Title

Methods of Analysis of BAS 850 H and its Relevant Metabolites in Water with Limit of Determination (LOD) Calculation (Method D1724/01)

Guidelines Covered

U.S. EPA Ecological Effects Test Guidelines, OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation
EC Guidance document:
SANCO/3029/99 rev 4 (11/07/2000); SANCO/825/00 rev 8.1, (16/11/2010)

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. D1724/01 used for the determination of residues of trifludimoxazin (BAS 850 H), including six metabolites, 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 held under refrigeration 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., 784160-5). The test system samples were assigned unique numbers and these were recorded in each analytical set or “Master Sheet” (e.g., water fortification sample 784160-05-04, from Master Sheet No. 784160-5). The actual sample numbers used for the analysis were identified in the raw data and in this final report.

2.2 Test and Reference Substances

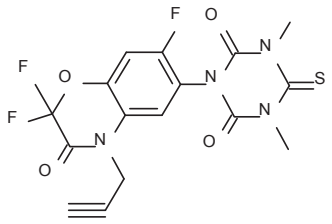
The test/reference standards, shown below, were synthesized by BASF Aktiengesellschaft (Limburgerhof, Germany) and were maintained at room temperature (for M850H004, refrigerator or freezer) until use in this study. BASF Aktiengesellschaft determined characterization and purity prior to the substances being used in this study. Details of these determinations are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany. BASF has retained a reserve sample of each chemical and has documentation at BASF Corporation, BASF Crop Protection (Research Triangle Park, North Carolina, USA). The certificate of analysis for each test substance is provided in Appendix A.

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 Trifludimoxazin

Common Name	Trifludimoxazin
BAS Code Name	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
Molecular Formula	$C_{16}H_{11}F_3N_4O_4S$
Molecular Weight	412.3
Lot No.	L84-130
Purity:	99.2%
Expiration Date	February 01, 2020

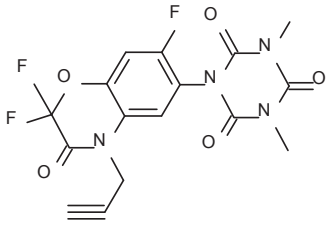
Chemical structure:



2.2.2 M850H001

Common Name	None
BAS Code Name	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
Molecular Formula	$C_{16}H_{11}F_3N_4O_5$
Molecular Weight	396.3
Lot No.	L85-52
Purity:	98.7%
Expiration Date	April 01, 2018

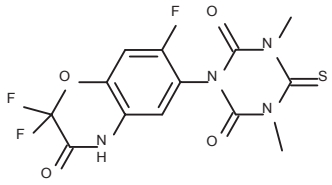
Chemical structure:



2.2.3 M850H002

Common Name	None
BAS Code Name	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
Molecular Formula	$C_{13}H_9F_3N_4O_4S$
Molecular Weight	374.3
Lot No.	L84-162
Purity:	96.8%
Expiration Date	February 01, 2020

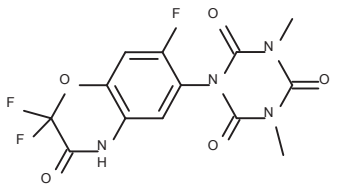
Chemical structure:



2.2.4 M850H003

Common Name	None
BAS Code Name	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
Molecular Formula	$C_{13}H_9F_3N_4O_5$
Molecular Weight	358.2
Lot No.	L85-70
Purity:	99.4%
Expiration Date	April 01, 2018

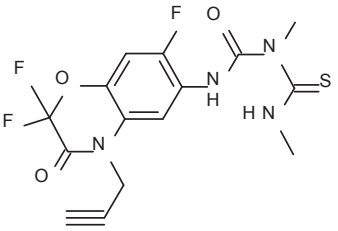
Chemical structure:



2.2.5 M850H004

Common Name	None
BAS Code Name	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
Molecular Formula	$C_{15}H_{13}F_3N_4O_3S$
Molecular Weight	386.4
Lot No.	L85-50
Purity:	99.5%
Expiration Date	April 01, 2018

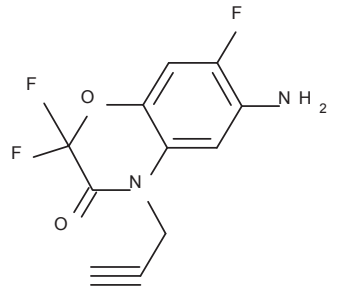
Chemical structure:



2.2.6 M850H012

Common Name	None
BAS Code Name	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
Molecular Formula	$C_{11}H_7F_3N_2O_2$
Molecular Weight	256.2
Lot No.	L85-66
Purity:	98.9%
Expiration Date	September 01, 2018

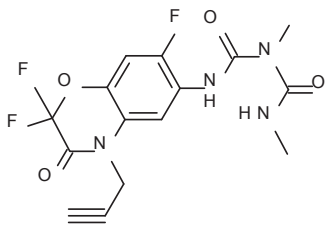
Chemical structure:



2.2.7 M850H035

Common Name	None
BAS Code Name	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
Molecular Formula	$C_{15}H_{13}F_3N_4O_4$
Molecular Weight	370.3
Lot No.	L2017-007
Purity:	100.0%
Expiration Date	February 01, 2019

Chemical structure:



Stock solutions of each analyte were prepared in methanol with 0.1% formic acid, or acetone (M850H035 only), and the mixed intermediate (fortification) solutions containing each analyte were prepared by diluting combined aliquots of the stock solutions using methanol with 0.1% formic acid. Solvent-based mixed calibration standards were prepared by serial dilution of the mixed intermediate standards using methanol:water (20:80, v/v) with 0.1% formic acid. The stability of each analyte, except M850H035, in standard solutions held under refrigeration has been previously determined (References 1-3); the stability of M850H035 in standard solutions was determined in conjunction with this study. Matrix-matched standards were prepared using control material as described in the method and were not stored longer than approximately 1 week. During the course of this study, the test/reference substance solutions were stored under refrigeration and were used within the demonstrated period of stability. Preparation and dilution data forms pertaining to the stock and working solutions are located in the analytical facility data and are archived periodically. Example standard dilution and use information, as performed in the subject study, are provided in Appendix L.

2.3 Route of Administration

In this method validation study, the test substances were applied to the test system as analytical standard solutions (in acidified methanol) by micropipette 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. D1724/01, residues of trifludimoxazin in water are cleaned-up by filtration or liquid/liquid partitioning and filtration, and then quantified using LC-MS/MS. The method procedures validated in this study are provided in Appendix B. Briefly, residues of trifludimoxazin in a 1 mL aliquot of water samples (10 mL each) are diluted with methanol containing 0.5% formic acid, filtered (0.45 µm PTFE) and analyzed by LC-MS/MS. M850H001 residues in a separate 5 mL aliquot of the water samples are acidified, partitioned with ethyl acetate:cyclohexane (10:90, v/v), and centrifuged; residues in an aliquot of the organic layer are evaporated to dryness, re-dissolved in a final volume of methanol:water with 0.1% formic acid (20:80 v/v), filtered (0.45 µm PTFE) and analyzed by LC-MS/MS.

2.4.2 Specificity/Selectivity

The residues of trifludimoxazin are determined by HPLC-MS/MS monitoring ion transitions m/z 413→74 for parent trifludimoxazin; m/z 397→141 and 397→134 for M850H001; m/z 373→323 and 373→193 for M850H002; m/z 357→307 and 357→193 for M850H003; m/z 387→131 and 387→74 for M850H004; m/z 257→163 and 257→116 for M850H012; or m/z 371→257 and 371→163 for M850H035. In lieu of a secondary (alternate) ion transition for trifludimoxazin confirmatory analysis is performed using a different LC column and gradient. The results are calculated by direct comparison of the sample peak responses to those of external standards. Two mass transitions are available for all analytes except parent trifludimoxazin. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary, except as discussed above for parent trifludimoxazin, for which a separate chromatographic technique is available for confirmatory quantitation. 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 typically consisted of a reagent blank, and for each matrix, two controls, five replicates fortified with each analyte at the method limit of quantitation, 30 ppt, and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 300 ppt. For each analyte, the two mass transitions or one mass transition with the additional confirmatory method described above were evaluated.

2.6 Influence of Matrix Effects on Analysis

In conjunction with the subject study, matrix-matched standards and solvent-based standards were analyzed in a separate experiment to evaluate any potential matrix effects on LC-MS/MS analysis. This involved comparing calibration standards prepared with control matrix against calibration standard solutions prepared with acidified methanol:water (20:80, v/v). The matrix-matched standards were prepared, using control sample material worked up through the method, to final concentration levels approximating 1/2XLOQ, 1XLOQ, and 2XLOQ. Each set of matrix-matched standards (for each water type) was bracketed by a block of calibration standards with additional injections of tested standard levels occurring as appropriate during the run.

