ABSTRACT

The purpose of this study is to validate BASF Analytical Method Number. D1508/01 for the analysis of the BF 500-3 (Reg.No.340266) in surface and drinking water using LC-MS/MS.

Principle of the method: The water samples (10 mL) were fortified with BF 500-3 in acetonitrile (0.03 mL) and thoroughly mixed. For control and blank, acetonitrile (0.03 mL) was added to the sample and thoroughly mixed. An aliquot of resulting solution is analysed to determine the residues of BF 500-3 in using LC-MS/MS. The transitions at *m/z* 358 → *m/z* 164 and at *m/z* 358 → *m/z* 132 was monitored in positive mode for primary and confirmation quantification, respectively. The results are calculated by direct comparison of the sample peak responses to those of external standards.

Test conditions: The method was validated at two fortification levels (0.03 and 0.3 µg/L) for BF 500-3 in surface and drinking water. For validation, untreated water samples (surface and drinking water) were fortified with BF 500-3 and analyzed according to the established method validation guidelines; The analytical sets for each matrix typically consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 0.03 µg/L (ppb), and five replicates fortified at a higher level, corresponding to ten times of the limit of quantitation, 0.3 µg/L (ppb). For each level, the two mass transitions described above were evaluated.

In conjunction with the subject study, matrix- and solvent-matched standards were analyzed in a separate experiment to evaluate any potential matrix effects. The stability of each analyte in extract solutions for each matrix of interest was also tested.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The LOQ was defined as the lowest fortification level tested. The LOQ for BF 500-3 in surface water and drinking water was 0.03 µg/L. The LOD for BF 500-3 in surface water and drinking water was set at 0.006 µg/L, which was 20% of the defined LOQ. The LOD for BF 500-3 in surface water and drinking water was defined as the absolute amount of analyte injected (0.00006 ng) into the LC-MS/MS when the lowest calibration standard was analyzed (0.0060 ng/mL) with acceptable signal to noise ratio (S/N is >3:1).

Selectivity. The method determines metabolite BF 500-3 residues in water matrices by using LC-MS/MS. No interfering peaks were found at the retention times for these analytes. The MRM transitions used to identify BF 500-3 were determined by product ion spectra.

The experiment to evaluate any potential matrix effects showed that the matrix load in the samples from the each commodity had no significant influence on analysis (matrix effects <20%); therefore, the validation samples were analyzed only using solvent-based calibration standard solutions.

Linearity. Acceptable linearity was observed for the standard range and the two mass transitions tested for each analyte: The method-detector response was linear over the 0.006-0.15 ng/mL range (r = ≥0.990).

ABSTRACT (continued)

Standard stability. Stock and intermediate (fortification) standards solutions of BF 500-3 were prepared in acetonitrile. The solution stability of fortification solution was determined in study BASF Study Number 98130. Analyte BF 500-3 exhibited stability up to 42 days in acetonitrile. A separate stability evaluation of the stock solution was not investigated as the stock and fortification standards solutions of BF 500-3 were prepared in same solution (acetonitrile). The calibration solutions were prepared in every month by serial dilution of the fortification standards solutions with water and were stored in refrigerator at an average temperature of 3°C and all solutions were used within the demonstrated time period of stability. The calibration solutions showed stability up to 30 days in water upon storage.

During the course of this study, the test/reference substance solutions were stored in refrigerator and all solutions were used within the demonstrated time period of stability.

Extract stability. The method validation fortification sample extracts were stored at refrigerator (if needed) prior to analysis and were analyzed within 0 to 7 days of extraction. The acceptable method recoveries obtained during analysis demonstrate the storage stability of residues of BF 500-3 in the extracts prior to analysis. In addition, the recoveries from stored solutions generated during extract stability experiments performed in conjunction with this study, which included tests on initial extracts and extracts at the final volume stage that was stored at refrigerator, indicated that residues of BF 500-3 are stable in extracts for at least the time period tested, 7 days, sufficient to support the storage intervals and conditions incurred by sample extracts in the study.

Recovery and Repeatability. The method validation was performed successfully for each surface and drinking water and the LC-MS/MS ion transitions (primary and secondary) available for the method, using solvent-based standards. The mean recovery values fell within the acceptable recovery range of 70-120%. The overall relative standard deviation for both fortification levels was below 20%. Additionally, the independent laboratory validation (ILV) was performed successfully for each water matrix using the LC-MS/MS ion transitions (primary and secondary) available for the method.

