FINAL REPORT

Title

Independent Laboratory Validation of BASF Analytical Method D1724/01:
"Method for the determination of BAS 850 H (Reg.No. 5654329) and M850H001 (Reg.No. 5749359), M850H002 (Reg.No. 5757725), M850H003 (Reg.No. 5757726), M850H004 (Reg.No. 5833884), M850H012 (Reg.No. 5797901), and M850H035 (Reg.No. 6070203) in Surface and Drinking Water by LC-MS/MS"

BASF Study Code

BASF Study Number: 826949
ADPEN Study Number: 17K0204

Guidelines

US Environmental Protection Agency (EPA) Ecological Effects Test Guideline:
OCSPP 850.6100 Environmental Chemistry Methods and Associated ILV

1. INTRODUCTION

1.1 Scope of the Method

BASF Analytical Method No. D1724/01 was developed to determine the residues of BAS850H, M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035 in water matrices using LC-MS/MS at BASF Crop Protection in Research Triangle Park, North Carolina. This method was independently validated at ADPEN Laboratories, Inc. during this study.

1.2 Principle of the Method

No sample extraction or clean-up was required for BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035 according to the method. A 10 mL of control sample was fortified and a 1 mL aliquot of the fortified control sample was mixed with 0.25 mL of acidified methanol (methanol + 0.5% formic acid). The solution was filtered through a 0.45 µm PTFE syringe filter into an HPLC vial after discarding the first ~0.1 mL that passed through the syringe filter for the LC-MS/MS analysis.

The metabolite M850H001 required an initial clean up where a 5 mL aliquot was taken from the sample and mixed with 0.05 mL of water with 10% formic acid. The sample was partitioned using 10 mL cyclohexane/ethyl acetate (90/10 v/v), vortexed for 2 minutes, and centrifuged for 5 minutes at 1500 rpm. An 8 mL aliquot was removed from the top organic layer into a separate culture tube and evaporated to dryness under nitrogen at 50 ºC. Residues were re-dissolved in 0.4 mL of methanol: water with 0.1% formic acid (50:50, v/v), followed by an addition of 0.6 mL of water with 0.1% formic acid and filtered using 0.45 µm PTFE syringe filter into an HPLC vial after discarding the first ~0.1 mL that passed through the syringe filter for the LC-MS/MS analysis.

2. REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test Systems

The test systems used in this study were surface and drinking water.

The control surface and drinking water samples used in this study were provided by BASF Crop Protection, and were received at ADPEN Laboratories, Inc. on November 29, 2017. Upon arrival at the laboratory, the samples were inspected and were assigned unique numbers through ADPEN's Laboratory Information Management System (LIMS). The samples were received frozen and in good condition, then stored in freezer E16 at an average temperature of -20 ºC and in refrigerator E57 at an average temperature of 4 ºC during the thawing process prior to its use for analyses. The characterization and stability data reports for the control samples are maintained by BASF Corporation. The characterization reports accompanied the control samples are presented in **Appendix A**.

2.2 Test and Reference Substances

The reference substances used in this study were provided by BASF Crop Protection and a reserve sample of these standards is retained at Research Triangle Park, North Carolina. Detailed information regarding the test substances, including the certificates of analysis are presented in **Appendix B**. A brief description of each analyte follows:

Code No.:	**BAS 850 H**	
Chemical Name (IUPAC):	1,5-dimethyl-6-thioxo-3-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4-dione	
CAS Registry No.:	1258836-72-4	Molecular Structure:
BASF Reg. No.:	5654329	
Molecular Formula:	$C_{16}H_{11}F_3N_4O_4S$	
Molecular Weight:	412.3 g/mol	
Batch No.:	L84-130	
Purity:	99.2 %	
Expiration Date:	February 01, 2020	
Storage:	Freezer	

Code No.:	**M850H001**	
Chemical Name (IUPAC):	1,3-dimethyl-5-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4,6-trione	
CAS Registry No.:	None	Molecular Structure:
BASF Reg. No.:	5749359	
Molecular Formula:	$C_{16}H_{11}F_3N_4O_5$	
Molecular Weight:	396.3 g/mol	
Batch No.:	L85-52	
Purity:	98.7 %	
Expiration Date:	April 01, 2018	
Storage:	Freezer	

Code No.:	**M850H002**	
Chemical Name (IUPAC):	1,5-dimethyl-6-thioxo-3-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4-dione	
CAS Registry No.:	None	Molecular Structure:
BASF Reg. No.:	5757725	
Molecular Formula:	$C_{13}H_9F_3N_4O_4S$	
Molecular Weight:	374.3 g/mol	
Batch No.:	L84-162	
Purity:	96.8 %	
Expiration Date:	February 01, 2020	
Storage:	Freezer	

Code No.:	**M850H003**	
Chemical Name (IUPAC):	1,3-dimethyl-5-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4,6-trione	
CAS Registry No.:	None	Molecular Structure:
BASF Reg. No.:	5757726	
Molecular Formula:	$C_{13}H_9F_3N_4O_5$	
Molecular Weight:	358.2 g/mol	
Batch No.:	L85-70	
Purity:	99.4 %	
Expiration Date:	April 01, 2018	
Storage:	Freezer	

Code No.:	**M850H004**	
Chemical Name (IUPAC):	N,N-dimethyl-N'-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-dicarbonimidothioicdiamide	
CAS Registry No.:	None	Molecular Structure:
BASF Reg. No.:	5833884	
Molecular Formula:	$C_{15}H_{13}F_3N_4O_3S$	
Molecular Weight:	386.4 g/mol	
Batch No.:	L85-50	
Purity:	99.5 %	
Expiration Date:	April 01, 2018	
Storage:	Freezer	

Code No.:	**M850H012**	
Chemical Name (IUPAC):	6-amino-2,2,7-trifluoro-4-(prop-2-yn-1-yl)-2H-1,4-benzoxazin-3(4H)-one	
CAS Registry No.:	None	Molecular Structure:
BASF Reg. No.:	5797901	
Molecular Formula:	$C_{11}H_7F_3N_2O_2$	
Molecular Weight:	256.2 g/mol	
Batch No.:	L85-66	
Purity:	98.9%	
Expiration Date:	September 1, 2018	
Storage:	Freezer	

Code No.:	**M850H035**	
Chemical Name (IUPAC):	N ,N-dimethyl-N'-[2 ,2, 7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-2-imidodicarbonic diamide	
CAS Registry No.:	None	Molecular Structure:
BASF Reg. No.:	6070203	
Molecular Formula:	$C_{15}H_{13}F_3N_4O_4$	
Molecular Weight:	370.3 g/mol	
Batch No.:	L2017-007	
Purity:	100.0%	
Expiration Date:	February 1, 2019	
Storage:	Freezer	

3. ANALYTICAL METHOD

BASF Analytical Method D1724/01 “Method for the Determination of Residues of BAS 850 H (Reg. No. 5654329) and its Metabolites M850H001 (Reg. No. 5749359), M850H002 (Reg. No. 5757725), M850H003 (Reg. No. 5757726), M850H004 (Reg. No. 5833884), M850H012 (Reg. No. 5797901), and M850H035 (Reg. No. 6070203) in Surface and Drinking Water by LC-MS/MS” (**Reference 1**) was used for the analysis of the samples. Method D1724/01 is presented in **Appendix D**.

