Abstract

The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method D1605/01 for the determination of BAS 750 F metabolites M750F002 (Reg.No. 6031465), M750F036 (Reg.No. 6055268), and M750F037 (Reg.No. 148502) in surface and drinking water by using LC-MS/MS.

Principle of the method. Residues of M750F002, M750F036 and M750F037 in water samples are acidified with formic acid and then analyzed by direct injection onto a high performance liquid chromatography (HPLC) column with detection by positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) monitoring the following ion transitions: m/z 246→70 and 246→149 for M750F002; m/z 252→70 for M750F036; and m/z 126→70 and 126→99 for M750F037. In lieu of secondary (alternate) ion transitions for M750F036, confirmatory analysis is performed using a different LC-MS/MS column (C18 and phenyl column options are available). The results are calculated by direct comparison of the sample peak responses to those of external standards.

Test conditions. For validation, untreated drinking (well) water and surface (lake) water samples were fortified with each analyte and analyzed according to the established method validation guidelines. The analytical sets for each water type typically consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 30 ng/L (30 ppt), and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 300 ng/L (300 ppt). For each analyte, the two mass transitions or confirmatory LC-MS/MS procedures 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.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The LOQ was defined by the lowest fortification level successfully tested. The validated LOQ for residues of M750F002, M750F036, and M750F037 in water is 30 ng/L (30 ppt), for each analyte. The limit of detection is set at 6 ng/L (6 ppt, or 20% of the LOQ). The LOD is defined as the absolute amount of analyte injected (0.0006 ng) into the LC-MS/MS using lowest standard solutions.

All standards were prepared without concentration correction of purity for M750F036 (87.1% pure) and M750F037 (86.7% pure). Since all analyses had sufficient signal-to-noise ratios (≥3:1 for LOD, ≥10:1 for LOQ), the method is considered validated at the stated LOQ of 30 ng/L with an LOD of 6 ng/L even though actual concentrations of the standards were 10-15% lower than nominal. Nominal concentration values for these analytes were used throughout the study and report.

Selectivity. The method determines M750F002, M750F036, and M750F037 residues in water by LC-MS/MS. No interfering peaks were found at the retention times for these analytes. The multiple reaction monitoring (MRM) transitions used to identify each analyte were determined by product ion spectra. The experiment to evaluate any potential matrix effects showed that the matrix load in the samples from each water type had no significant influence on analysis (matrix effects <20%) with the exception of M750F036 in surface water, and then only for the confirmatory method, which required validation using matrix-matched standards exclusively.

1 Introduction

1.1 Background and Purpose of Study

The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method No. D1605/01, used for the determination of residues of M750F002, M750F036, and M750F037 in water by LC-MS/MS.

2 Materials and Methods

2.1 Test Systems

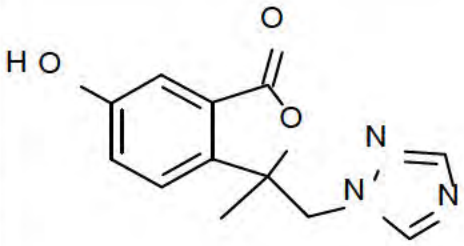
The water samples used in this study were drinking (well) water and surface (lake) water samples, which were characterized by AGVISE Laboratories. The GLP water characterization reports are provided in Appendix K. The samples were refrigerated during the experimental period. Each analysis set was uniquely identified with a Master Sheet Number, which consisted of the study number plus a unique number (e.g., 788121-1). The test system samples were assigned unique numbers and these were recorded in each analytical set or "Master Sheet" (e.g., water fortification sample 788121-1-4, from Master Sheet No. 788121-1). 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 substances were maintained according to the recommended storage conditions set forth on the certifcates of analysis (see Appendix A, page 27) until use in this study. BASF Aktiengesellschaft determined characterization and purity prior to the substance being used in this study. Details of these determinations are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

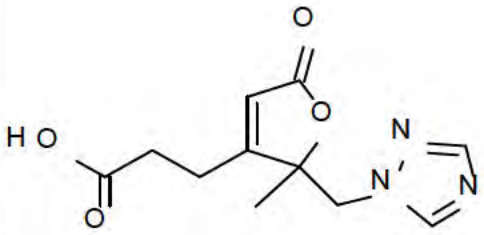
2.2.1 M750F002

Internal-Code	M750F002
Common Name	-----
IUPAC Name	6-hydroxy-3-methyl-3-(1H-1,2,4-triazol-1-ylmethyl)-2-benzofuran-1(3H)-one
BASF Reg. No.	6031465
CAS-No.	-----
Molecular Formula	$C_{12}H_{11}N_3O_3$
Molecular Weight	245.2
Lot Number	L85-138
Purity	96.8%
Expiration Date	March 1, 2018