Apparent residues of BF 500-3 were below the method limit of detection (0.006 ng/L) in all of the control in water samples.

A summary of the recovery data obtained is shown on the next page.

Conclusion. The results of this method validation study demonstrate that BASF Analytical Method D1508/01 fulfils the requirements with regard to specificity, repeatability, limit of quantification, and recoveries and is, therefore, applicable to correctly determine BF 500-3 residues (Reg. No. 340266) in water matrices (surface and drinking water).

1. INTRODUCTION

1.1 Background and Purpose of Study

BAS 500 F is a fungicide used in several crops. A residue analytical method (D1508/01), for the analysis of the Pyraclostrobin metabolite, BF 500-3, in surface and drinking water was validated at BASF Crop Protection in Research Triangle Park, North Carolina.

The purpose of this study is to validate BASF Analytical Method Number. D1508/01 for the analysis of the BF 500-3 (Reg.No.340266) in surface and drinking water using LC-MS/MS.

1.2 Principle of the Method

The water samples (10 mL) were fortified with BF 500-3 in acetonitrile (0.03 mL) and thoroughly mixed. For control and blank, acetonitrile (0.03 mL) was added to the sample and thoroughly mixed. An aliquot of resulting solution is analysed to determine the residues of BF 500-3 in using LC-MS/MS. The transitions at *m/z* 358 → *m/z* 164 and at *m/z* 358 → *m/z* 132 was monitored in positive mode for primary and confirmation quantification, respectively.

1.3 Specificity

To demonstrate the specificity of the analytical method, one additional mass transition (*m/z* 358 → *m/z* 132) was monitored simultaneous to the primary quantitation transition (*m/z* 358 → *m/z* 164) for analysis of BF 500-3. The method was able to accurately determine residues of BF 500-3 and no interference was observed at the retention time of the analyte peak. No matrix suppression or enhancement was found to affect the analyte.

2. MATERIALS AND METHODS

2.1 Test Systems

The test systems considered in this study were surface water (ID No. 201441104) and drinking (tap) water (ID No. 201441105).

The test systems were characterized at AGVISE Laboratories (604 Highway 15 West, Northwood, ND 58267). A copy of these characterization data for both water samples is provided in the **Appendix A.**

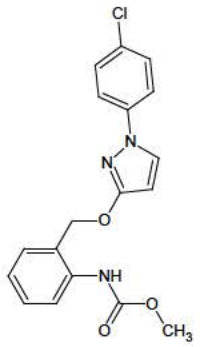
Each analysis set was uniquely identified with a Master Sheet Number, which consisted of the study number plus a unique number (e.g., 762712-1). The test system samples were assigned unique numbers according to SOP 10.04.XX and these were recorded in each analytical set or "Master Sheet" [e.g., Sample matrix (surface water, 762712-03-4, from Master Sheet No. 762712-03]. The actual sample numbers used for the analysis were identified in the raw data and in this final report.

2.2 Test and Reference Substances

The test/reference standards shown below were synthesized by BASF Aktiengesellschaft (Limburgerhof, Germany) and used during the analytical portion of this study. The test/reference items were maintained frozen until use in this study. BASF Aktiengesellschaft determined characterization and purity prior to the substances being used in this study. Details

of these determinations are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

The certificate of analysis is presented in **Appendix B**. A detailed summary of the reference substance is presented below

Code Name	BF 500-3 (500M07)	Chemical structure: 
BASF Reg. No.	340266	
CAS No.	512165-96-7	
Molecular Formula	$C_{18}H_{16}ClN_3O_3$	
Molecular Weight	357.8 g/mol	
IUPAC Name	Methyl N-(2-{[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxymethyl}phenyl) carbamate	
Lot Number	L74-118	
Purity	99.9 %	
Storage	Refrigerated or frozen	
Expiration Date	October 1, 2016	

The test/reference items in solution were used in the study to generate data for both instrument and method performance. Quantitation of residues in all samples was achieved using calibration curves calculated by linear regression of instrument responses for the reference items. The performance of the instrument was evaluated during each injection set. The solution stability detail is provided in **Section 4.3**.

2.3 Route of Administration

In this method validation study, the test items were applied to the test system as analytical standard solutions (in acetonitrile) by micropipette to ensure precise delivery of a small amount of the test items.