Final determination on BAS 850 H, M850H001, M850H002, M850H003, M850H004, M850H012 and M850H035 was conducted using the following transition ions.

Analyte	Polarity	Quantitation (*m/z*)	Confirmation (*m/z*)
BAS 850 H	Positive	413→74	413→74
M850H001	Positive	397→141	397→134
M850H002	Negative	373→193	373→323
M850H003	Negative	357→307	357→137
M850H004	Positive	387→131	387→74
M850H0012	Positive	257→163	257→116
M850H0035	Positive	371→257	371→163

3.1 Validation of Method

For validation, untreated water samples were fortified with BAS 850 H, M850H001, M850H002, M850H003, M850H004, M850H012 and M850H035 then analyzed according to the established method guidelines. To test the repeatability of the method, the analytical sets for each matrix consisted of a reagent blank, two controls, five replicates fortified with analytes at the method LOQ (0.03 ppb) and five replicates fortified at a higher level, corresponding to 10xLOQ (0.3 ppb). Summaries of the method instrument condition and parameters used in this study are provided in Tables 30 through 33. The example of recovery calculation is provided in Table 34. The validation data including the detail analytical data for each matrix types are provided in **Appendix C**.

Minor modifications in gradient were required to successfully analyze M850H004 analyte in surface water. Instrument parameters detailing the changes are referenced in Table 33. Additionally, when equal amount of formic acid was added in the matrix matched standards as well as samples; the low recovery issue of M850H004 in surface water was addressed successfully.

3.2 Influence of Matrix Effects on Analysis

Matrix effect was not evaluated during the conduct of this study, however; matrix matched standards were used in this ILV per guidelines in the analytical method.

STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included simple descriptive statistics, such as determinations of averages, standard deviation, and/or percent relative standard deviation (%RSD) for the procedural recoveries. Calibration standards and samples were analyzed using an LC/MS/MS system, which included an Agilent 1290 HPLC and ABSciex 6500 and 6500+ mass spectrometers. Representative chromatograms of control and fortified samples are provided in the Figures section. Calibration curves and residue values were calculated using Analyst 1.6.2 data processing software using mean response factors with linear 1/x weighting and correlation coefficient ($r \geq 0.99$). The statistical calculations throughout this report were performed using 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 ADPEN 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 a secured laboratory area.

5. SUMMARY OF METHOD

Type of Method LC-MS/MS

Test System Surface water and Drinking water

Selected mass transitions (m/z)

Analyte	Polarity	Quantitation (*m/z*)	Confirmation (*m/z*)
BAS 850 H	Positive	413→74	413→74
M850H001	Positive	397→141	397→134
M850H002	Negative	373→193	373→323
M850H003	Negative	357→307	357→137
M850H004	Positive	387→131	387→74
M850H0012	Positive	257→163	257→116
M850H0035	Positive	371→257	371→163

Analytical Procedure BASF Analytical Method D1724/01 "Method for the determination of BAS 850 H (Reg. No. 5654329), M850H001 (Reg. No. 5749359), M850H002 (Reg. No. 5757725), M850H003 (Reg. No. 5757726), M850H004 (Reg. No. 5833884), M850H012 (Reg. No. 5797901), and M850H035 (Reg. No. 6070203) in Surface and Drinking Water by LC-MS/MS"

Confirmation Technique A secondary MRM transition was used for confirmation.

Method of Quantitation The quantitation is based on the monitoring of one mass transition for BAS 850 H analyte and two mass transitions for M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035. Recovery data were reported for each mass transition considered.

LOD 0.006 ppb

LOQ 0.03 ppb (lowest fortification level)

Levels of Fortification 0.03 ppb and 0.3 ppb

Time Required A set of 13 samples requires approximately 2-3 hours of work (LC-MS/MS analysis and calculation of the results excluded).

6. DISCUSSION

Linearity
Good linearity (r ≥0.99) was observed in the range of 0.005 – 0.1250 ng/mL for BAS 850 H and its metabolites, except for M850H001 where the range was 0.025 – 0.5 ng/mL.

Standard Stability
Standard stability data is listed in the method validation report (**Reference 1**). Stock and fortification solutions (intermediate standards) of BAS 850 H, M850H001, M850H002, M850H003, M850H004 and M850H012 were prepared in methanol with 0.1% formic acid. Stock solutions of M850H035 were prepared in acetone and fortification solutions were prepared using methanol with 0.1% formic acid. Calibration standard solutions were prepared by serial dilution of an intermediate standard solutions using methanol-water with 0.1% formic acid (20:80, v/v). During the course of this study, the test/reference substance solutions were stored under refrigerated conditions and all solutions were used within the demonstrated time period of stability.

All analytes have been shown to be stable in stock and fortification solutions prepared in methanol with 0.1% formic acid for at least 31 days when stored under refrigeration. Each analyte has been shown to be stable in calibration standard solutions prepared by serial dilution of the intermediate standard solutions with methanol-water with 0.1% formic acid (20:80 v/v) and held under refrigeration for at least 27 days.

Extract Stability
Extract stability for water matrices was not established in this study. Extract stability in the final volume solution (20:80 methanol-water with 0.1% formic acid, v/v) was established in the method validation study (**Reference 1**), 7 days for surface water and 6 days for drinking water.

Specificity
Method D1724/01 determines residues of BAS 850 H, M850H001, M850H002, M850H003, M850H004, M850H012 and M850H035 in water. No interfering peaks were found at the retention time for any of the analytes.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)
The LOQ was defined as the lowest fortification level tested. The LOQ for BAS850H, M850H001, M850H002, M850H003, M850H004, M850H012 and M850H035 in water matrices was 0.03 ppb. The LOD in water matrices was set at 0.006 ppb, which was 20% of the defined LOQ. The LOD is defined as the absolute amount of analyte injected (0.0005 ng) into the LC-MS/MS when the lowest calibration standard was analyzed (0.005 ng/mL) for all analytes with acceptable signal to noise ratio (S/N > 3:1).

Repeatability

The ILV was successful for both ion transitions (quantitation and confirmation) using matrix matched standards for surface and drinking water as per the validation method guidelines.

The independent laboratory validation (ILV) was performed successfully in surface water for BAS 850 H, M850H002, M850H003, M850H012 and M850H035 analytes during the first trial. The M850H004 analyte did not run during the first trial. The first trial was repeated and the recovery results were high. After removing matrix effects with minor modifications, low recoveries were observed and failed the second trial. It was then successfully performed in the third trial after additional minor modifications. The independent laboratory validation was performed successfully for M850H001 in surface water during the second trial.

The independent laboratory validation was performed successfully in drinking water for BAS 850 H, M850H002, M850H003, and M850H012 during the first trial. The independent laboratory validation was performed successfully for M850H001, M850H004 and M850H035 in surface water and drinking water during the second trial.

7. RECOMMENDATIONS/CONCLUSIONS FROM ILV

The independent laboratory validation of BASF method (D1724/01) was successfully completed for all analytes in drinking water and surface water except for M850H004 in surface water which required 3 trials because of the reasons listed below:

a. Trial 1 for M850H004 in surface water was artificially enhanced and the issue was not resolved before the extract aged beyond proven stability.
b. Trial 2 for M850H004 in surface water failed with low recovery due to possible compromised formic acid.
c. Trial 3 was successfully completed for M850H004 in surface water after a new bottle of formic acid was used and the formic acid concentration in the matrix-matched calibration standards was increased to 0.1% to match the concentration in control and recovery samples.