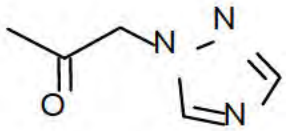
2.2.2 M750F036

Internal-Code	M750F036
Common Name	----
IUPAC Name	3-[2-methyl-5-oxo-2-(1 H-1,2,4-triazol-1-ylmethyl)-2,5-dihydrofuran-3-yl)propanoic acid
BASF Reg. No.	6055268
CAS-No.	-----
Molecular Formula	$C_{11}H_{13}N_3O_4$
Molecular Weight	251.2
Lot Number	L85-190
Purity	87.1%
Expiration Date	May 1, 2018



2.2.3 M750F037

Internal-Code	M750F037
Common Name	-----
IUPAC Name	1 -(1 H-1,2,4-triazol-1-yl)propan-2-one
BASF Reg. No.	148502
CAS-No.	64882-52-6
Molecular Formula	$C_5H_7N_3O$
Molecular Weight	125.1
Lot Number	L85-164
Purity	86.7%
Expiration Date	January 1, 2018



Stock solutions of analytes were prepared in water. The mixed intermediate/fortification solutions containing each analyte were prepared by combining aliquots of the stock solutions for each analyte and diluting with water. The calibration standards were prepared by serial dilution of the intermediate standards using water with 0.1% formic acid. The stability of the analytes in standard solutions was determined in conjunction with this study by analyzing aged standards containing each analyte against freshly prepared standard solutions. During the course of this study, the test/reference substance solutions were stored under refrigeration. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

2.3 Route of Administration

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

2.4 Analytical Method

2.4.1 Principle of the Method

Using BASF Analytical Method No. D1605/01, residues of M750F002, M750F036, and M750F037 in water are quantified using LC-MS/MS. The method procedures validated in this

study are provided in Appendix B. A description of the methodology follows: Briefly, residues in water samples (10 mL each) are acidified with formic acid and then analyzed by HPLC/MS/MS.

2.4.2 Specificity/Selectivity

Residues of M750F002, M750F036 and M750F037 are determined by HPLC-MS/MS, in positive mode, monitoring the following ion transitions: m/z 246→70 and 246→149 for M750F002; m/z 252→70 for M750F036; and m/z 126→70 and 126→99 for M750F037. In lieu of secondary (alternate) ion transitions for M750F036, confirmatory analysis is performed using a different LC-MS/MS column (C18 and phenyl column options are available). The results are calculated by direct comparison of the sample peak responses to those of external standards. Two mass transitions are available for M750F002 and M750F037. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary for these analytes. As discussed above, for M750F036 a confirmatory technique is available using a phenyl column. The multiple reaction monitoring (MRM) transitions used to identify each analyte were determined by product ion scan (see Appendix J).

2.5 Validation of Method

For validation, untreated drinking (well) water and surface (lake) water samples were fortified with each analyte and analyzed according to the established method validation guidelines. To test the repeatability of the method, the analytical sets consisted of a reagent blank, and for each matrix, two controls, five replicates fortified with each analyte at the method limit of quantitation, 30 ppt, and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 300 ppt. For each analyte, the two mass transitions or one mass transition with the additional confirmatory method described above were evaluated.

2.6 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 HPLC water with 0.1% formic acid. The matrix-matched standards were prepared by diluting mixed standards of each analyte with control drinking or surface water to 0.015, 0.03, and 0.06 ng/mL. Each set of matrix-matched standards (for each water type) was bracketed by a block of solvent-based calibration standards and included additional single injections of the tested standard levels during the run.

The data generated were evaluated by comparing the average area response of the standards for three or more injections of each type (with and without matrix) for the three standard concentration levels. Acceptability (i.e., matrices had no significant influence on the analysis) requires a difference in area of <20%, calculated as the "Mean Area Change (%)". For each matrix, an overall average "Mean Area Change (%)" across the two tested concentrations was calculated to make a general assessment of acceptability with respect to matrix effects.

2.7 Stability of Extracts

As the method does not consist of a typical “extraction” – the water samples are diluted and analyzed – “extracts” and “final volume” are used interchangeably in this report. The stability of each analyte in stored “extract” solutions was determined in conjunction with the subject method validation study. The stability in the final volume, the solution prepared for LC-MS/MS injection, was established for each matrix by reanalyzing a control and five recovery samples which had

been stored under refrigeration at the final volume stage. Quantification of the analytes in the stored samples for this experiment was performed for the primary mass transitions.

4 CALCULATIONS AND RAW DATA

An example calculation is included in Appendix C. Detailed analytical data such as supporting raw data necessary for re-calculations, standards and calibration curve data are provided in Appendix F. Example standard curves are provided in Appendix H. Example chromatographs are provided in Appendix I.

