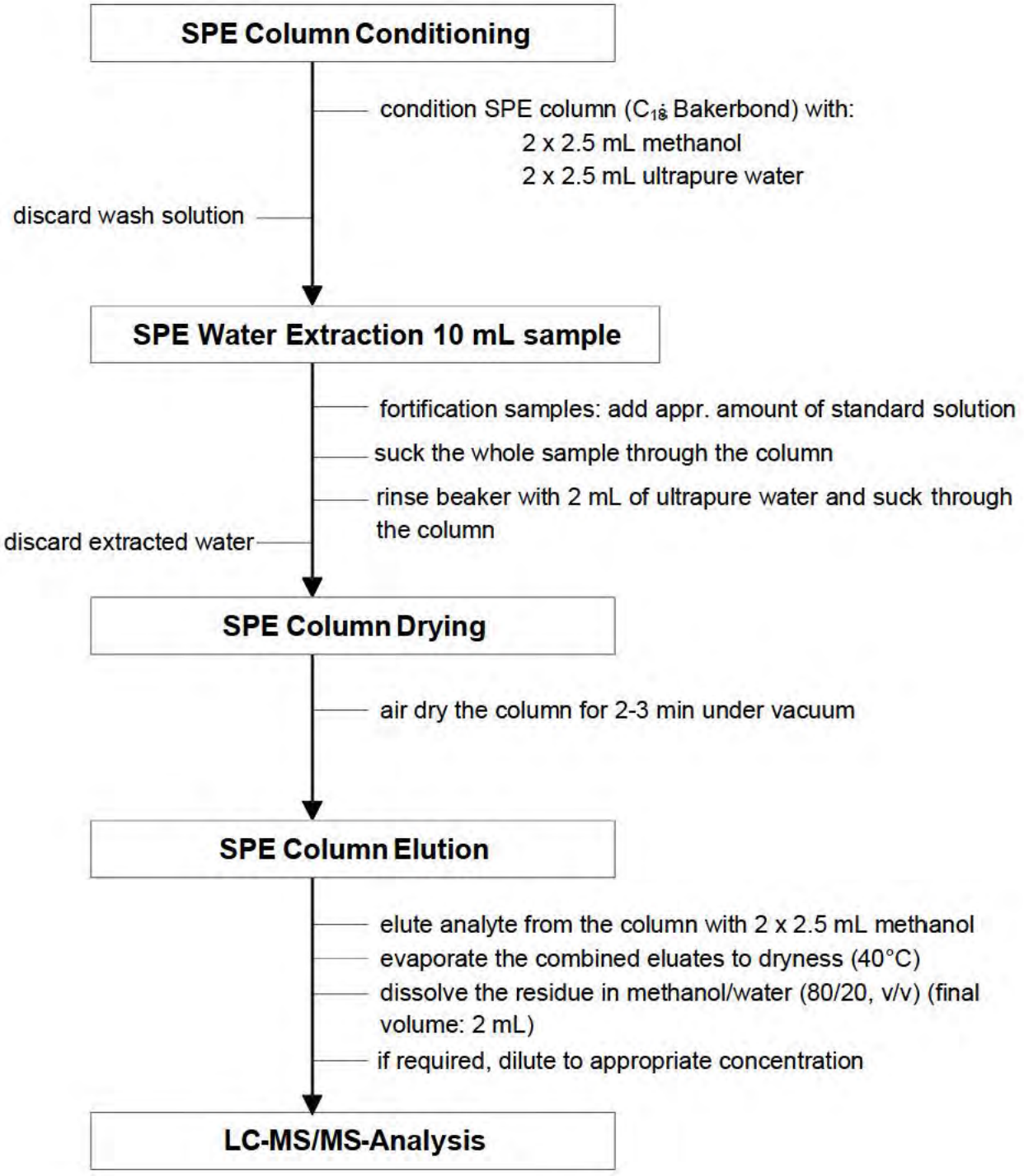
Boscalid:



M510F47:


SPE Column Conditioning
condition SPE column (OASIS HLB) with:
2 x 6 mL methanol
2 x 6 mL ultrapure water
discard wash solution
SPE Water Extraction 50 mL sample
fortification samples: add appr. amount of standard solution after acidification to pH < 2
suck the whole sample through the column
rinse beaker with 4.5 mL of ultrapure water and suck through the column
discard extracted water
SPE Column Drying
air dry the column for 5 min under vacuum
SPE Column Elution
elute analyte from the column with 2 x 4.5 mL methanol
evaporate the combined eluates to dryness (40 °C)
dissolve the residue in ultrapure water (final volume: 1 mL)
if required, dilute to appropriate concentration
LC-MS/MS-Analysis

M510F49:


SPE Column Conditioning
condition SPE column (C_{18} Bakerbond) with:
2 x 2.5 mL methanol
2 x 2.5 mL ultrapure water
discard wash solution
SPE Water Extraction 10 mL sample
fortification samples: add appr. amount of standard solution
suck the whole sample through the column
rinse beaker with 2 mL of ultrapure water and suck through the column
discard extracted water
SPE Column Drying
air dry the column for 2-3 min under vacuum
SPE Column Elution
elute analyte from the column with 2 x 2.5 mL methanol
evaporate the combined eluates to dryness (40°C)
dissolve the residue in methanol/water (80/20, v/v) (final volume: 2 mL)
if required, dilute to appropriate concentration
LC-MS/MS-Analysis