In addition, it is recommended to make the following changes to the analytical method to improve ruggedness:

1. The organic rinse and equilibrium times were extended for two minutes each (section 4.2). The additional rinse and equilibration steps may help to reduce matrix components at the time of analyte elution and allow for full analytical column re-equilibration for some LC systems.
2. The formic acid concentration in the matrix-matched calibration standards for BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035 (method section 3.7.1.c) was increased to 0.1% to match the concentration in control and recovery samples. Matrix matched calibration standards prepared as per the method have a concentration of 0.02% formic acid while samples are in 0.1% formic acid. The pre-calibration standards were made at the same levels as stated in the method except 0.5% formic acid in methanol was used instead of the 0.1% formic acid in methanol as stated in the method. Then 0.25 mL of the pre-calibration standard was added to 1.0 mL of the sample to make the final standard solvent 0.1% formic acid in 80/20 water/methanol.

All recovery results and statistical data demonstrated that the BASF Analytical Method D1724/01 can be performed successfully for quantitation of BAS 850 H and its metabolites, M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035 in drinking and surface water matrices provided the above changes be incorporated into the method.

Table 29 Example Standard Solutions Preparation and Dilution Data

Stock Standard Solutions

Standard ID#	Analyte	Parent Standard ID#	Adjusted Net Weight (mg)	Dilution Volume (mL)[a]	Final Conc. (ng/µL)	Prep. Date
C8881	BAS 850 H	P5424	3.6506	5	730.11	12/8/17
C8812	M850H001	P5619	2.7600	5	553.00	10/18/17
C8885	M850H002	P5702	10.0962	10	1009.62	12/8/17
C8884	M850H003	P5762	9.9798	10	997.98	12/8/17
C8871	M850H004	P5618	9.9401	10	994.01	12/8/17
C8883	M850H012	P5703	9.9691	10	996.91	12/8/17
C8880	M850H035	P5763	9.9700	10*	997.00	12/8/17

[a] Prepared in methanol + 0.1% Formic acid
* Prepared in Acetone

Fortification and Pre calibration Standard Solutions

Standard ID#	Analytes	Parent Standard ID#	Parent Std. Conc. (µg/mL)	Aliquot Volume (mL)	Dilution Volume (mL)[b]	Final Conc. (ng/µL)	Prep. Date
I9565 and I9566	BAS 850 H	C8881	730.11	0.342	25	10	12/8/17
	M850H001	C8812	553.00	0.452			
	M850H002	C8885	1009.62	0.248			
	M850H003	C8884	997.98	0.251			
	M850H004	C8871	994.01	0.252			
	M850H012	C8883	996.91	0.251			
	M850H035	C8880	997.00	0.251			
Standard ID#	**Analytes**	**Parent Standard ID#**	**Parent Std. Conc. (ng/µL)**	**Aliquot Volume (mL)**	**Dilution Volume (mL)[b]**	**Final Conc. (ng/µL)**	**Prep. Date**
W14622-1	BAS 850 H (7-mix)	I9566	10	1.0	10	1	12/14/2017
W14622-2		W14622-1	1	2.5	25	0.1	
W14622-3		W14622-2	0.1	2.5	25	0.01	
W14622-4		W14622-3	0.01	0.25	10	0.00025	

[b] Prepared in methanol + 0.1% Formic acid

Calibration Standard Solutions (Matrix Matched)

Standard ID#	Analyte	Parent Standard ID#	Parent Conc. (ng/mL)	Aliquot Volume (mL)	Dilution Volume (mL)[c]	Final Conc. (ng/mL)	Prep. Date
W14630-1	BAS 850 H (7-Mix)	W14622-2	100	1.25	50	2.5	12/18/2017
W14630-2			100	0.625		1.25	
W14630-3		W14630-1	2.5	5.0	20	0.625	
W14630-4				5.0	50	0.250	
W14630-5				2.5		0.125	
W14630-6				1.25		0.0625	
W14630-7				0.50		0.025	

[c] Prepared in methanol + 0.1% Formic acid

Table 30 Instrument Conditions and Parameters for BAS 850 H, M850H002, M850H003, M850H004, M850H012 and M850H035

HPLC Conditions			
Chromatographic System:	Agilent 1290 system		
Column:	HSS T3 1.8 µm 2.1 x 100 mm		
Temperature:	50 °C		
Flow rate (µL/min):	500		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	85	15
	0.25	60	40
	4.25	30	70
	4.50	5	95
	5.50	5	95
	5.75	85	15
	8.00	85	15
Mobile Phase A:	1% formic acid in water		
Mobile Phase B:	0.1% formic acid in methanol		
Injection Volume:	0.0999 mL		

MS/MS Conditions							
Detection System:	ABSciex 6500 Triple Quad						
Ionization:	ESI						
Polarity:	Positive: BAS 850 H, M850H004, M850H012 and M850H035 Negative: M850H002 and M850H003						
Curtain Gas (CUR, psi):	20						
Collision Gas (CAD):	9						
Temperature (TEM, °C):	650						
Ion Source Gas 1 (GS1, psi):	55						
Ion Source Gas 2 (GS2, psi):	45						
Ion Source Voltage (IS, V):	5500 (-4500 for M850H002 and M850H003)						
MRM Conditions	Transition (*m/z*)	Dwell (msec)	DP	EP	CE (V)	CXP (V)	Retention Time (min)
BAS 850 H	413→74	50	119	10	69	8	~5.7
M850H002	373→193	25	-150	-5.7	-54	-9	~5.6
	373→323			-11.2	-34	-13	
M850H003	357→307	50	-86	-8	-33	-12	~3.96
	357→137				-50	-8	
M850H004	387→131	50	72	10	16.5	12	~5.51
	387→74				37	12.5	
M850H0012	257→163	50	96	11	23	20	~3.79
	257→116				35	14	
M850H035	371→257	50	54	10	19.8	12.9	~5.22
	371→163				37	7.5	

Table 31 Instrument Conditions and Parameters for BAS 850 H (Confirmation)

HPLC Conditions			
Chromatographic System:	Agilent 1290 system		
Column:	BEH C18 1.7 µm 2.1 x 50 mm		
Temperature:	50 °C		
Flow rate (µL min):	600		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	85	15
	6.25	40	60
	6.50	5	95
	7.50	5	95
	7.75	85	15
	8.00	85	15
Mobile Phase A:	1% formic acid in water		
Mobile Phase B:	0.1% formic acid in methanol		
Injection Volume:	0.0999 mL		

MS/MS Conditions							
Detection System:	Sciex 6500 Triple Quad						
Ionization:	ESI						
Polarity:	Positive						
Curtain Gas (CUR, psi):	20						
Collision Gas (CAD):	9						
Temperature (TEM, °C):	650						
Ion Source Gas 1 (GS1, psi):	55						
Ion Source Gas 2 (GS2, psi):	45						
Ion Source Voltage (IS, V):	5500						
MRM Conditions	Transition (*m/z*)	Dwell (msec)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
BAS 850 H*	413 → 74	50	119	10	69	8	~7.2