The data generated were evaluated by comparing the average area response of the standards for typically three injections of each type (with and without matrix) for each of 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/ion transition, an overall average "Mean Area Change (%)" across the three tested concentrations was calculated to make a general assessment of acceptability with respect to matrix effects.

2.7 Stability of Extracts

The stability of residues of trifludimoxazin in stored extract solutions was determined in conjunction with the subject method validation study. To establish stability, one control and multiple method validation recovery samples for each fortification level (n=5 total recovery samples) that had been stored under refrigeration at the final volume stage were re-analyzed. Quantification of each analyte in the stored samples for this experiment was performed using the primary ion transition (or chromatographic technique in the case of trifludimoxazin).

3. RESULTS

3.2 Influence of Matrix Effects

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. The results of the extensive testing on each water matrix (for both ion transitions) demonstrated that the matrix load in the water samples had no significant influence on the analysis. Matrix effects, calculated as the overall mean percent area count difference between matrix-matched standards and solvent-based standards, at three standard concentration levels, were less than 20% (Table 2).

3.3 Solution Storage Stability

Standards. The available standard solution storage stability data, summarized indicate that trifludimoxazin, M850H001, M850H002, M850H003, M850H004, and M850H012 are stable in stock standards prepared in acidified methanol (0.1% formic acid in methanol) for at least 3 months (≥81 days), and indicate that these analytes are also stable in mixed intermediate (fortification) standards prepared in acidified methanol and in mixed calibration standards prepared by serial dilution of the intermediate standards using acidified methanol:water (20:80, v/v with 0.1% formic acid) for at least 2 months ≥61 days), each when held under refrigeration.

In this study, M850H035 was shown to be stable in stock solution prepared in acetone, in intermediate standard solutions prepared in 0.1% formic acid in methanol, and in calibration standards prepared by serial dilution of the intermediate standards using acidified methanol:water (20:80, v/v with 0.1% formic acid), for at least 1 month (27-43 days), each when held under refrigeration (Table 3). During the course of this study, the test/reference substance solutions were stored under refrigeration and all solutions were used within the demonstrated time period of stability.

Extracts. The method validation fortification sample extracts were analyzed within 2 days of extraction. The acceptable method recoveries obtained during analysis demonstrate the storage stability of residues of trifludimoxazin 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 stored under refrigeration, indicated that residues of trifludimoxazin are stable in water matrix extracts for approximately 1 week, the longest interval tested, sufficient to support the storage intervals and conditions incurred by the extracts in the subject study,

4. CALCULATIONS AND RAW DATA

An example calculation is included in Appendix C (page 66). Detailed analytical data such as supporting raw data necessary for re-calculations, standards and calibration curve data are provided in Appendix D (page 68).

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 and refrigerator temperatures were continuously monitored by electronic means.

6. SUMMARY OF METHOD

Summaries of the method parameters and characteristics are provided in Table 5 and Table 6.

7. INDEPENDENT LABORATORY VALIDATION

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.

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.

Linearity:	Acceptable linearity was observed for the standard ranges tested: The method-detector response was linear over the 0.005 to 0.125 ng/mL range ($r \geq 0.9989$), or for M850H001, over the 0.025 to 0.5 ng/mL range ($r \geq 0.9979$), for the definitive method validation sets.
Specificity:	The control chromatograms generally have no peaks above the chromatographic background and the spiked sample chromatograms contain only the analyte peak of interest. Peaks were well-defined and symmetrical. There appeared to be no carryover to the following chromatograms.
Limit of Quantification:	The LOQ of the method was set at 30 ng/L (30 ppt) for BAS 850 H and its metabolites, M850H001, M850H002, M850H003, M850H004, M850H012, and M850H035, and was also defined as the lowest fortification level tested. Additionally the eco-toxicology endpoints in water (NOEC) were considered for all analytes. For parent (BAS 850 H), the NOEC is 23 ng/L which is close to the set LOQ of 30 ng/L. For all other metabolites, the LOQ was lower than the lowest relevant eco-toxicology endpoint in water (NOEC M850H001: 0.37 µg/L; M850H002: 0.63 µg/L;M850H004: 0.5 µg/L).
Limit of Detection:	The LOD for all analytes was set at 6 ng/L (6 ppt), which was 20% of the defined LOQ. The LOD was shown to be detectable as the absolute amount of analyte injected (0.5 pg on column for trifludimoxazin and metabolites except for M850H001 which was 2.5 pg on column) into the LC-MS/MS when the lowest calibration standard was analyzed (0.005 ng/mL for trifludimoxazin and metabolites except for M850H001 which was 0.025 ng/mL) with acceptable signal to noise ratio (S/N) greater than 3:1.
Repeatability	Overall relative standard deviations (%RSD) for all fortification levels were below 20%.
Reproducibility	Reproducibility of the method was not determined within this validation study.