5 STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included simple descriptive statistics, such as determinations of averages, standard deviation and/or RSD for the procedural recoveries and area counts and calculation of the calibration curve and correlation coefficient (r) by linear regression of the instrument responses for the reference standards. The statistical calculations throughout this report were performed using an automated computer spreadsheet (Microsoft Excel®) and were rounded for presentation purposes. Slight differences may be noted in hand calculations using the recoveries presented in the tables. These are due to rounding and have no effect on the scientific conclusions presented in this report. The detailed analytical data may be consulted for confirmation of the calculated results.

Several measures were taken to ensure the quality of the study results. The quality assurance unit at BASF inspected the analytical procedures for compliance with Good Laboratory Practices that included adherence to the protocol. The dates inspected are detailed in the quality assurance unit statement. Study samples and test and reference items were maintained in secured (i.e. pad-locked) storage with limited access. Freezer temperatures were continuously monitored by electronic means.

6 SUMMARY OF METHOD

Summaries of the method parameters and characteristics are provided in Appendix B.

7 INDEPENDENT LABORATORY VALIDATION

This independent laboratory validation was successfully completed on the first trial at ADPEN Laboratories, Inc (reference 1). Recovery results and statistical data demonstrate BASF Analytical Method D1605/01 can be performed successfully for quantitation of M750F002, M750F036 and M750F037 in surface and drinking water.

ADPEN provided the following comments on the method:

The method is well-written and contains a fair amount of comments to guide the analyst through the procedure for the first time. Recommendations for improvement of the analytical method along with findings from the ILV are presented in below and it is recommended that they be incorporated into the method.

The following recommendations should be incorporated into the technical procedure:

1. Section 4.2: Instrumental Analysis

   Some method modifications were done to successfully complete this ILV. Please see below.

   i. For analysis of M750F036 using the XBridge BEH Phenyl LC column, an injection volume of 40 µL was used (versus 100 µL as stated in the method).

ii. For analysis of all analytes using the XSelect HSS T3 LC column, an injection volume of 80 µL was used (versus 100 µL as stated in the method).

There were no findings in this ILV. Method D1605/01 was run with the following changes:

a. The ILV used the transition *m/z* 245.99 → 149.00 for the quantitation transition and *m/z* 245.99 → 70.00 for the confirmation transition for M750F002. The transition's sensitivity was opposite of that in the validation and showed to be more sensitive and rugged with less interference. Both transitions have been validated and either can be used for data generation.
b. The following columns were used during the validation:
    i. Xselect HSS T3, 150 × 3 mm, 2.5µm
    ii. Xbridge BEH Phenyl, 150 × 3 mm, 2.5 µm
c. The following columns were used during the ILV:
    i. XSelect HSS T3, 150 mm × 2.1mm, 2.5 µm
    ii. XBridge BEH Phenyl, 100 × 2.1 mm, 2.5 µm

The difference in the column dimension had no adverse effect on recoveries, but did give different retention times for the analytes. The differences are captured in the tables below:

Primary Chromatographic Conditions for all Analytes (XSelect HSS T3)

MRM Conditions	Transition (m/z)	Retention Time (min) Validation	Retention Time (min) ILV
M750F002	245.99 → 149.00	4.75	2.6
	245.99 → 70.00		
M750F036	252.05 → 70.00	4.05	2.0
M750F037	126.00 → 70.00 126.00 → 98.90	2.90	1.0

Secondary Column for M750F036 Using Alternate Chromatographic Conditions (XBridge BEH Phenyl)

MRM Conditions	Transition (m/z)	Retention Time (min) Validation	Retention Time (min) ILV
M750F036	252.05 → 70.00	3.90	1.5

8 DISCUSSION

The method validation was performed successfully for each water matrix and the LC-MS/MS ion transitions (primary and secondary) available for the method, using solvent-based standards. The overall results are summarized below.

Table 3. Summary Parameters for the Analytical Method Used for the Quantitation of Residues of M750F002, M750F036, and M750F037 in Water

Method ID	BASF Analytical Method No. D1605/01
Analyte(s)	Residues of M750F002, M750F036, and M750F037 in water
Extraction solvent/technique	None. Residues of M750F002, M750F036, and M750F037 in water samples (10 mL each) are acidified with formic acid and mixed.
Cleanup strategies	None
Instrument/Detector	high performance liquid chromatography (HPLC) column with detection by positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) monitoring the following ion transitions: m/z 246→70 and 246→149 for M750F002; m/z 252→70 for M750F036; m/z 126→70 and 126→99 for M750F037. In lieu of secondary (alternate) ion transitions for M750F036, confirmatory analysis is performed using a different LC-MS/MS column (C_{18} and phenyl column options are available). All analyses are performed using a Waters Aquity UPLC system equipped with an XSelect HSS T3 C18 column (150 x 3 mm, 2.5µm particle size) or, for confirmatory purposes for M750F036, an Xbridge BEH phenyl column (150 x 3 mm, 2.5µm particle size) using a mobile phase gradient of water:acetonitrile, each acidified with 0.1% formic acid (flow rate 800 uL/minute). Detection is obtained with a AB Sciex API 6500 Mass Spectrometer.
Standardization method	Direct comparison of the sample peak responses to those of external standards
Stability of std solutions	The stability of the analytes in standard solutions has been determined. The storage stability data indicate that stock and fortification solutions of each analyte prepared in water are stable held under refrigeration for at least 1 month, and that calibration standards prepared in water + 0.1% formic acid have been demonstrated stable when held under refrigeration for 1 month. During the course of this study, the test/reference substance solutions were stored in a refrigerator and all solutions were used within the demonstrated time period of stability.
Retention times	See Appendix B. for typical retention times