*Confirmatory ion

Table 32 Instrument Conditions and Parameters for M850H001

HPLC Conditions			
Chromatographic System:	Agilent 1290 system		
Column:	BEH C18 1.7 µm 2.1 x 50 mm		
Temperature:	50 °C		
Flow rate (µL/min):	600		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	85	15
	6.25	40	60
	6.50	5	95
	7.50	5	95
	7.75	85	15
	8.00	85	15
Mobile Phase A:	1% formic acid in water		
Mobile Phase B:	0.1% formic acid in methanol		
Injection Volume:	0.0999 mL		

MS/MS Conditions					
Detection System:	ABSciex 6500 Triple Quad				
Ionization:	ESI				
Polarity:	Positive				
Curtain Gas (CUR, psi):	20				
Collision Gas (CAD):	9				
Temperature (TEM, °C):	650				
Ion Source Gas 1 (GS1, psi):	55				
Ion Source Gas 2 (GS2, psi):	45				
Ion Source Voltage (IS, V):	5500				
De-clustering Potential (DP)	62				
Entrance Potential (EP)	10				
MRM Conditions	Transition (*m/z*)	Dwell (msec)	CE (V)	CXP (V)	Retention Time (min)
M850H001	397→141	50	54.00	17.70	~4.8
	397→134		64.00	16.20	

Table 33 Alternate Instrument Conditions and Parameters for M850H004 in Surface Water

HPLC Conditions			
Chromatographic System:	Agilent 1290 system		
Column:	HSS T3 1.8 µm 2.1 x 100 mm		
Temperature:	50 °C		
Flow rate (µL/min):	600		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	85	15
	0.25	60	40
	4.25	30	70
	4.50	5	95
	7.50	5	95
	8.00	85	15
	10.00	85	15
Mobile Phase A:	1% formic acid in water		
Mobile Phase B:	0.1% formic acid in methanol		
Injection Volume:	0.04 mL		

MS/MS Conditions					
Detection System:	ABSciex 6500 Plus Triple Quad				
Ionization:	ESI				
Polarity:	Positive				
Curtain Gas (CUR, psi):	20				
Collision Gas (CAD):	9				
Temperature (TEM, °C):	650				
Ion Source Gas 1 (GS1, psi):	50				
Ion Source Gas 2 (GS2, psi):	45				
Ion Source Voltage (IS, V):	5500				
De-clustering Potential (DP)	30				
Entrance Potential (EP)	10				
MRM Conditions	Transition (*m/z*)	Dwell (msec)	CE (V)	CXP (V)	Retention Time (min)
M850H004	387→131	100	15.00	10.0	~5.5
	387→74		40.00	8.0	

Table 34 Example Residue Calculations for Recovery in Drinking Water

Quantitation of residues in all samples was achieved using an external calibration curve calculated by linear regression of instrument responses for the reference substances at multiple concentrations. The performance of the instrument was evaluated during each injection set. The correlation coefficient for each calibration curve was found to be $r \geq 0.99$. A standard curve was prepared by injecting standard solutions at appropriate concentrations for each analyte. Calibration standard concentrations for BAS 850 H and its metabolites ranged from 0.005 - 0.125 ng/mL, except for M850H001 which was 0.025 to 0.5 ng/mL. A calibration standard was typically injected every five to six sample injections. Data processing software, Analyst ® 1.6.2, created the standard curve based on linear regression, using 1/x weighting. The regression functions were used to calculate the best-fit line by plotting the amount found (ng) on the x-axis versus the detector's peak response (peak area ratio) on the y-axis.

The following equations are used for residue and recovery calculations for BAS 850 H and its metabolites in drinking water:

a) Calibration curve $y = mx + b$: Solving for x: $x = \frac{y - b}{m}$

Where, m = Slope

b = y-intercept
x = Amount found (ng)
y = Peak area of the analyte

b) Final sample volume = [(extract volume ÷ aliquot volume) × final extract volume × DF]

c) Amount of sample injected (mL) = (sample volume × injection size) / final sample volume

d) Amount found (ppb) = (ng found / mL of sample inj.)

e) Percent recovery (%) = $\frac{\text{Residue Found in Spike (ppb)} \times 100}{\text{Fortification Level (ppb)}}$

As an example, calculation to obtain the percent recovery in drinking water sample fortified at the LOQ (0.03 ppb) with BAS 850 H is shown below. Similar calculation scheme was used to calculate the recovery of all metabolites in a given set:

Sample ID: CM15-030-029
Lab Code: 17121110-Recovery1-2
Set name: (WO-17121110)

where: y = (6.68e+006) x + 484
Analyte peak area = 13272

a) ng found = $\frac{13272 - 484}{6.68e+006}$ = 0.001914371 ng

b) Final sample volume (mL) = [(10 mL ÷ 1 mL) × 1.25 mL × 1] = 12.5 mL

c) Amount of sample injected (mL) = (10 mL × 0.0999 mL) / 12.5 mL = 0.07992 mL

d) Amount found (ppb) = (0.001914371 ng/ 0.07992 mL) = 0.0239536 ppb

There was no residue found in the corresponding control sample (lab code: 171130002-002A)

e) Percent recovery (%) = $\frac{0.0239536 \text{ ppb} \times 100\%}{0.03 \text{ ppb}}$ = **79.8**%

Statistical treatment of the data included calculation of means, standard deviations (SD), and percent relative standard deviations (%RSD). These calculations were performed using Microsoft® Excel®. Results were rounded only for reporting purposes. No calculations were made with rounded numbers.

Appendix D BASF Analytical Method D1724/01

Technical Procedure:

Method for the Determination of Residues of BAS 850 H (Reg. No. 5654329) and its metabolites M850H001 (Reg. No. 5749359), M850H002 (Reg. No. 5757725), M850H003 (Reg. No. 5757726), M850H004 (Reg. No. 5833884), M850H012 (Reg No. 5797901), and M850H035 (Reg. No. 6070203) in Surface and Drinking Water by LC-MS/MS

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 (known as blank or 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 850 H is a PPO herbicide, and is developed by BASF to be used for a broad spectrum of crops in the US. For registration of this herbicide and for establishing the tolerance for these use patterns, residue analytical method D1724/01, for the active ingredient and its metabolites in surface and drinking water, was developed by BASF.

BASF Method Number D1724/01 was successfully tested during method development in surface and drinking water.

The method has a limit of quantitation of 30 ng/L (30 ppt), in surface and drinking water, for each analyte. The limit of detection in water for each analyte is 6 ng/L (6 ppt, 20% of LOQ).

2 MATERIALS

2.1 Safety

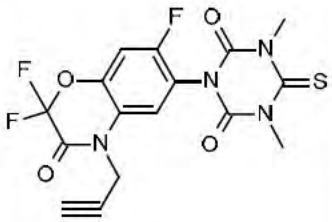
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Ensure that 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

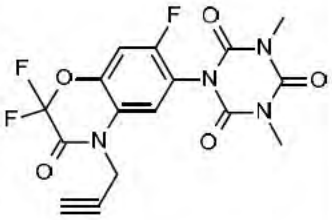
Standard substances are stored in a freezer (≤-5°C) until use.

