Abstract

The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method D1705/01 for the determination of BAS 450 I metabolites S(Br-OH)-8007 (Reg.No. 5959595), AB-Oxa (Reg.No. 5959600), and MFBA (Reg.No. 6088668) in surface and drinking water using LC-MS/MS.

Principle of the method. Residues of S(Br-OH)-8007, AB-Oxa and MFBA in water samples are diluted with methanol 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 601→256 and 601→581 for S(Br-OH)-8007; m/z 583→444 and 583→494 for AB-Oxa; and m/z 274→254 for MFBA. In lieu of secondary (alternate) ion transitions for MFBA, confirmatory analysis is performed using a different LC-MS/MS column and gradient. 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, 25 ng/L (25 ppt), and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 250 ng/L (250 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 of the method was set at 25 ng/L (25 ppt) in water for S(Br-OH)-8007, AB-Oxa, and MFBA which was lower than the lowest relevant endpoint in water ecotoxicology (NOEC is 6.3 ng/L for the parent, BAS 450 I) and also defined as the lowest fortification level for each analyte. The limit of detection is set at 5 ng/L (5 ppt, or 20% of the LOQ). The LOD is defined as the absolute amount of analyte injected (0.00005 ng on-column) into the LC-MS/MS using lowest standard solutions.

Selectivity. The method determines S(Br-OH)-8007, AB-Oxa, and MFBA 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%).

Linearity. Acceptable linearity was observed for the standard range tested for each analyte: The method-detector response, for the method validation sets, was linear over the 0.0025 to 0.05 ng/mL range ($r \geq 0.9978$).

Standard stability. The stability of the analytes in standard solutions was established in this study. The storage stability data indicate that stock and fortification solutions of each analyte prepared in acetonitrile are stable when refrigerated for at least 65 days, and that calibration standards prepared in methanol water (50:50, v/v) have been demonstrated stable when held under refrigeration for 62 days. 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.

Extract stability. Stability in final volume (extract) prepared for LC-MS/MS analysis was determined. The recoveries from stored solutions generated during extract stability experiments performed in conjunction with this study, which included tests on the final volume held under refrigeration, indicated that each analyte is stable in extracts of surface water for at least 7 days.

Recovery and Repeatability. The method validation was performed successfully for each water type and the LC-MS/MS ion transitions (primary and secondary) or confirmatory procedures available for the method, using solvent-based standards. Mean overall recoveries of S(Br-OH)-8007, AB-Oxa, and MFBA from drinking (well) water and surface (lake) samples fortified with each analyte at 25 and 250 ng/L ranged within 70 to 110% (RSD ≤ 20%) considering results obtained using both the primary and secondary transitions and/or confirmatory techniques. Apparent residues of each analyte were below the method limit of detection (< 5 ng/L) in all of the control water samples.

Conclusion. The results of this method validation study demonstrate that BASF Analytical Method No. D1705/01 fulfils the requirements with regard to specificity, repeatability, limit of quantification, and recoveries and is, therefore, applicable to correctly determine S(Br-OH)-8007, AB-Oxa, and MFBA residues in water.

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. D1705/01, used for the determination of residues of S(Br-OH)-8007, AB-Oxa, and MFBA 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., 838397-2). The test system samples were assigned unique numbers and these were recorded in each analytical set or "Master Sheet" (e.g., water fortification sample CM16-016-LF1, from Master Sheet No. 838397-2 using control matrix sample CM16-016). The actual sample numbers used for the analysis were identified in the raw data and in this final report.