Table 4. Characteristics for the Analytical Method Used for the Quantitation of Residues of M750F002, M750F036, and M750F037 in Water Matrices

Analyte	Residues of M750F002, M750F036, M750F037 in water
Equipment ID	Waters Aquity UPLC system equipped with an XSelect HSS T3 C18 column (150 x 3 mm, 2.5µm particle size) or, for confirmatory purposes for M750F036, an Xbridge BEH phenyl column (150 x 3 mm, 2.5µm particle size) using a mobile phase gradient of water:acetonitrile, each acidified with 0.1% formic acid (flow rate 800 uL/minute). Detection is obtained with a AB Sciex API 6500 Mass Spectrometer.
Limit of quantitation (LOQ)	The validated LOQ for residues of M750F002, M750F036, and M750F037 in water is 30 ppt for each analyte, which corresponds to a concentration in the final volume of 0.03 ng/mL.
Limit of detection (LOD)	6 ppt (The LOD was set at 20% of the LOQ), which corresponds to a concentration in the final volume of 0.006 ng/mL.
Accuracy/Precision	The range of percent recoveries and coefficient of variation for each analyte, water type (drinking and surface), transition (primary and secondary), chromatographic method, and standard type (solvent- or matrix-matched) tested indicate acceptable accuracy/precision (overall mean, generally within 70 to 110%, RSD ≤20%) in the range of spiking levels (30 & 300 ppt).
Reliability of the Method/ [ILV]	A successful independent laboratory validation [ILV] has been conducted for BASF Analytical Method No. D1605/01 for the determination of residues of M750F002, M750F036, and M750F037 in water. The values obtained are indicative of the reliability of Method No. D1605/01.
Linearity	The method-detector response, for the method validation sets, was linear over the 0.006 to 0.2 ng/mL range (r = ≥0.9953).
Specificity/ Selectivity	The control chromatograms generally have no peaks above the chromatographic background and the spiked sample chromatograms contain only the analyte peak of interest. Peaks were well-defined and symmetrical. There appeared to be no carryover to the following chromatograms.
	An experiment to evaluate any potential matrix effects showed that the matrix load in the samples from each water type had no significant influence on analysis (matrix effects <20%), with isolated exceptions (M750F036 confirmatory method in surface ater); therefore, the validation samples were analyzed primarily using solvent-based calibration standard solutions, and matrix-matched standards, where appropriate.
Confirmatory technique	Two mass transitions are available for M750F002, and M750F037. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary for these analytes. In lieu of secondary (alternate) ion transitions for M750F036, a confirmatory technique is available using a different LC-MS/MS column (C_{18} and phenyl column options are available).
Time required	A set of 13 samples (one reagent blank, two controls, and 10 fortified samples for recovery experiments) requires about 8 hours of work (calculation of the results included).

ABSTRACT

BASF Method D1605/01 is developed to determine the residues of BAS 750 F metabolites M750F002, M750F036, M750F037 in surface and drinking water using LC-MS/MS at BASF Crop Protection, Research Triangle Park, N.C.

Short description of the method:

Formic acid is added to a 10 mL water sample to achieve a 0.1% formic acid in water mixture. The sample is then ready for analysis using LC-MS/MS.

The method has a limit of quantitation of 30 ng/L (30 ppt) in water for each analyte. The limit of detection in water for each analyte is 6 ng/L.

1 INTRODUCTION

BAS 750 F is a fungicide used against several diseases in various crops. The analytical method D1605/01 offers the possibility to determine residues of M750F002, M750F036, and M750F037 in water. Method D1605/01 was successfully tested during method development in surface and drinking water for all analytes.

This method was developed at BASF Crop Protection, RTP, NC.