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

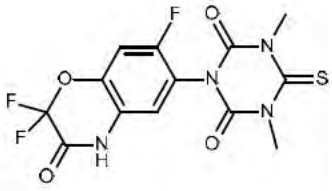
BAS-Code	BAS 850 H
IUPAC Name	1,5-dimethyl-6-thioxo-3-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5654329
CAS-No.	1258836-72-4
Molecular Formula	$C_{16}H_{11}F_3N_4O_4S$
Molecular Weight	412.3



BAS-Code	M850H001
IUPAC Name	1,3-dimethyl-5-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4,6-trione
BASF Reg. No.	5749359
CAS-No.	N/A
Molecular Formula	$C_{16}H_{11}F_3N_4O_5$
Molecular Weight	396.3

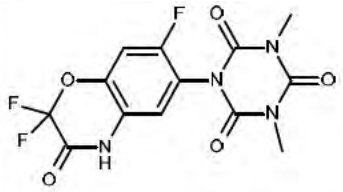


BAS-Code	M850H002
IUPAC Name	1,5-dimethyl-6-thioxo-3- (2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5757725
CAS-No.	N/A
Molecular Formula	$C_{13}H_9F_3N_4O_4S$
Molecular Weight	374.3

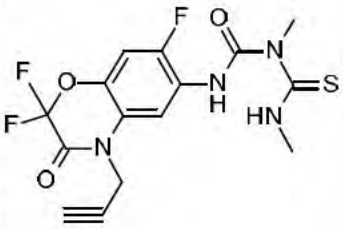


2.2 Test and Reference Items (Cont.)

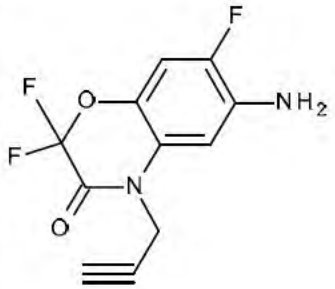
BAS-Code	M850H003
IUPAC Name	1,3-dimethyl-5-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4,6-trione
BASF Reg. No.	5757726
CAS-No.	N/A
Molecular Formula	$C_{13}H_9F_3N_4O_5$
Molecular Weight	358.2



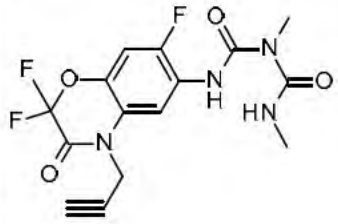
BAS-Code	M850H004
IUPAC Name	N,N-dimethyl-N'-[2,2,7-trifluroro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl] dicarbonimidothioicdiamide
BASF Reg. No.	5833884
CAS-No.	N/A
Molecular Formula	$C_{15}H_{13}F_3N_4O_3S$
Molecular Weight	386.4



BAS-Code	M850H012
IUPAC Name	6-amino-2,2,7-trifluoro-4-(prop-2-yn-1-yl)-2H-1,4-benzoxazin-3(4H)-one
BASF Reg. No.	5797901
CAS-No.	N/A
Molecular Formula	$C_{11}H_7F_3N_2O_2$
Molecular Weight	256.2



BAS-Code	M850H035
IUPAC Name	N,N-dimethyl-N'-[2,2,7-trifluroro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-2-imidodicarbonic diamide
BASF Reg. No.	6070203
CAS-No.	N/A
Molecular Formula	$C_{15}H_{13}F_3N_4O_4$
Molecular Weight	370.3



2.3. Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Analytical	Model AT100	Mettler	----
Balance, Top Loader	Model PJ3600	Mettler DeltaRange	----
Beakers	Various sizes	PYREX Brand, VWR Scientific Products	13922-029
Bottle, Amber glass	Qorpak, 2 oz and 4 oz with Teflon®-lined cap	VWR Scientific Products Boston Round, Amber	89042-908
Culture tube caps	16 mm	VWR	60828-768
Culture Tubes	Glass, disposable, 16x100mm size	Fisher	14-961-29
Cylinder, Graduated	Various sizes	Various	----
Flasks	Various sizes	Various	----
French Square Bottles, Wide Mouth, Qorpak® with PTFE Lined Caps	240 mL / 8 oz. 43-400 Cap	Berlin Packaging	GLC-01331
Glass Centrifuge Tubes	50 mL	VWR	8422-50
Plastic syringe	1 mL	Various	----
Positive Displacement Pipette and tips	1000 µL, 250 µL, 25 µL	Gilson Microman Fisher Scientific	----
Repeater Pipette and tips	50 mL	BrandTec Scientific	----
Syringe filter	PTFE Acrodisc® 0.45 µm pore size	Pall Gelman	4543
Volumetric, pipettes	0.5 mL, 1 mL, 2.5 mL, 5 mL, 10mL, 20 mL	Various – Class A	----
Volumetric flask	Various sizes	Various – Class A	----
Centrifuge	Allegra 6	Beckman Coulter	----
Mechanical shaker	KS401 Digital	IKA Labortechnik	----
Nitrogen evaporator	N-EVAP 112	Organomation Associates, Inc.	----
Ultrasonic Bath	Branson 1210	Branson	----
Vortex	Genie 2	VWR Scientific Products	14216-184
LC System	Acquity UPLC I-Class System	Waters	----
Mass Spectrometer	Sciex 6500 Mass Spectrometer	Sciex	----
HPLC Column	Acquity HSS T3, 2.1x100 mm, 1.8 µm	Waters	186003539
HPLC Column	Acquity BEH C18, 2.1x50 mm, 1.7 um	Waters	186002350

Note: The equipment and instrumentation listed above may be substituted by that of similar specifications. The applicability is confirmed if the recoveries of the fortification experiments are in the expected concentration range.

2.4 Reagents

2.4.1 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Formic acid	98% GR ACS	MilliporeSigma	FX0440-7
Formic acid (LC Mobile Phase Use)	95%	Sigma Aldrich	F0507-100 mL
Methanol	HPLC Grade	MilliporeSigma	MX0475P-1
Water	HPLC Grade	BDH Aristar Plus	87003-652
Cyclohexane	HPLC Grade	Fischer	C620-4
Ethyl Acetate	HPLC Grade	MilliporeSigma	EX0245-1
Acetone	HPLC Grade	MilliporeSigma	AX0115P-1

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

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Solvent	**S1**	Methanol with 0.1% Formic Acid Add 1 mL of formic acid to 1000 mL of methanol into an appropriate container and mix well to ensure complete homogeneous solution.
Solvent	**S2**	Water with 0.1 % Formic Acid Add 1 mL of formic acid to 1000 mL of water into an appropriate container and mix well to ensure complete homogeneous solution.
Liquid-Liquid Extraction Solvent	**S3**	Cyclohexane-Ethyl Acetate mixture (90:10, v/v) Add 900 mL of cyclohexane to 100 mL of ethyl acetate into an appropriate container and mix well to ensure complete homogeneous solution.
Final Volume Solution	**S4**	Methanol-Water mixture with 0.1% Formic Acid (20:80, v/v) Add 200 mL of **S1** to 800 mL of **S2** into an appropriate container and mix well to ensure complete homogeneous solution.
Solvent	**S5**	Methanol with 0.5% Formic Acid Add 0.5 mL of formic acid to 100 mL of methanol into an appropriate container and mix well to ensure a complete homogeneous solution.
Solvent	**S6**	Water with 10 % Formic Acid Add 10 mL of formic acid to 90 mL of water into an appropriate container and mix well to ensure a complete homogeneous solution.
Solvent	**S7**	Methanol-Water mixture with 0.1% Formic Acid (50:50, v/v) Add 100 mL of **S1** to 100 mL of **S2** into an appropriate container and mix well to ensure complete homogeneous solution.
Mobile Phase A	**LC1**	1% Formic Acid in Water Add 990 mL of water to 10 mL of concentrated formic acid into an appropriate container and mix well to ensure complete homogeneous solution.
Mobile Phase B	**LC2**	0.1% Formic Acid in Methanol Add 1 mL of formic acid to 1 L of methanol into an appropriate container and mix well to ensure complete homogeneous solution.