3. ANALYTICAL METHOD

3.1 Principle of the Method

Using BASF Analytical Method No. D1508/01, residues of BF 500-3 from water matrices are determined using direct injection after mixing with trace amount of acetonitrile using LC-MS/MS. The working method validated in this study is provided in **Appendix C**. A brief description of the methodogy as follows.

The water samples (10 mL) were fortified with BF 500-3 in acetonitrile (0.03 mL) and thoroughly mixed. For control and blank, acetonitrile (0.03 mL) was added to the sample and thoroughly mixed. An aliquot of resulting solution is analysed to determine the residues of BF 500-3 in using LC-MS/MS. The transitions at *m/z* 358 → *m/z* 164 and at *m/z* 358 → *m/z* 132 was monitored in positive mode for primary and confirmation quantification, respectively..

3.2 Specificity/Selectivity

The residues BF 500-3 are determined by LC-MS/MS, monitoring (in the positive mode) ion transitions at *m/z* 358 → *m/z* 164 (proposed as the primary transition for quantitation) and *m/z* 358 → *m/z* 132 (typically for confirmatory purposes). The results are calculated by direct comparison of the sample peak responses to those of external standards. The MRM transitions

used to identify BF 500-3 were determined by product ion spectra (see **Appendix J**). As LC-MS/MS is regarded as a highly-specific detection method when two ion transitions have been validated, an additional confirmatory method or technique is not necessary.

3.3 Validation of Method

For validation, untreated water samples (surface and drinking) were fortified with BF 500-3 and analyzed according to the established method validation guidelines; To test the repeatability of the method, the analytical sets for each matrix typically consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 0.03 μg/L (ppb), and five replicates fortified at a higher level, corresponding to 10 X the limit of quantitation, 0.3 μg/L (ppb),. For each analyte, the two mass transitions described above were evaluated. The validation data for each matrix types are provided in **Appendix E.**

3.4 Influence of Matrix Effects on Analysis

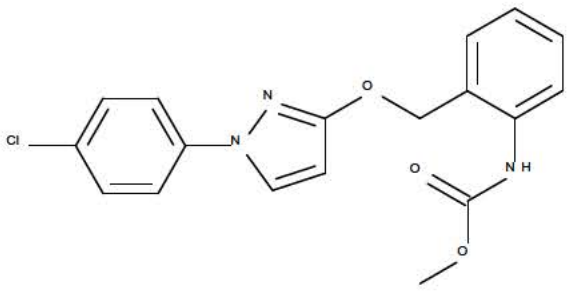
In conjunction with the subject study, matrix-matched standards and solvent-based standards were analyzed in a separate experiment to evaluate any potential matrix effects on LC-MS/MS analysis. This involved comparing calibration standards prepared in control matrix against calibration standard solutions prepared with pure solvent (water). The matrix-matched standards were made by diluting an aliquot each of three solvent-based standards with control water matrix (worked-up through the method) to the desired matrix-matched standards concentration. Each set of matrix-matched standards (for each water matrix) was bracketed by a block of calibration standards and was to have an additional single injection of each of the tested standard levels occur during the run. Only the standards which immediately bracket a matrix set, and all standard injections within that matrix set, were used in calculations involving matrix effects.

The data generated were evaluated by comparing the average area response of the standards for three injections without matrix and three injection with matrix, for each of the three standard concentration levels. Acceptability (i.e., matrices had no significant influence on the analysis) required a difference in area of <20%, calculated as the "Mean Area Change (%)". For each analyte/matrix/ion transition, an overall average "Mean Area Change (%)" across the three tested concentrations was calculated to make a general assessment of acceptability with respect to matrix effects. The detail data from matrix effect evaluation is provided in **Appendix F.**

2.2 Test and Reference Items

Standard substances are stored in a freezer (≤-5°C) until use.

BASF has retained a reserve sample of this chemical, and has documentation specifying the location of the synthesis and characterization information for this compound and is available to the BASF Research Triangle Park, North Carolina.