2 MATERIALS

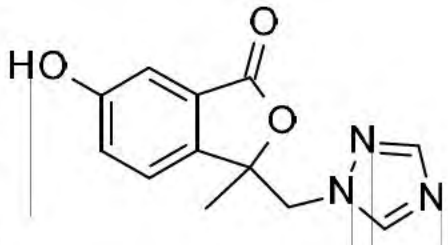
2.1 Safety

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. Store work clothing separately. Keep away from food, drink and animal feed stuffs. 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 Safety Data Sheets (SDS) 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.

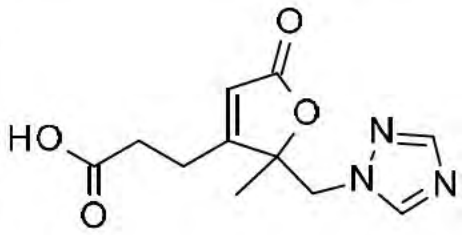
2.2 Test and Reference Items

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

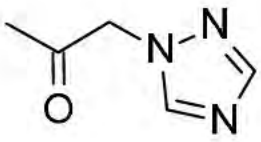
Internal-Code	M750F002
Common Name	-----
IUPAC Name	6-hydroxy-3-methyl-3-(1H-1,2,4-triazol-1-ylmethyl)-2-benzofuran-1(3H)-one
BASF Reg. No.	6031465
CAS-No.	-----
Molecular Formula	$C_{12}H_{11}N_3O_3$
Molecular Weight	245.2



Internal-Code	M750F036
Common Name	----
IUPAC Name	3-[2-methyl-5-oxo-2-(1 H-1,2,4-triazol-1-ylmethyl)-2,5-dihydrofuran-3-yl)propanoic acid
BASF Reg. No.	6055268
CAS-No.	-----
Molecular Formula	$C_{11}H_{13}N_3O_4$
Molecular Weight	251.2



Internal-Code	M750F037
Common Name	-----
IUPAC Name	1 -(1 H-1,2,4-triazol-1-yl)propan-2-one
BASF Reg. No.	148502
CAS-No.	64882-52-6
Molecular Formula	$C_5H_7N_3O$
Molecular Weight	125.1



2.3 Equipment:

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Analytical	Model AT100	Mettler	----
Beakers	Various Sizes	PYREX Brand, VWR Scientific Products	13922-029
Amber HDPE Bottles	25 mL	VWR	414004-116
Centrifuge Tubes, Polypropylene	15 mL	VWR	89039-666
Cylinder, Graduated	Various sizes	Various	----
HPLC Column : Xselect HSS T3 C18	150 x 3 mm, 2.5 µm particle size	Waters	186006737
HPLC Column : Xbridge BEH Phenyl	150 x 3 mm, 2.5 µm particle size	Waters	186006719
LC	Acquity UPLC	Waters	---
LC Vials	2 mL injection vials	National Scientific	C400-79
MicroMan pipettes	10-1000 µL	Gilson	M-25, M-50, M-250, M-1000
MS/MS	API 6500	AB Sciex	---
Mechanical shaker	KS501 digital	IKA Labortechnik	---
Ultrasonic Bath	Model FS 7652H	Fisher Scientific	---
Various Flask, Volumetric	100, 50, 25 ,10 and 5 mL	Various	---
Volumetric pipettes	Various sizes	VWR	---
Vortex mixer	Genie 2	VWR	58816-121

Note: The equipment and instrumentation listed above represents typical laboratory equipment and can be substituted by equipment of similar technical specifications. Suitability of the entire set of equipment 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.
Acetonitrile	HPLC	EMD	AX0145P-1
Formic acid	98%	EMD	Fx0440-6
Methanol	HPLC Grade	EMD	MX0475P-1
Water, e.g. Baker® or Millipore®	Gradient Grade	BDH ARISTAR PLUS	87003-652

Note: Equivalent reagents and chemicals from other suppliers may be used. If not stated otherwise, common laboratory grade chemicals are used.

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Final Volume	S1	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 A	LC1	0.1% Formic Acid in Water Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	0.1% Formic Acid in Acetonitrile Add 1000 mL of Acetonitrile and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

Note: The total volume of solutions / mixtures prepared can vary depending on the required total amounts; however, mixture ratios have to be kept as described. 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 Solution

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

For example, weigh 10 mg of M750F002 into a 10-mL volumetric flask. Dissolve and dilute to mark with water. This creates a solution containing 1 mg/mL of M750F002. Ensure a complete homogeneous solution (e.g., by sonication and/or vortexing).

Note: Sonication is required for the dissolution of M750F002, and the sample should be repeatedly sonicated in 3 minute intervals until fully dissolved.

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

1. Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
2. 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.

Fortification Solutions

Prepare mixed standard solutions for fortification with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Preparation of mixed Fortification solutions for M750F002, M750F036, and M750F037.

Take solution (µg/mL)	Aliquot Volume (mL)	Dilute with water to a final volume of (mL)	Final Concentration (µg/mL)
1000	0.1 (of each solution)	10	10
10	1	100	0.1
0.1	1	10	0.01

Note: Different concentration schemes can be used, if different fortification levels are required.