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

2.5 Standard Solutions

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

Stock Solutions (BAS 850 H, M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035) Preparation

Prepare a 1.0 mg/mL stock solution individually by weighing an appropriate amount of an analyte into a flask and add the required volume of **S1** (methanol with 0.1% formic acid), except for M850H035 which should be prepared in acetone.

For example, weigh 10 mg BAS 850 H into a 10 mL volumetric flask. Dissolve and dilute to mark with **S1**. This creates a solution containing 1 mg/mL of BAS 850 H in **S1**. Ensure a complete homogeneous solution (e.g. by sonication and/or vortexing). The stock solutions for all other analytes are made in a similar fashion.

Independence of standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved for example using one of the following approaches:

- Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
- Fortification and calibration standard solutions should be prepared from one stock solution in separate dilution series.

For subsequent preparations of solutions, freshly prepared solutions can be compared directly to previous standard solutions.

A correction for purity is done if the purity is ≤ 95%. If the purity is > 95 % correction is optional.

Fortification Solutions (BAS 850 H, M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035)

Prepare mixed standard solutions for fortification by combining stock solutions of each analyte (see above) in a flask. 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 S1 to a final volume of (mL)	Concentration (µg/mL)
1000	0.25	25	10
10	1.0	10	1
1	2.5	25	0.1
1	0.25	25	0.01

Note: A different concentration scheme may be used. If necessary, the volume of solution prepared may be changed.

Calibration Standard Solutions (BAS 850 H, M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035)

Prepare mixed standard calibration solutions for LC-MS/MS analysis, in flasks, by using the 10 ng/mL solution that was prepared in Section "Fortification Solutions (BAS 850 H, M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035)". Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by vortexing).

While the calibration solutions contain all analytes, the following concentrations of the solutions were used for the analysis as described in the tables below:

Preparation of Mixed Solvent Standard Calibration Solutions

Take solution (ng/mL)	Volume (mL)	Dilute with S4 to a final volume of (mL)	Concentration (ng/mL)	Solution No.†
10*	2.5	50	0.5	1
10*	1.25	50	0.25	2
10*	0.625	50	0.125	3
0.5	5.0	50	0.05	4
0.5	2.5	50	0.025	5
0.5	1.25	50	0.0125	6
0.5	0.5	50	0.005	7

*This solution was made in the "Fortification Solutions" section.
†Typically, solutions 1-5 are used for the analysis of M850H001 while solutions 3-7 are used for the analysis of BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035.

In case matrix-matched standards are needed for successful analysis, see Section 3.7.1 for their preparation.

Note: A different concentration scheme may be used. If necessary, the volume of solution prepared may be changed.

2.5.1 Stability of Standard Solutions

BASF recommends that stock solutions (1 mg/mL) of all analytes be made fresh every three months.

Stock and fortification solutions of BAS 850 H, M850H001, M850H002, M850H003, M850H004, and M850H012 in methanol with 0.1% formic acid have been shown to be stable for up to **90** days when stored refrigerated. Stability of M850H035 in methanol with 0.1% formic acid and acetone will be assessed during method validation.

Calibration solutions of BAS 850 H, M850H001, M850H002, M850H003, M850H004, and M850H012 in methanol-water with 0.1% formic acid (20:80 v/v) have been shown to be stable for up to **30** days when stored refrigerated. Stability of M850H035 in methanol-water with 0.1% formic acid (20:80 v/v) will be assessed during method validation.

3 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Sample homogenization is not needed for water samples. However, samples should be fully thawed and mixed before removing an aliquot for analysis.

3.2 Sample Storage

Water samples are to be kept frozen until analysis.

3.3 Weighing and Fortification

For fortified samples, measure 10 ± 0.1 mL of control sample into a glass container suitable to allow proper mixing and add fortification solutions according to the table below and then proceed to Section 3.4.

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

***** Limit of quantification

3.4 Extraction

No extraction is necessary for the analysis of BAS 850 H and its metabolites in water. Proceed to Section 3.5.

3.5 Sample Clean-up

3.5.1 Sample Clean-up for BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035

No sample clean-up is required. Proceed to Section 3.6.1 to prepare the samples for measurement.

3.5.2 Sample Clean-up for M850H001

a) Transfer an exact 5 mL aliquot from the sample in Step 3.4 to a glass centrifuge tube; add exactly 0.05 mL of **S6** (water with 10% formic acid) and mix well.
b) Add 10 mL of **S3** (cyclohexane-ethyl acetate, 90:10, v/v) to the tube above and vortex mix for 2 minutes. Centrifuge the tube for about 5 minutes at ~1500 rpm.
c) Remove exactly 8 mL of the top organic layer into a separate culture tube and evaporate to dryness under nitrogen at 50°C.

Proceed to Section 3.6.2 to prepare the samples for measurement.

Note:	Samples should be shaken, instead of vortexed, if there is too much liquid volume in the culture tube to form a proper vortex.

3.6 Preparation for Measurement

3.6.1 For Analysis of BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035

Transfer an exact 1 mL aliquot from the sample from Step 3.5.1 to a culture tube; add exactly 0.25 mL of **S5** (methanol with 0.5% formic acid) and mix well. Filter all samples through a 0.45 µm PTFE syringe filter into an HPLC vial. Discard the first ~0.1 mL that passes through the syringe filter.

For all samples, samples are ready for injection.

In case of residues higher than the calibration curve, dilute the samples with **S4** (or control final volume from this subsection, if matrix matched standards are used) as needed to fit into the calibration curve.

See Section 4.2.1 and 4.2.2 for LC-MS/MS conditions.

3.6.2 For Analysis of M850H001

a) Add exactly 0.4 mL of **S7** to the dry residue in the culture tube from Section 3.5.2[f]. Cap and vortex the tubes, being sure to wet the sides of the tube, and then sonicate for 2 minutes.
b) Remove the cap and add exactly 0.6 mL **S2** to the tubes above and vortex thoroughly to ensure homogenous solution.
c) Filter all samples through a 0.45 µm PTFE syringe filter in an HPLC vial. Discard the first 0.1 mL that passes through the syringe.

For all samples, samples are ready for injection.

In case of residues higher than the calibration curve, dilute the samples with **S4** (or control final volume from this subsection, if matrix matched standards are used) as needed to fit into the calibration curve.

See Section 4.2.3 for LC-MS/MS conditions.

3.7 Influence of Matrix Effects on Analysis

In some water matrices, matrix effects have been found to cause significant suppression of analytes when analyzed with LC-MS/MS. If significant suppression occurs, matrix-matched standards may be utilized. Matrix-matched calibration standards are used for quantitation when signal suppression or enhancement is >20% compared to the response for standards prepared in calibration solution alone.