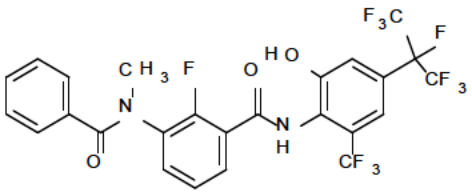
2.2 Test and Reference Substances

The test/reference standards, shown below, were synthesized by Mitsui Chemical Agro, Inc. (MCAG, Tokyo, Japan) and were maintained at room temperture until use in this study. Japan Analytical Chemistry Consultants Co., Ltd., on behalf of MCAG, determined characterization and purity prior to the substances being used in this study for S(Br-OH)-8007 and AB-Oxa. MCAG determined characterization and purity prior to the substances being used in this study for MFBA. Copies of the certificate of analysis (COAs) are presented in Appendix A. Details of these determinations are available to BASF and are located at Japan Analytical Chemistry Consultants Co., Ltd. (Funado Itabashi, Tokyo, Japan). BASF has retained a reserve sample of these chemicals and has documentation at BASF Corporation, BASF Crop Protection (Research Triangle Park, North Carolina, USA).

The test/reference substances 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 (1/x weighting) of instrument responses for the reference substances. The performance of the instrument was evaluated during each injection set.

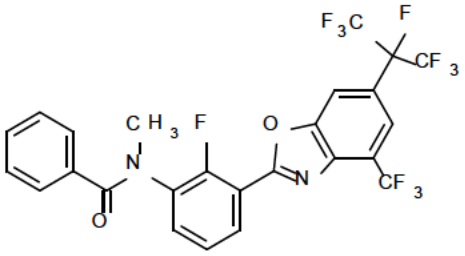
2.2.1 S(Br-OH)-8007

Common Name	S(Br-OH)-8007
Chemical Name	2-fluoro-*N*-[2-hydroxy-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-3-(*N*-methylbenzamido) benzamide
BASF Reg. No.	5959595
Molecular Formula	$C_{25}H_{15}F_{11}N_2O_3$
Molecular Weight	600.4
Lot No.	296-012-016-1
Purity:	98.38%
Expiration Date	June 28, 2017



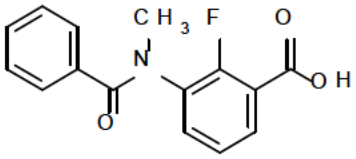
2.2.2 AB-Oxa

Common Name	AB-Oxa
Chemical Name	*N*-{2-fluoro-3-[6-perfluoropropan-2-yl)-4-(trifluoromethyl)-1,3-benzooxazol-2-yl]phenyl}-*N*-methylbenzamide
BASF Reg. No.	5959600
Molecular Formula	$C_{25}H_{13}F_{11}N_2O_2$
Molecular Weight	582.4
Lot No.	296-012-012-1
Purity:	98.89%
Expiration Date	June 28, 2017



2.2.3 MFBA

Common Name	MFBA
Chemical Name	2-fluoro-3-(N-methylbenzamido) benzoic acid
BASF Reg. No.	6088668
Molecular Formula	$C_{15}H_{12}FNO_3$
Molecular Weight	273.26
Lot No.	N4145911-146
Purity:	99.87%
Expiration Date	October 11, 2019



Stock solutions of analytes were prepared in acetonitrile. The mixed intermediate/fortification solutions containing each analyte were prepared by combining aliquots of the stock solutions for each analyte and diluting with acetonitrile. The calibration standards were prepared by serial dilution of the intermediate standards using methanol-water (50:50, v/v). 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. D1705/01, residues of S(Br-OH)-8007, AB-Oxa, and MFBA 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 diluted with 10 mL methanol and shaken for 30 min on a mechanical shaker at 300 rpm. Samples are filtered then analyzed by HPLC/MS/MS.

2.4.2 Specificity/Selectivity

Residues of S(Br-OH)-8007, AB-Oxa and MFBA are determined by HPLC-MS/MS, in positive mode, monitoring the following ion transitions: m/z 601→256 and 601→581 for S(Br-OH)-8007; m/z 583→444 and 583→494 for AB-Oxa; and m/z 274→254 for MFBA. In lieu of secondary (alternate) ion transitions for MFBA, confirmatory analysis is performed using a different LC-MS/MS column and gradient. The results are calculated by direct comparison of the sample peak responses to those of external standards. Two mass transitions are used for S(Br-OH)-8007 and AB-Oxa. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary for these analytes. As discussed above, an alternative chromatographic method is used for confirmation of MFBA. 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, 25 ng/L, and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 250 ng/L. 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 in 50/50 methanol/water. The matrix-matched standards were prepared by diluting mixed standards of each analyte with control drinking or surface water to 0.00625, 0.0125, and 0.025 ng/mL and compared to solvent-based calibration standards at the same concentrations. 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.