Calibration Standard Solutions

Prepare mixed standard calibration solutions for LC-MS/MS analysis by using the solutions that were prepared in Section "stock solutions" or "fortification solutions" in flasks. Prepare the calibration standards as exemplified in the table below and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Preparation of standard solutions for calibration for M750F002, M750F036, and M750F037.

Initial Concentration (ng/mL)	Aliquot Volume (mL)	Dilute with S1 to a final volume of (mL)	Final Concentration (ng/mL)
*100	1.5	50	3*
10	2	100	0.2
0.2	15	50	0.06
0.2	7.5	50	0.03
0.2	7.5	100	0.015
0.2	3	100	0.006

* Not intended to be a calibration standard but needed to prepare subsequent calibration solutions.

Note: Different concentration schemes can be used, if different fortification levels are required.

Matrix-matched standards are required for this method when determining residues in surface and drinking water matrices. Standard solutions may be used for evaluation of matrix-effects.

Depending on the matrix, significant matrix effects may interfere with the analysis of the samples. If significant matrix-effects occur, 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. In this case, calibration standard solution are prepared in matrix solution, i.e., using a final volume mixture from multiple control samples or using a large batch of 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. See section 3.5 for details on matrix-matched standards preparation.

2.4.4 Stability of Standard Solutions

BASF recommends that stock solutions (1 mg/mL) in water are prepared freshly every 3 months. Dilutions of stock solutions should be stored refrigerated no longer than one month or according to their established storage stability in the particular solvent.

The stability of M750F002, M750F036, and M750F037 in stock, fortification and calibration solutions will be established during the method validation.

3 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Sample homogenization is not needed for water samples.

3.2 Sample Storage

Water samples are stored frozen in clean amber Nalgene (HDPE) bottles.

3.3 Weighing and Preparation of Fortified /Treated Samples

For treated samples and control samples, measure 10 ±0.1 g (or 10 mL) of water sample into a HDPE Nalgene bottle.

For fortified samples, measure 10 ±0.1 g (or 10 mL) of water sample into a HDPE Nalgene bottle. Fortify the solution with analyte(s) and shake for approximately 15 minutes to ensure homogenization.

The following scheme may be used:

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	10 g (or mL)	-	-	0.00 ng/L
Fortification (**LOQ**)	10 g (or mL)	10 ng/mL	0.03 mL	30 ng/L * (30 ppt)
Fortification (**10xLOQ**)	10 g (or mL)	100 ng/mL	0.03 mL	300 ng/L (300 ppt)
Treated	10 g (or mL)	-	-	-

* limit of quantification

Note: Different concentration schemes can be used, if different fortification levels are required.

Total volume of solutions prepared can be changed if overall ratios are maintained. Volume of spiking solution added should not exceed 1% of sample volume.

3.4 Preparation for Measurement

Add 10 µL of formic acid to the water sample and shake/vortex for approximately 1 minute to ensure homogenization. Transfer an aliquot to LC vial for analysis. High fortification and high residue treated samples may need to be diluted further with control matrix (see section 3.5).

3.5 Influence of Matrix Effects on Analysis

Depending on the matrix, significant matrix effects may interfere with the analysis of the samples. If significant matrix-effects occur, matrix-matched standards may be utilized.

a) Prepare precursor standard solutions for matrix-matched calibration standards according to the following table:

Preparation of Precursor Solutions for Matrix-Matched Standards for M750F002, M750F036, and M750F037.

Initial Concentration (ng/mL)	Aliquot Volume (mL)	Dilute with S1 to a final volume of (mL)	Final Concentration (ng/mL)
100	10	50	20
100	3	50	6
100	1.5	50	3
10	7.5	50	1.5
10	3	50	0.6

b) When preparing 5 matrix-matched standards, prepare at least two extra control samples by completing all steps through 3.4.
c) Combine all samples prepared according to 3.5 [b] above into one sample container and vortex to ensure homogeneity.
d) Prepare matrix-matched calibration standards according to the table below using precursor standard solutions prepared in 3.5 [a] and control matrix in 3.5 [c]:

Preparation of Matrix-Matched Standards for M750F002, M750F036, and M750F037.

Take Precursor Solution (ng/mL)	Volume of Precursor Solution (mL)	Dilute with Control Matrix to a final volume of (mL)	Final Concentration (ng/mL)
20	0.01	1	0.2
6	0.01	1	0.06
3	0.01	1	0.03
1.5	0.01	1	0.015
0.6	0.01	1	0.006

e) Dilute high residue samples to an appropriate concentration with control matrix prepared in 3.5 [c].

3.6 Stability in Sample Matrix

Stability in surface and well water will be tested during the method validation.