3.7.1 Matrix Matched Standards

Matrix matched standards should be used for both sets of standards. Matrix effects have been observed in both drinking water and surface water on a highly sensitive instrument (Sciex 6500). Therefore, it may be necessary to test matrix effects against calibration standards even if a highly sensitive instrument is used.

Use the tables below, for each type of standard, to make the matrix matched standards. BASF recommends making at least 5 levels of standards. Matrix-matched calibration standard solutions are prepared in the following manner:

a) Prepare precursor standards for matrix matched calibration standards in the following manner from the respective fortification solutions found in Section 2.5:

Preparation of Mixed Calibration Precursor Solutions:

Take solution (ng/mL) in **S1**	Volume (mL)	Dilute with **S1**‡ to a final volume of (mL)	Concentration (ng/mL)	Solution No.†
100*	1.25	50	2.5	1
100	0.625	50	1.25	2
2.5	10	40	0.625	3
2.5	5	50	0.25	4
2.5	2.5	50	0.125	5
2.5	1.25	50	0.0625	6
2.5	0.5	50	0.025	7

* This solution is prepared in Section 2.5

† Typically, solutions 1-5 are used for the analysis of M850H001 while solutions 3-7 are used for the analysis of BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035.

‡ Precursor solutions for BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035 may be made in methanol with 0.5% formic acid (**S5**) (as used in ILV for M850H004 analysis).

Note: A different concentration scheme may be used. If necessary, the volume of solution prepared may be changed.

b) Preparation of matrix matched calibration standards for M850H001
   i. Prepare at least five (5) extra control aliquots through section 3.5.2 (additional control samples may be prepared to dilute samples with residues higher than LOQ).
   ii. Add exactly 0.2 mL of precursor solution from section 3.7.1[a] and 0.2 mL of **S2** to the dry control residue in the culture tube from Section 3.5.2[c].
   iii. Cap and vortex the tubes, being sure to wet the sides of the tube, and then sonicate for 2 minutes.
   iv. Remove the cap and add exactly 0.6 mL **S2** to the tubes above and vortex thoroughly to ensure homogenous solution.
   v. Filter all matrix matched standards through a 0.45 µm PTFE syringe filter into an HPLC vial. Discard the first ~0.1 mL that passes through the syringe filter.

c) Preparation of matrix matched calibration standards for BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035.
   i. Combine exactly 1 mL of control water with exactly 0.25 mL of precursor solution from section 3.7.1[a].
   ii. Cap and vortex tubes to ensure homogenous solution.
   iii. Filter all matrix matched standards through a 0.45 µm PTFE syringe filter into an HPLC vial. Discard the first ~0.1 mL that passes through the syringe filter.

3.8 Stability in Final Volumes

Stability of BAS 850 H and metabolites in final volume solutions will be determined 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 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 (BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035)

	Parameter		
Chromatographic System	Waters Acquity UPLC system**		
Analytical-column	Acquity HSS T3, 2.1 x 100 mm, 1.8 µm particle size		
Column Temperature	50 ºC		
Injection Volume	100 µL (can be raised or lowered depending on sensitivity)		
Mobile Phase A Mobile Phase B	Water with 1.0% formic acid (**LC1**) Methanol with 0.1% formic acid (**LC2**)		
Flow Rate	500 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00 0.25 4.25 4.50 5.50 5.75 6.50	85 60 30 5 5 85 85	15 40 70 95 95 15 15
Detection System	AB Sciex 6500 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	650 °C		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
BAS 850 H	413 → 74*	Positive	~ 5.0 min
M850H002	373 → 323* 373 → 193	Negative	~ 4.7 min
M850H003	357 → 307* 357 → 193	Negative	~ 3.2 min
M850H004	387 → 131* 387 → 74	Positive	~ 4.7 min
M850H012	257 → 163* 257 → 116	Positive	~ 3.0 min
M850H035	371 → 257* 371 → 163	Positive	~ 4.4 min

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

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

Note: Polarity switching is necessary. Multiple periods may be necessary and should be adjusted to ensure an adequate number of data points to appropriately define each chromatographic peak.

Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of the fortification experiments are in the acceptable range.

In general, a divert valve is used to reduce the matrix load on the detection system.

Instrument conditions, e.g. injection volume, ionization temperature, column, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the fortification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the instrument used.

4.2.2 Instrumentation and Conditions (BAS 850 H Confirmatory)

	Parameter		
Chromatographic System	Waters Acquity UPLC system**		
Analytical-column	Waters Acquity BEH C18, 2.1 x 50 mm, 1.7 µm particle size		
Column Temperature	50 ºC		
Injection Volume	100 µL (can be raised or lowered depending on sensitivity)		
Mobile Phase A Mobile Phase B	Water with 1.0% formic acid (**LC1**) Methanol with 0.1% formic acid (**LC2**)		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00 6.25 6.50 7.50 7.75 8.00	85 40 5 5 85 85	15 60 95 95 15 15
Detection System	AB Sciex 6500 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	650 °C		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
BAS 850 H	413 → 74	Positive	~ 6.1 min

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

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of the fortification experiments are in the acceptable range.

In general, a divert valve is used to reduce the matrix load on the detection system.

Instrument conditions, e.g. injection volume, ionization temperature, column, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the fortification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the instrument used.

4.2.3 Instrumentation and Conditions (M850H001)

	Parameter		
Chromatographic System	Waters Acquity UPLC system**		
Analytical-column	Waters Acquity BEH C18, 2.1 x 50 mm, 1.7 µm particle size		
Column Temperature	50 ºC		
Injection Volume	100 µL (can be raised or lowered depending on sensitivity)		
Mobile Phase A Mobile Phase B	Water with 1.0% formic acid (**LC1**) Methanol with 0.1% formic acid (**LC2**)		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00 6.25 6.50 7.50 7.75 8.00	85 40 5 5 85 85	15 60 95 95 15 15
Detection System	AB Sciex 6500 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	650 °C		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
M850H001	397 → 141* 397 → 134	Positive	~ 4.3 min

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

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

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of the fortification experiments are in the acceptable range.

In general, a divert valve is used to reduce the matrix load on the detection system.

Instrument conditions, e.g. injection volume, ionization temperature, column, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the fortification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the instrument used.

4.3 Calibration procedures

Calculation of results is based on peak area measurements using a calibration curve. At least 5 calibration levels need to be injected (e.g., required for enforcement).

One calibration curve is obtained by direct injection of BAS 850 H, M850H002, M850H003 M850H004, M850H012, and M850H035 on the LC-MS/MS in the range of 0.005 ng/mL to 0.125 ng/mL. The second calibration curve is obtained by direct injection of M850H001 on the LC-MS/MS in the range of 0.025 ng/mL to 0.5 ng/mL. In a given injection run, the same injection volume is used for all samples and standards.

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

4.4 Calculation of Residues and Recoveries

Calculation of results is based on area measurements.

For the procedural recoveries, the sample volume will be considered 10 mL in the final calculation of residues [ng/L]. The method requires that the sample weight to be 10 ± 0.1 mL for fortification samples. The recovery is the percentage of the fortified amount (µg or ng), which is recovered through the method and the weights cancels out, as shown in the equation below, during the final calculation step.