Extracts. The method validation fortification sample extracts were analyzed within 1 day of extraction. The generally acceptable method recoveries obtained during analyses demonstrate the storage stability of residues of S(Br-OH)-8007, AB-Oxa, and MFBA in the extracts in the brief period 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 the HPLC final volume held under refrigeration, indicated that each analyte, for the representative water type tested (surface water) is stable in extracts for at least the time period tested, 7 days, as shown in Appendix D.

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 chromatograms 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

Primera provided the following comments on the method:

This independent laboratory validation was successfully completed on the second try at Primera Analytical Solution Corp for both surface water and drinking water. The chemist made a mistake on the first try. In the acceptable trial, solvent standards were used for drinking water analysis while matrix matched standards were used for surface water. Recovery results and statistical data demonstrate BASF Analytical Method D1705/01 can be performed successfully for quantitation of S(Br-OH)-8007 (Reg. No. 5959595), AB-Oxa (Reg. No. 5959600), and MFBA (Reg. No. 6088668) in surface and drinking water.

10 PROTOCOL, AMENDMENTS, AND DEVIATIONS

The study was conducted according to a study protocol. All protocol deviations that occurred during the conduct of this study were reported and reviewed by the Study Director. A list of all protocol amendments and deviations is provided in Appendix M. None of the changes had an impact on the validity of the study.

11 REFERENCES

1. Xu, A. (2017). Independent Laboratory Validation of “Method for the Determination of S(Br-OH)-8007 (Reg. No. 5959595), AB-Oxa (Reg. No. 5959600), and MFBA (Reg. No. 6088668) in Surface and Drinking Water by LC-MS/MS” (BASF Method Number D1705/01). BASF Study Number: 838398. BASF Registration Document Number 2017/7012334

Table 2. Summary Parameters for the Analytical Method Used for the Quantitation of Residues of S(Br-OH)-8007, AB-Oxa, and MFBA in Water

Method ID	BASF Analytical Method No. D1705/01
Analyte(s)	Residues of S(Br-OH)-8007, AB-Oxa, and MFBA in water
Extraction solvent/technique	None. Residues of S(Br-OH)-8007, AB-Oxa, and MFBA in water samples (10 mL each) are diluted with methanol 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 601→256 and 601→581 for S(Br-OH)-8007; m/z 583→444 and 583→494 for AB-Oxa; and m/z 274→254 for MFBA. In lieu of secondary (alternate) ion transitions for MFBA, confirmatory analysis is performed using a different LC-MS/MS column (C18 and phenyl column options are available). All analyses are performed using a Waters Aquity UPLC system equipped with an XBridge BEH Phenyl column (100 x 2.1 mm, 2.5µm particle size) or, for confirmatory purposes for MFBA, an Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm particle size) using a mobile phase gradient of water:methanol, each acidified with 0.1% formic acid (flow rate 600 uL/minute). Detection is obtained with a AB Sciex API 5500 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 acetonitrile are stable held under refrigeration for at least 65 days, and that calibration standards prepared in 50/50 methanol/water have been demonstrated stable when held under refrigeration for 2 months (62 days). 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 3. Characteristics for the Analytical Method Used for the Quantitation of Residues of S(Br-OH)-8007, AB-Oxa, and MFBA in Water Matrices

Analyte	Residues of S(Br-OH)-8007, AB-Oxa, MFBA in water
Equipment ID	Waters Aquity UPLC system equipped with an XBridge BEH Phenyl column (100 x 2.1 mm, 2.5µm particle size) or, for confirmatory purposes for MFBA, an Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm particle size) using a mobile phase gradient of water:methanol, each acidified with 0.1% formic acid (flow rate 600 uL/minute). Detection is obtained with a AB Sciex API 5500 Mass Spectrometer.
Limit of quantitation (LOQ)	The validated LOQ for residues of S(Br-OH)-8007, AB-Oxa, and MFBA in water is 25 ng/L for each analyte, which corresponds to a concentration in the final volume of 0.0125 ng/mL.
Limit of detection (LOD)	5 ng/L (The LOD was set at 20% of the LOQ), which corresponds to a concentration in the final volume of 0.0025 ng/mL.