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 considered 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 at least be injected twice. At least 5 calibration levels are needed.

4.2 Instrumental analysis

4.2.1 Instrumentation and Conditions

Reg. No.'s 6031465, 6055268 and 148502	Parameter		
Chromatographic System	Waters Acquity UPLC System		
Analytical-column	Xselect HSS T3 C18, 150 x 3 mm, 2.5µm particle size		
Column Temperature	50°C		
Injection Volume	100 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	95	5
	0.25	95	5
	4.25	50	50
	5.50	1	99
	6.99	1	99
	7.00	95	5
	8.00	95	5
Detection System	AB Sciex API 6500 Mass Spectrometer		
Ionisation	Turbo Spray (ESI) Source Temp.: 700°C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Reg. No. 6031465 **M750F002**	246 --> 70* 246 --> 149	positive	Approx 4.0 min
Reg. No. 6055268 **M750F036**	252 --> 70*	positive	approx. 3.3 min.
Reg. No. 148502 **M750F037**	126 --> 70* 126 --> 99	positive	approx. 2.2 min.

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

[1] The system is a UPLC instrument. However, the method operates under HPLC conditions (<400 bar).

4.2.2 Confirmatory Instrumentation and Conditions (for Reg. No. 6055268)

Reg. No. 6055268	Parameter		
Chromatographic System	Waters Acquity UPLC System		
Analytical-column	Xbridge BEH Phenyl, 150 x 3 mm, 2.5 µm particle size		
Column Temperature	50°C		
Injection Volume	100 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Acetonitrile / formic acid, 1000/1, v/v		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	95	5
	0.25	95	5
	4.00	70	30
	6.50	1	99
	7.35	1	99
	7.40	95	5
	8.00	95	5
Detection System	AB Sciex API 6500 Mass Spectrometer		
Ionisation	Turbo Spray (ESI) Source Temp.: 700ºC		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Reg. No. 6055268 **M750F036**	252 --> 70	positive	approx. 3.4 min.

[1] The system is a UPLC instrument. However, the method operates under HPLC conditions (<400 bar).

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.

A divert valve can be used to reduce the matrix load on the detection system.

Instrument conditions, e.g. injection volume, column, gradient steps may be modified; however changes have to be documented in the raw data. Changes are acceptable, if the recoveries of the fortification experiments are in the acceptable range of the required guidelines.

If the use of different analytical columns (different stationary phase) is required, then methodology has to be validated by analyzing at least five replicates of fortified samples prepared at e.g. LOQ and 10xLOQ. Assessment of matrix impact by preparation of at least one concentration level of a matrix matched standard is also required.

The same applies to different mass transitions used: Validation of the methodology is required as described above (fortification and assessment of matrix effect).

Other parameters, such as ion source gas flows and voltages, are highly specific of the equipment used and therefore not listed. Those parameters may need to be adapted to the actual instrument.

4.2.3 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). The calibration curve is obtained by direct injection of standards for LC-MS/MS in the range of 0.2 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), this should be fully justified.

4.2.4 Calculation of Residues and Recoveries

Calculation of results is based on area measurements.
For the procedural recoveries, a sample volume of 10 g (or 10 mL) will be considered in the final calculation of residues [ng/L]. This approach requires that the sample volume has to be within a measuring precision of 10 ± 0.1 g (or mL) for fortification samples (matrix). The recovery is the percentage of the fortified amount of the analyte (µg or ng), which is recovered after the entire sample work-up steps.

Calculation is described by the equation given below:

The residues of BAS 750 F in ng/L are calculated as shown in equations **I** and **II**:

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

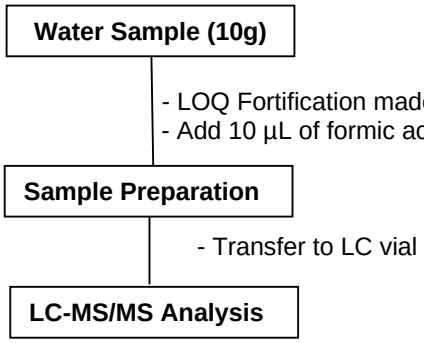
II. **Residue [ng/L]** $= \dfrac{V_{end} \times C_A}{G \times A_F}$

V_{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 = Volume of sample extracted in L
A_F = Aliquot factor (1 for this method)

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

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

5 FLOWCHART



6 METHOD MANAGEMENT AND TIME REQUIREMENTS

The analysis of one series of samples (= 13 total samples, 1 reagent blank, 2 controls, 5 fortified samples at LOQ and 5 fortified samples at 10x LOQ) requires 1 working day (8 hours) to complete. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

7 CONCLUSION AND METHOD CAPABILITIES

Recoveries, Chromatograms, and Calibration Curves
Recovery data will be provided in the validation report of the analytical method D1605/01.