The residues of BAS 850 H in ng/L are calculated as shown in equations **I** and **II**:

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

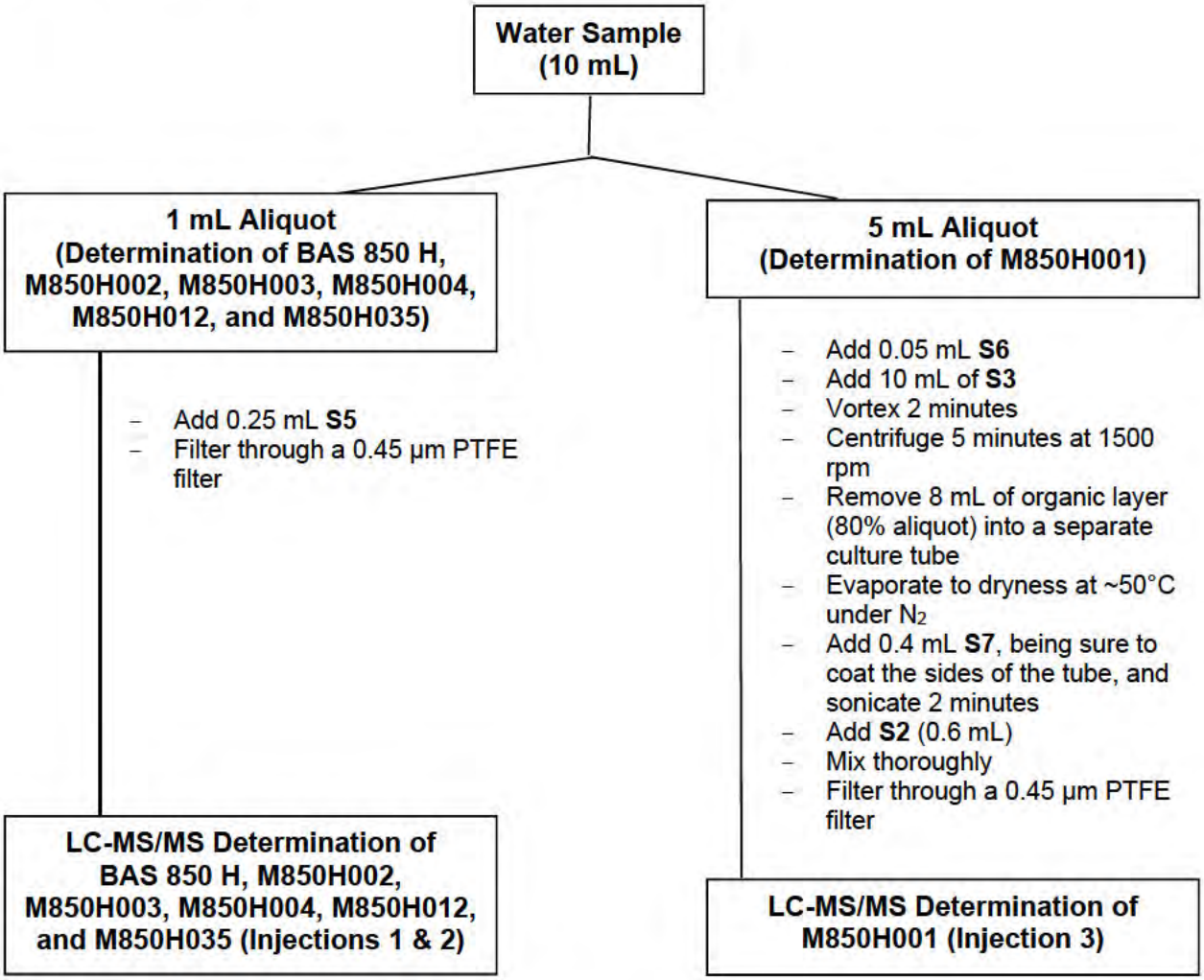
II. **Residue [ng/L]** $= \frac{V_{end} \times C_A \times 1000}{G \times A_F}$

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 [mL]
A_F	=	Aliquotation factor
1000	=	Factor remaining after all unit conversions

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

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

5 FLOWCHART



Note: Injection 2 is for confirmation of BAS 850 H only.

S2 = Water with 0.1% Formic Acid
S3 = 10:90 Ethyl Acetate-Cyclohexane (v/v)
S5 = Methanol with 0.5% Formic Acid
S6 = Water with 10% Formic Acid
S7 = 50:50 Methanol-Water with 0.1% Formic Acid (v/v)

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 0.75 working days (6 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
This information will be provided in the validation part of the analytical method D1724/01.

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The limit of quantification is defined as the lowest fortification level successfully tested.

The method has a limit of quantitation of 30 ng/L, in water, for each analyte. The limit of detection for each analyte is approximately 6 ng/L. All analytes are determined individually. The limit of detection was estimated at approximately 20% of the limit of quantification 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 method was able to accurately determine residues of BAS 850 H and its metabolites, and no interferences were observed at the retention time of the analyte peaks. Sufficient matrix suppression (>20%) was found to influence the analysis of most analytes, therefore matrix matched standards are used. LC-MS/MS is a highly-specific and selective detection method that uses two ion transitions and retention time for all analytes except for BAS 850 H, which has a single ion transition and a confirmatory chromatographic method.

Confirmatory Techniques
The HPLC-MS/MS final determination is a highly selective detection technique. For M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035, the quantitation is possible at two different transitions. Therefore, no additional confirmatory technique is required.

Based on the sensitivity of the instrument, a secondary transition for BAS 850 H does not have a strong enough signal-to-noise ratio in the lowest standard. Therefore a confirmatory method, Section 4.2.2, is provided as an alternative chromatographic technique for its primary transition.

Potential Problems
The glassware used for the method should be thoroughly rinsed with methanol to prevent contamination. Only glass and Teflon containers should be used during the extraction in this method to prevent interference from the containers. Test tube caps and filter syringes may be plastic.

7.1 Example Calculations

Example: BAS 850 H, *m/z* 413 → 74; water sample fortified at 30 ng/L:

Concentration in the final volume [ng/mL]

$$\textbf{Concentration [ng/mL]} = \frac{\text{Response} - \textit{Intercept}}{\textit{Slope}} = C_A$$

Residue in the sample [ng/L]

$$\textbf{Residue [ng/L]} = \frac{V_{end} \times C_A \times 1000}{G \times A_F}$$

$$\textbf{Recovery \%} = \frac{\text{Residue in fortified sample} - \text{Residue in control} \times 100}{\text{Amount of analyte fortified}}$$

The following values were used in this calculation:

Response of fortified sample	14715
Response of control sample	0.000
Slope:	497000
Intercept:	2130
Sample Volume (G):	10 mL
Final Volume (V_{end}):	1.25 mL
Aliquotation factor A_F:	0.1 (=10%)
Conversion factor mL → L:	1000

Aliquotation factor A_F

$$\frac{\text{Aliquot from Sample (mL)}}{\text{Total Sample Volume (mL)}} = \frac{1}{10} = 0.1$$

$$\textbf{Concentration (ng/mL),}\ C_A = \frac{14715 - 2130}{497000} = 0.0253\ ng/mL$$

$$\textbf{Residue (ng/L)} = \frac{1.25\,mL \times 0.0253\,ng/mL \times 1000}{10\,mL \times 0.1} = 0.0316\,ng/L$$

$$\textbf{Recovery \%} = \frac{(0.0316\,ng/L - 0.0000\,ng/L) \times 100}{0.0300\,ng/L} = 105\%$$