BAS Code	BF 500-3	
IUPAC Name	methyl N-(2-{[1-(4-chlorophenyl)-1H-pyrazol-3-y]oxymethl}phenyl) carbamate	
BASF Reg. No.	340266	
CAS-No.	512165-96-7	
Molecular Formula	$C_{18}H_{16}ClN_3O_3$	
Molecular Weight	357.8	

2.3 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Analytical	Model AT100	Mettler	----
Balance, Top Loader	Model PJ3600	Mettler DeltaRange	----
Beakers	Various sizes	PYREX Brand, VWR Scientific Products	13922-029
Bottle, Amber glass	Qorpak , 4 oz with Teflon®-lined cap	VWR Scientific Products Boston Round, Amber	89042-908
Centrifuge	Allegra 6	Bechman Coulter	----
Cylinder, Graduated	Various sizes	Various	----
Flask, Erlenmeyer, 24/40	1000 mL	Various	----
LC Vials	2 mL	Waters	600000669CV
Repeater Pipette	1000 µL, 250 µL, 25 µL	Gilson Microman	F148506G
Ultrasonic Bath	Branson 1210	Branson	----
Volumetric flask	10 mL, 25 mL, 50 mL	VWR – Class A	89041-924
Volumetric pipettes	0.5 mL, 1 mL, 2.5 mL, 5 mL, 10mL, 20 mL, 25 mL	VWR – Class A	13-650-2A
Vortex	Genie 2	VWR Scientific Products	14216-184
UPLC	Acquity UPLC Classic System	Waters	----
Mass Spectrometer	Sciex 5500 Mass Spectrometer	Sciex	----
HPLC Column	Xbridge BEH C18, 2.1 x 50 mm, 2.5 µm	Waters	186003085

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 (LC Mobile Phase Use)	Reagent Grade ≥95%	Sigma Aldrich	F0507-100 mL
Methanol	HPLC Grade	EMD	MX0475P-1
Acetonitrile	HPLC Grade	EMD	AX0145P-1
Water	HPLC Grade	BDH Aristar Plus	87003-652

Note: Equivalent reagents and chemicals from other suppliers may be substituted.

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Mobile Phase A	**LC1**	0.1% Formic Acid in Water Add 1 mL of formic acid to 1 L of water into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Mobile Phase B	**LC2**	0.1% Formic Acid in Methanol Add 1 mL of formic acid to 1 L of methanol 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.

2.5 Standard Solutions

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

2.5.1 Stock Solution (BF 500-3)

Prepare a 1.0 mg/mL stock solution individually by weighing an appropriate amount of analyte into a flask and add the required volume of **acetonitrile**.

For example, weigh 10 mg BF 500-3 into a 10 mL volumetric flask. Dissolve and dilute to mark with **acetonitrile**. This creates a solution containing 1 mg/mL of BF 500-3 in **acetonitrile**. Ensure a complete homogeneous solution (e.g. by sonication and/or vortexing). The stock solutions for all other analytes are made in a similar fashion.

Independence of standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved for example 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 is ≤ 95%. If the purity is > 95 % correction is optional.

2.5.2 Fortification Solutions (BF 500-3)

Prepare fortification solutions by diluting the stock solution made above. Dilute volumetrically with **acetonitrile** and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Take solution (µg/mL)	Volume (mL)	Dilute with acetonitrile to a final volume of (mL)	Concentration (µg/mL)
1000	0.5	50	10
10	5	50	1
1	5	50	0.1
0.1	5	50	0.01

Note: Use only volumetric glassware for the preparation of any solutions; no plastic may be used.

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.

2.5.3 Calibration Standard Solutions (BF 500-3)

Prepare calibration solutions by diluting the fortification solutions made above. Dilute volumetrically with **water** and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Take solution (ng/mL)	Volume (mL)	Dilute with water to a final volume of (mL)	Concentration (ng/mL)
100*	0.5	50	1**
1	7.5	50	0.15
0.15	20	50	0.06
0.06	25	50	0.03
0.03	25	50	0.015
0.015	20	50	0.006

*This solution was made in the “Fortification Solutions” section under the name, “0.1 µg/mL”
** This solution is not included in the standard curve, but is needed to make the subsequent calibration solutions

Note:	Use only volumetric glassware for the preparation of any solutions; no plastic may be used. 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.

Additional Information:

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

2.5.4 Stability of Standard Solutions

Stock and fortification solutions in acetonitrile were shown to be stable (less than 10% decline) for 42 days. (Reference 1)

During method development, it was shown that calibration solutions in water (HPLC Grade) were stable (less than 10% decline) for at least 30 days. An official evaluation of storage stability is conducted during the validation of this method.

3 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples have to be sufficiently homogenized beforehand, in order to assure that the aliquot taken for residue analysis is representative for the whole sample.