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The limit of quantification is defined as the lowest fortification level successfully tested. The limit of quantification is 30 ng/L (30 ppt) for all analytes. The limit of detection is estimated to be 20% of the limit of quantification, equivalent to 6 ng/L for all analytes. The lowest standard for each analyte in the calibration curve has good sensitivity, hence a signal to noise ratio greater than 3:1.

Selectivity
The tested untreated surface and well water samples showed no significant interferences (<30 %) at the retention time of the analytes of interest.

Confirmatory Techniques
The HPLC-MS/MS determination for M750F002 and M750F037 is a highly selective detection technique, and quantitation is possible at two different mass transitions. For M750F036 a secondary chromatographic technique using a different stationary phase is included for confirmation.

DEFINITIONS AND ACRONYMS

Sample Set:	A group of samples that are extracted and cleaned up at the same time using the same method represented.
Untreated Sample:	A sample that has not been treated with the test substance.
Control Sample:	Usually an untreated sample used for fortification experiments (can be acquired from same study or from a different source).
Unknown Sample:	The samples with unknown residues.
Treated Sample:	A sample that has been treated with the test substance.
Blank:	Solvent, solution or mobile phase injected together with a sample set.
Reagent Blank:	A complete analysis conducted using solvents and reagents only in absence of any sample. Also known as blank of reagents or procedural blank. This sample is analyzed within the sample set in order to evaluate possible contamination on chemicals/reagents.
Procedural Recovery:	A control sample to which a known amount of analyte has been added before sample work up. This sample is then carried through the method and analyzed with the unknown samples in order to determine the reliability of the method.
Instrument Recovery:	A control sample which is carried through the method and to which a known amount of analyte has been added before injection. This sample is analyzed within the sample set in order to evaluate the matrix effect in the instrument.
Analytical Run:	A group of samples that undergo a determinative measurement on an analytical instrument (such as GC, HPLC, CE, GC/MS, or LC/MS/MS) in a defined and continuous sequence under identical instrumental conditions.
Limit of Quantitation (LOQ):	Lowest tested concentration of the analyte in a sample that can be determined with acceptable accuracy and precision according to the method which is also known as reporting limit.
Limit of Detection (LOD):	Concentration of analyte equivalent to a defined percentage of the limit of quantitation of the method (e.g 20% of LOQ). At this concentration, the analyte must be qualitatively detectable in sample matrix (analyte peak height at least 3-5 x baseline noise).

Typical Recovery Calculation for LC/MS/MS Quantitation

Sample No. 788121-2-4. Control surface water sample fortified at the LOQ with M750F002, M750F036, and M750F037, Master Sheet No. 788121-2.

$$\text{Concentration of analyte (ng/mL)} = \frac{\text{peak area - intercept}}{\text{slope}}$$

	M750F002
Peak Area =	23429
Intercept =	-1464.3787
Slope =	960198.3807
Conc. (ng/mL) =	0.0259

The concentration of analyte in ng/kg (ppt) is calculated as shown in equation:

$$\text{Residue [ppt]} = \frac{V_{end} \times C_A}{G \times A_F}$$

Where:

V_{end} = Final volume [mL]
C_A = Concentration of analyte as read from the calibration curve [ng/mL]
G = Weight of the sample extracted
A_F = Aliquotation factor

	M750F002
V_{end} =	10 mL
A_F =	100%
G =	10.0
Conc. (ng/mL) =	0.0259
Residue (ppt) =	25.9

Net residue (ppt of analyte) = Residue (ppt of analyte) - Residue in Control (ppt)

$$\text{Recovery of analyte (\%)} = \frac{\text{Residue (ppt of analyte) - Residue in Control (ppt)}}{\text{Amount Fortified (ppt)}} \times 100$$

	M750F002
Amount fortified (ppt) =	30
Residue (ppt) =	25.9
Residue in control =	0.0000
%Recovery	86%

Use full calculator precision in any intermediate calculations. Round only the final value.

1) All standards were prepared without concentration correction of purity for M750F036 (87.1% pure) and M750F037 (86.7% pure). Since all analyses had sufficient signal-to-noise ratios (≥3:1 for LOD, ≥10:1 for LOQ), the method is considered validated at the stated LOQ of 30 ng/L with an LOD of 6 ng/L even though actual concentrations of the standards were 10-15% lower than nominal. Nominal concentration values for these analytes were used throughout the study and report.

2) There were only two injections for the matrix test of 788121-3-D. Section 4 of the protocol states there should be ‘at least three injections of each.

3) Corrected the secondary transition for M750F037 to 126→99 due to a typographical error in the method.

4) Updated purity and expiration date for M750F002 to reflect new COA.

5) The title of the technical procedure in the original protocol is incorrect. Updated title.

None of these changes had an adverse effect on the outcome of this study.