We create chemistry

Working Procedure:

Method for the Determination of S(Br-OH)-8007 (Reg. No. 5959595), AB-Oxa (Reg. No. 5959600), and MFBA (Reg. No. 6088668) in Surface and Drinking Water by LC-MS/MS

BASF Method Number D1705/01

Final

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Number of Pages
17

ABSTRACT

BASF Method D1705/01 is developed to determine the residues of BAS 450 I metabolites S(Br-OH)-8007 (Reg. No. 5959595), AB-Oxa (Reg. No. 5959600), and MFBA (Reg. No. 6088668) in surface and drinking water using LC-MS/MS at BASF Crop Protection, Research Triangle Park, N.C.

Brief description of the method:

10 mL methanol is added to a 10 mL water sample and mixed. After filtration, the sample is ready for analysis by LC-MS/MS.

The method has a limit of quantitation (LOQ) of 25 ng/L (25 ppt) in water. The limit of detection (LOD) in water is 5 ng/L.

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.
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).

1 INTRODUCTION

BAS 450 I is a new insecticide that will be used for various crops. The analytical method D1705/01 offers the possibility to determine residues of S(Br-OH)-8007 (Reg. No. 5959595), AB-Oxa (Reg. No. 5959600), and MFBA (Reg. No. 6088668), metabolites of BAS 450 I, in water. Method D1705/01 was successfully validated in surface and drinking water for all analytes.

This method was developed at BASF Crop Protection, Research Triangle Park, NC.

2 MATERIALS

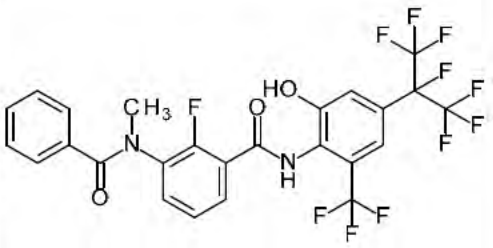
2.1 Safety

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. Ensure work clothing is stored 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 Materials Safety Data Sheets (MSDS) of the individual substances. All procedures involving organic solvents should be performed in a well-ventilated hood.
Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

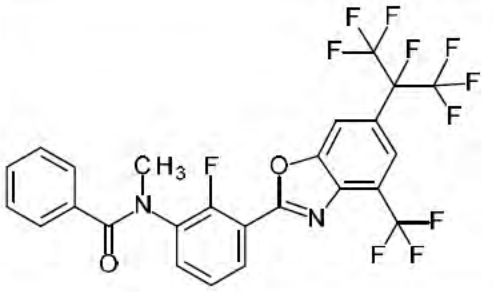
2.2 Test and Reference Items

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

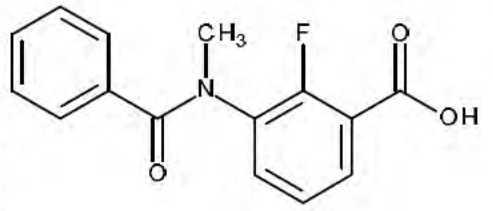
Common Name	S(Br-OH)-8007
Chemical Name	2-fluoro-*N*-[2-hydroxy-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-3-(*N*-methylbenzamido) benzamide
BASF Reg. No.	5959595
CAS-No.	None
Molecular Formula	$C_{25}H_{15}F_{11}N_2O_3$
Molecular Weight	600.4



Common Name	AB-Oxa
Chemical Name	N-{2-fluoro-3-[6-perfluoropropan-2-yl)-4-(trifluoromethyl)-1,3-benzooxazol-2-yl]phenyl}-N-methylbenzamide
BASF Reg. No.	5959600
CAS-No.	None
Molecular Formula	$C_{24}H_{13}F_{11}N_2O_2$
Molecular Weight	582.4