3.2 Sample Storage

Water samples are to be kept frozen until analysis. Freezer storage stability of this analyte in water matrices will be conducted in separate study, if required.

3.3 Weighing and Fortification

For control and treated samples, measure 10.0 mL of water sample into a 10 mL volumetric flask. Add 0.03 mL of acetonitrile and proceed to section 3.4.

For fortified samples, measure 10.0 mL control sample into a 10 mL volumetric flask and add fortification solutions in acetonitrile according to the table below and then proceed to section 3.4.

Sample Type	Sample Volume	Concentration of Fortification Solution	Volume of Fortification Solution	Level of Fortification
Control	10 mL	-	-	0.00 µg/L
Fortification (LOQ) *	10 mL	0.01 µg/mL	0.03 mL	0.03 µg/L
Fortification (10xLOQ)	10 mL	0.1 µg/mL	0.03 mL	0.3 µg/L
Fortification (100xLOQ)	10 mL	1 µg/mL	0.03 mL	3 µg/L
Treated	10 mL	-	-	-

* Limit of quantification

Note: During method development it was shown that fortification solutions in pure acetonitrile will not adsorb to the plastic Microman (Glison) pipette tips. They may be used to spike the small volumes needed for the fortification levels.

3.4 Extraction

a) Add a glass stopper to all samples and vortex to mix thoroughly (about 10 seconds).
b) Proceed to Section 3.6.

Note: BF 500-3 is known to adsorb to plastics in the presence of water. Only volumetric glassware should be used.

3.5 Sample Clean-up

Sample clean-up is not necessary. Proceed to directly from Section 3.4 to Section 3.6 to prepare the samples for measurement.

3.6 Preparation for Measurement

a) Remove all glass stoppers from the volumetric flasks.
b) Add about of 1 ml of each sample to an LC vial, with a glass Pasteur Pipette, for LC-MS/MS determination.

For all samples, samples are ready for injection.

In case of residues higher than the calibration curve, dilute the samples with **water** as needed to fit into the calibration curve. Use only volumetric glassware to dilute the samples.

See Section 4.2 for LC-MS/MS conditions.

Note:	In the presence of water, BF 500-3 is known to adsorb to plastic. Mircoman (Gilson) pipette tips or any other type of plastic should not be exposed to the samples.

3.7 Influence of Matrix Effects on Analysis

During validation no significant matrix effects were observed for surface or drinking water. If significant suppression occurs, matrix-matched standards may be utilized. Matrix-matched calibration standards are used for quantitation when signal suppression or enhancement is >20% compared to the response for standards prepared in calibration solution alone. Use the following tables below to prepare matrix matched standards if necessary.

a) Prepare precursor standards for matrix matched calibration standards in the following manner from the respective fortification solutions found in Section 2.5.2:

Take solution (ng/mL) in acetonitrile	Volume (mL)	Dilute with acetonitrile to a final volume of (mL)	Concentration (ng/mL)
100*	7.5	50	15
15	20	50	6
6	25	50	3
3	25	50	1.5
1.5	20	50	0.6

*This solution is prepared in Section 2.5.2 under the name “0.1 µg/mL”

b) When preparing five matrix match calibration standards, prepare at least five extra control samples by completing all steps through Section 3.6 (additional control matrix may be prepared to dilute samples with residues higher than LOQ).
c) Combine all the extracts from Section 3.7 [b] above into one culture tube and vortex to ensure homogeneity.
d) Prepare the matrix matched calibration standards according to the tables below, using the combined control extract from Section 3.7 [c] and the precursor standards from Section 3.7 [a]:

Take Precursor Solution (ng/mL) in acetonitrile	Volume of Precursor Standard (mL)	Control Extract Taken (mL)	Matrix Matched Standard Concentration
15	0.01	0.99	0.15
6	0.01	0.99	0.06
3	0.01	0.99	0.03
1.5	0.01	0.99	0.015
0.6	0.01	0.99	0.006

3.8 Stability of Extracts and Final Volumes

During validation, stability of the extracts and final volume solutions has been proven for 7 days. Procedural recoveries was used to prove the stability over a longer time interval.