Common Name	MFBA
Chemical Name	2-fluoro-3-(N-methylbenzamido) benzoic acid
BASF Reg. No.	6088668
CAS-No.	None
Molecular Formula	$C_{15}H_{12}FNO_3$
Molecular Weight	273.26



2.3 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Amber Bottles	60 mL, Boston Round bottle with PTFE-faced PE lined cap attached	VWR	89042-908
Balance, Top-Load	150 g, CP153	Sartorius	
Beakers	Various Sizes	PYREX Brand, VWR Scientific Products	13922-029
Centrifuge Tubes (disposable)	50 mL	VWR	89039-660
Filters, Syringe Tip	13mm Syringe Filter, 0.45 µm PTFE membrane	PALL Life Sciences	4555
Graduated Cylinder	10 mL, PYREX	VWR	89090-636
LC-MS/MS injection vials	1.5 mL, Target DP	VWR, Thermo Scientific	00162506
LC Column	XBridge BEH Phenyl, 2.5 µm, 2.1x100 mm	Waters	186006067
LC Column (confirmation)	Acquity BEH C18, 1.7 µm, 2.1x50 mm	Waters	186002350
LC System	Acquity	Waters	
Mass Spectrometer	API 5500	Sciex	
Microman Pipettes	1000 µL 250 µL 50 µL	Gilson	M1000 M250 M50
Microman Pipette tips	1000 µL tips 250 µL tips 50 µL tips	Gilson	CP1000 CP250 CP50
Pasteur Pipettes, disposable	2 mL, 14.6 cm Borosilicate Glass	VWR	14673-010
Scintillation Vials	20 mL	VWR	66022-060
Shaker	KS501 digital	IKA Labortechnik	0002526401
Spatula		Various	
Syringes, Disposable	1 mL	Thermo Scientific	S7510-1
Volumetric Flasks	10 mL, 50 mL, 100 mL	Various	
Volumetric Pipettes	Various, class-A	Various	
Vortex Mixer	Genie 2	Fisher Scientific Co	12-812
Vortexer	Multi-tube vortexer, VX-2500	VWR	444-7063

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.
Acetonitrile	HPLC Grade	EMD	AX0145P-1
Methanol	HPLC Grade	EMD	MX0475P-1
Water	HPLC Grade	BDH ARISTAR PLUS	87003-652
Formic Acid	≥95%	Sigma-Aldrich	F0507

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

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Final Volume Solvent	FV1	**Methanol-water, 50:50, v/v** Add 500 mL of methanol and 500 mL of water into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase A	LC1	**0.1% Formic Acid in Water** Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	**0.1% Formic Acid in Methanol** Add 1000 mL of Methanol and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

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

2.4.3 Standard Solutions

Stock Solutions

Prepare a 1.0 mg/mL stock solution individually by weighing an appropriate amount of the analyte into a flask and add the required volume.
For example, to prepare 10 mL of 1.0 mg/mL stock solution of S(Br-OH)-8007 in acetonitrile, weigh 10 mg of S(Br-OH)-8007 into a 10 mL volumetric flask. Dissolve and dilute to mark with acetonitrile. Ensure a complete homogeneous solution (e.g. by sonication 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.

Fortification Solutions

Prepare standard solutions for fortification by dilution of the above stock solution. Dilute volumetrically 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

Take solution (µg/mL)	Volume (mL)	Dilute with acetonitrile to a final volume of (mL)	Concentration (µg/mL)
1000	0.5	10	50
50	0.05	50	0.050
0.050	5	50	0.005

Note: A different concentration scheme may be used, if other fortification levels are needed for the analysis. If necessary, the volume of solution prepared may be changed.

Calibration Standard Solutions

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

Preparation of standard solutions for calibration

Take solution (ng/mL)	Volume (mL)	Dilute with FV1* to a final volume of (mL)	Concentration (ng/mL)
5.0	5.0	50	0.50 †
0.50	10	100	0.05
0.05	25	50	0.025
0.05	12.5	50	0.0125
0.05	5.0	50	0.005
0.05	2.5	50	0.0025

† Not intended to be a calibration standard but needed to prepare subsequent calibration standards.

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

Note: A different concentration scheme may be used and additional standards may be prepared as needed. If necessary, the volume of solution prepared may be changed.

Additional Information:

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

2.4.4 Stability of Standard Solutions

Stability for solutions in acetonitrile (stock and fortification solutions) is 65 days for all analytes when stored under refrigerated conditions. Stability of calibration standard solutions (methanol-water, 50:50, v:v) is 62 days for all analytes when stored under refrigerated conditions.

3 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Sample homogenization is not needed for water samples.

S(Br-OH)-8007, AB-Oxa, and MFBA have the potential to adhere to container walls. As a result, any water samples to be analyzed (of unknown volume) must be transferred to a new container (while measuring sample volume), such as a graduated cylinder. An equal volume of methanol should be added to the original container; shake methanol in containers for 15 minutes at 300 rpm on a mechanical shaker, ensuring that solvent contacts all interior surfaces of the container. The methanol should then be transferred to the new container that is holding the sample. (Be sure the new container used has adequate capacity to contain both the sample and the methanol to be added as well as allow adequate mixing.) The diluted sample should then be mixed, filtered, and analyzed as specified below.

3.2 Sample Storage

Water samples are to be kept frozen until analysis.

3.3 Weighing and Fortification

For treated samples and control samples, measure 10 ± 0.1 g (or 10 mL) of water sample into a disposable tube (such as 50 mL plastic centrifuge tube).

For fortification samples, measure 10 ± 0.1 g (or 10 mL) of water sample into a disposable tube (such as 50 mL plastic centrifuge tube). Fortify the solution with analytes and shake/vortex for approximately 1 minute to ensure sample homogeneity.

The following scheme may be used:

Sample Type	Sample Weight	Concentration of Spiking Solution†	Volume of Spiking Solution	Level of Fortification†
Control	0.010 L	-	-	0.00 ng/L
Fortification (LOQ*)	0.010 L	5.0 ng/mL	0.05 mL	25 ng/L (ppt)
Fortification (10xLOQ)	0.010 L	50 ng/mL	0.05 mL	250 ng/L (ppt)
Treated	0.010 L	-	-	-

* limit of quantification

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

For fortified samples, 0.05 mL solvent is added that is not added to control or treated samples. This additional volume is considered insignificant and will not be considered in recovery calculations.

3.4 Preparation for Measurement

Add 10 mL methanol to all samples and shake for 30 minutes at 300 rpm on a mechanical shaker to ensure homogeneity. Syringe filter all samples using 0.45µm PTFE syringe filters directly into HPLC injection vials, passing the first approximately 0.2 – 0.3 mL to waste. Samples are ready for injection.

High fortification and high residue samples - further dilute with **FV1** (methanol-water, 50:50, v/v) as necessary, to fit in the calibration curve.

3.5 Influence of matrix effects on analysis

During method validation, it was demonstrated that the matrix load in the samples from the water matrices had no significant influence on the analysis (i.e., matrix effects < 20%). Therefore, samples can be analyzed using calibration standard solutions prepared in solvent FV1 (see 2.4.3).

3.6 Stability of Extracts / Final Volumes

Each analyte has been shown to be stable in extracts for at least the time period tested, 7 days for all analytes in surface water.

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

4.2.1 Instrumentation and Conditions for S(Br-OH)-8007, AB-Oxa, and MFBA

	Parameter		
Chromatographic System	Waters Acquity		
Analytical-column	XBridge BEH Phenyl 2.5µm, 2.1x100mm		
Column Temperature	50°C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Methanol / formic acid, 1000/1, v/v		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	70	30
	0.10	70	30
	1.10	45	55
	1.20	30	70
	3.20	5	95
	4.20	5	95
	4.25	70	30
	5.00	70	30
Detection System	Sciex 5500		
Ionisation	Electrospray (ESI)		
Ionisation Temperature	700 °C		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
S(Br-OH)-8007 (Reg. No.5959595)	601 → 256* 601 → 581	positive	approx. 2.6 min
AB-Oxa (Reg. No. 5959600)	583 → 444* 583 → 494	positive	approx. 3.3 min
MFBA (Reg. No. 6088668)	274 → 254*	positive	approx. 1.9 min