4 QUANTIFICATION AND CALCULATION

4.1 Set-up of the Analytical Run

A sequence for measurement generally consists of:
- Calibration standards
- Control samples
- Procedural recovery samples
- Unknown samples
- Instrument recovery sample

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 and Conditions

	Parameter		
Chromatographic System	Waters UPLC Acquity system*		
Analytical-column	Xbridge BEH C18, 2.1 x 50 mm, 2.5 µm		
Pre-column	None		
Column Temperature	50 ºC		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	Water with 0.1% formic acid (**LC1**) Methanol with 0.1% formic acid (**LC2**)		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00 0.25 3.75 4.45 4.50 5.00	85 85 1 1 85 85	15 15 99 99 15 15
Detection System	Sciex 5500 Mass Spectrometer		
Ionization	Turbospray (ESI)		
Ionization Temperature	550 °C		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
BF 500-3	358 → 164** 358 → 132	positive	~ 3.5 min

*The above gradient is appropriate for the hardware profile listed. Different instrument combinations may require additional equilibration time at the end of the LC conditions to prepare the system and/or column for the next injection.

**Proposed as primary quantification transition. Any of these transitions could be used for quantitation 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.

4.3 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).

A calibration curve is obtained by direct injection of BF 500-3 on the LC-MS/MS in the range of 0.15 ng/mL to 0.006 ng/mL. In a given injection run, the same injection volume is used for all samples and standards.

Linear calibration functions are preferred for evaluation. If other functions are used (e.g. quadratic, 1/x), the new procedures need to be fully justified.

4.4 Calculation of Residues and Recoveries

Calculation of results is based on area measurements.

For the procedural recoveries, the sample weight will be considered 10 g in the final calculation of residues [ng/kg]. The method requires that the sample weight to be 10 ± 0.1 g for fortification samples. The recovery is the percentage of the fortified amount (µg or 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 BF 500-3 in mg/kg are calculated as shown in equations **I** and **II**:

Residues found in the final volume [ng] (C_A)

$$= \frac{\text{Response} - \text{Intercept}}{\text{Slope}} \times \text{Injection Volume} \times \frac{1\ \text{mL}}{1000\ \mu L}$$

Matrix injected per sample [L] (M_A)

$$= \frac{\text{Sample Weight X Injection Volume X Aliquot Factor X Dilution Factor}}{\text{Final Volume}} \times \frac{1\ \text{kg}}{1000\ g} \times \frac{1\ \text{mL}}{1000\ \mu L} \times Density_{water}$$

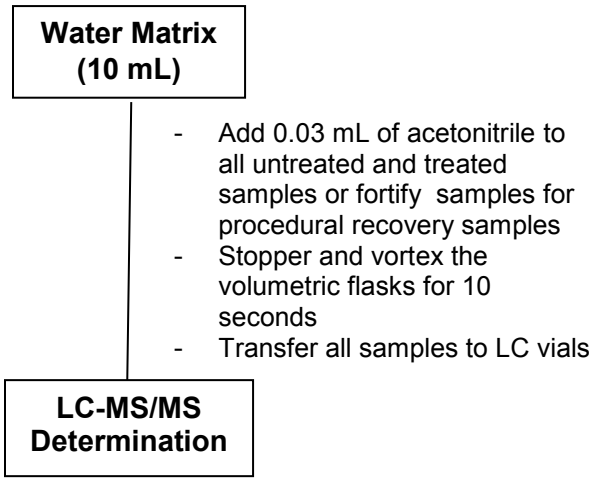
Residues Found (ng/L, ppt)

$$= \frac{\text{Residues Found in Final Volume } (C_A)}{\text{Matrix Injected per Sample } (M_A)}$$

Recovery %

$$= \frac{\text{Residues Found in Sample (ppt)} - \text{Residues Found in Control (ppt)}}{\text{Amount Fortified (ppt)}} \times 100$$

5 FLOWCHART


Water Matrix
(10 mL)
- Add 0.03 mL of acetonitrile to all untreated and treated samples or fortify samples for procedural recovery samples
- Stopper and vortex the volumetric flasks for 10 seconds
- Transfer all samples to LC vials
LC-MS/MS
Determination

Appendix L. Protocol Amendments and Deviations

Following changes will be incorporated to the study protocol

Amendment (1): The data from the first analysis set will were not used. This is due to presence of large interference peak generated using plastic cap in the culture tube. The analysis of the rest of the sets in study were conducted using the Volumetric flask instead of culture tube with plastic caps

Deviation (2): The product ion spectra was not generated within the study as stated originally in Section 5 of the protocol.

None of the amendment and deviation noted above affect the validity of the study.