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

4.2.2 Confirmatory Instrumentation and Conditions for MFBA

	Parameter		
Chromatographic System	Waters Acquity		
Analytical-column	Acquity UPLC BEH C18 1.7µm, 2.1x50mm		
Column Temperature	50°C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Methanol / formic acid, 1000/1, v/v		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	90	10
	0.10	70	30
	1.10	45	55
	1.30	5	95
	1.90	5	95
	2.00	90	10
	2.50	90	10
Detection System	Sciex 5500		
Ionisation	Electrospray (ESI)		
Ionisation Temperature	700 °C		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
MFBA (Reg. No. 6088668)	274 → 254*	positive	approx. 1.4 min

* proposed as 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.

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 must 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 must 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 (in the range of 0.05 ng/mL to 0.0025 ng/mL) for LC-MS/MS. 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, the 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.

The residues of S(Br-OH)-8007, AB-Oxa, and MFBA in mg/L are calculated as shown in equations **I** and **II**:

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

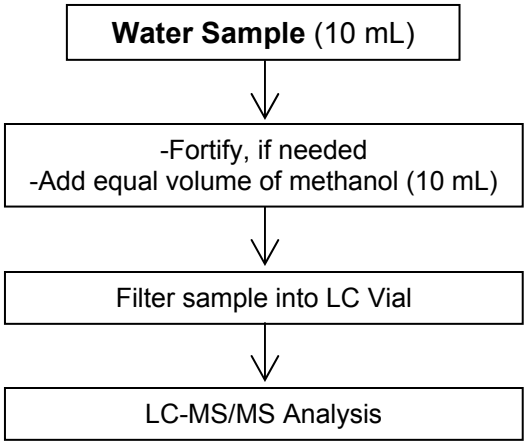
II. **Residue [ng/L]** $= \dfrac{V_{end} \times C_A}{G \times A_F}$

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 the 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 unknown samples, 2 fortified samples for recovery experiments, 1 blank sample) requires 0.5 working day (4 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

7 CONCLUSION AND METHOD CAPABILITIES

Recoveries, Chromatograms, and Calibration Curves
Recovery data will be provided in the validation report of the analytical method D1705/01.

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 25 ng/L (25 ppt) for all analytes. The limit of detection is estimated to be 20% of the limit of quantification, equivalent to 5 ng/L for all analytes. The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

Selectivity
The tested untreated surface and drinking water samples showed no significant interferences (< 20%) at the retention time of the analytes.

Confirmatory Techniques
The LC-MS/MS final determination for S(Br-OH)-8007 and AB-Oxa is a highly selective detection technique and quantitation is possible at two different mass transitions. For MFBA, a secondary chromatographic technique using a different stationary phase is included for confirmation.

Potential Problems
A PVDF filter is not suitable for use with this method, however, GHP and nylon filters may be found to be acceptable.

The glassware used for the method should be thoroughly rinsed with methanol followed by acetone to prevent contamination.

S(Br-OH)-8007, AB-Oxa, and MFBA have the potential to adhere to container walls. As a result, any water samples to be analyzed (of unknown volume) must be transferred to a new container (while measuring sample volume), such as a graduated cylinder. An equal volume of methanol should be added to the original container; shake methanol in containers for 15 minutes at 300 rpm on a mechanical shaker, ensuring that solvent contacts all interior surfaces of the container. The methanol should then be transferred to the new container that is holding the sample. (Be sure the new container used has adequate capacity to contain both the sample and the methanol to be added as well as allow adequate mixing.) The diluted sample should then be mixed, filtered, and analyzed as specified below.

Typical Recovery Calculation for LC/MS/MS Quantitation

Sample No. C16-016-LF1. Control surface water sample fortified at the LOQ with S(Br-OH)-8007, AB-Oxa, and MFBA, Master Sheet No. 838397-2.

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

	S(Br-OH)-8007
Peak Area =	17832
Intercept =	-184
Slope =	1760000
Conc. (ng/mL) =	0.0102

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

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

Where:

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

	S(Br-OH)-8007
V_{end} =	20 mL
A_F =	100%
G =	10.0 mL
Conc. (ng/mL) =	0.0102
Residue (ng/L) =	20.4

Net residue (ng/L of analyte) = Residue (ng/L of analyte) - Residue in Control (ng/L)

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

	S(Br-OH)-8007
Amount fortified (ng/L) =	25
Residue (ng/L) =	20.4
Residue in control (ng/L) =	0.0000
%Recovery	82%

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

Typical analytical standards dilution and use records for Analytes

Standard Number	Analyte	Standard (Lot # used)	Amount Weighed / Volume	Final Dilution Vol. (mL)	Final Conc. [1]	Solvent[2]	Prep. Date	Expiry Date
Stock solutions								
ERS17-0830	S(Br-OH)-8007	296-012-016-1	10.0 mg	10	1 mg/mL	Acetonitrile	18-Apr-17	28-Jun-17
ERS17-0832	AB-Oxa	296-012-012-1	10.0 mg	10	1 mg/mL	Acetonitrile	18-Apr-17	28-Jun-17
ERS17-0805	MFBA	N4145911-146	10.0 mg	10	1 mg/mL	Acetonitrile	17-Apr-17	17-Jul-17
Serial dilutions								
ERS17-0881	All	ERS17-0830 ERS17-0832 ERS17-0805	0.5 mL 0.5 mL 0.5 mL	10	50 µg/mL	Acetonitrile	21-Apr-17	21-May-17
ERS17-0882	All	ERS17-0881	0.05 mL	50	0.05 µg/mL	Acetonitrile	21-Apr-17	21-May-17
ERS17-0883	All	ERS17-0882	5 mL	50	0.005 µg/mL	Acetonitrile	21-Apr-17	21-May-17
Calibration Standards								
ERS17-0884	All	ERS17-0883	5 mL	50	0.5 ng/mL	Mixture1	21-Apr-17	21-May-17
ERS17-0885	All	ERS17-0884	10 mL	100	0.05 ng/mL	Mixture1	21-Apr-17	21-May-17
ERS17-0886	All	ERS17-0885	25 mL	50	0.025 ng/mL	Mixture1	21-Apr-17	21-May-17
ERS17-0887	All	ERS17-0885	12.5 mL	50	0.0125ng/mL	Mixture1	21-Apr-17	21-May-17
ERS17-0888	All	ERS17-0885	5 mL	50	0.005 ng/mL	Mixture1	21-Apr-17	21-May-17
ERS17-0889	All	ERS17-0885	2.5 mL	50	0.0025 ng/mL	Mixture1	21-Apr-17	21-May-17

1. The concentration for each analyte shown in “Analyte” column.
2. Mixture1 = Methanol-Water (50:50, v/v)

Example fortification scheme used for analysis (Master Sheet No. 838397-2)

SAMPLE DATA	FORTIFICATION DATA						FINAL VOL (mL)
	Weight (mL)	PPB Fortified	COMPOUND	VOLUME (mL)	STD CONC	STD NO.	
838397-02-1	10	0	Reagent Blank	NA	NA	NA	10
CM16-016a	10	0	Control	NA	NA	NA	10
CM16-016-LF1	10	0.025	All 3 analytes	0.05	5 ng/mL	ERS17-0883	20
CM16-016-HF1	10	0.25	All 3 analytes	0.05	50 ng/mL	ERS17-0882	200

Appendix M. Protocol Amendments and Deviations

1) Analyte MFBA was added to the validation (plus a new technical procedure including MFBA). The title of the study and the method were updated to reflect this change.

2) Correct typographical error in the type of column used in the confirmatory chromatographic technique for MFBA: An “Acquity UPLC BEH C18” column was used rather than an “XSelect BEH C18”. All other column dimentions are correct. Corrected a typographical error listing the lot number for MFBA.

These changes did not have an adverse effect on the outcome of this study.