Table 4. Storage Stability of Trifludimoxazin and Metabolites in Extracts

Analyte	Solution Tested	Conditions	Limit of Demonstrated Storage Stability[1]
Trifludimoxazin, M850H001, M850H002, M850H003, M850H004, M850H012 M850H035	Final volume (methanol:water, 20:80 v/v, with 0.1% formic acid)	Refrigerated (in the dark in glass autosampler vials)	Surface and Drinking Water, ~1 week (7, 6 days)

1. The stability criteria: ±20% difference between initial result (time-zero analysis) for the selected recovery sample and the stored-fortified recovery result obtained upon re-analysis by LC-MS/MS.

Table 5. Summary Parameters for the Analytical Method Used for the Quantitation of Residues of Trifludimoxazin in Water

Method ID	BASF Analytical Method No. D1724/01
Analyte(s)	Residues of trifludimoxazin (BAS 850 H) and its metabolites M850H001, M850H002, M850H003, M850H004, M850H012 and M850H035 in drinking and surface water
Extraction solvent/technique	Briefly, residues of trifludimoxazin and metabolites (except M850H001) in water samples (10 mL each) are diluted with acidified methanol, filtered (0.45 µm PTFE) and analyzed by LC-MS/MS. M850H001 residues in a separate aliquot of the water samples are acidified, partitioned with ethyl acetate:cyclohexane (10:90, v/v), and centrifuged; residues in an aliquot of the organic layer are then evaporated to dryness, re-dissolved in a final volume of methanol:water with 0.1% formic acid (20:80 v/v), filtered (0.45 µm PTFE) and analyzed by LC-MS/MS.
Cleanup strategies	Centrifugation; liquid/liquid partition; filtration.
Instrument/Detector	Liquid chromatography (LC) electrospray ionization tandem mass spectrometry (ESI-MS/MS) monitoring ion transitions m/z 413→74 for parent trifludimoxazin; m/z 397→141 and 397→134 for M850H001; m/z 373→323 and 373→193 for M850H002; m/z 357→307 and 357→193 for M850H003; m/z 387→131 and 387→74 for M850H004; m/z 257→163 and 257→116 for M850H012; and m/z 371→257 and 371→163 for M850H035. In lieu of a secondary (alternate) ion transition for parent trifludimoxazin confirmatory analysis is performed using a different LC-MS/MS column and gradient. Analyses for most of the analytes are performed using a Waters Aquity UPLC system equipped with a Acquity HSS T3 column (100 x 2.1 mm, 1.8 µm particle size) and a Sciex Instruments API 6500 detector and using a mobile phase gradient of water with 1% formic acid:methanol with 0.1% formic acid 85:15, 60:40, 30:70, 5:95, to 85:15, v/v, over 6.5 minutes (flow rate 500 uL/minute). For the analysis of M850H001 (both ion transitions) and confirmatory analysis for parent trifludimoxazin are conducted with a separate chromatographic technique, in separate injections for each analyte, using the same UPLC system and detector equipped with a Waters Acquity BEH C_{18} column (50 x 2.1 mm, 1.7 µm particle size) using a mobile phase gradient of water with 1% formic acid:methanol with 0.1% formic acid, 85:15 to 40:60, v/v, over 6.25 minutes (flow rate 600 uL/minute).
Standardization method	Linear regression (1/x weighting). Direct comparison of the sample peak area responses to those of external standards.
Stability of std solutions	The available storage stability data indicate that each analyte is stable in stock solutions prepared in 0.1% formic acid in methanol for at least 3 months (≥81 days), or acetone in the case of M850H035 for at least 1 month (43 days), when held under refrigeration. In addition, the data indicate that each analyte is stable in mixed intermediate (fortification) standards prepared by diluting combined aliquots of the stock solutions with 0.1% formic acid in methanol and in mixed calibration standards prepared by serial dilution of the intermediate standards using methanol:water (20:80, v/v) with 0.1% formic acid for at least 1 month (≥27 days), each when held under refrigeration. During the course of this study, the test/reference substance solutions were stored under refrigeration and all solutions were used within the demonstrated time period of stability.
Expected retention times (minutes)	Parent trifludimoxazin, ~5.0 (for alternate chromatographic technique, ~6.1); M850H002, ~4.7; M850H003, ~3.2; M850H004, ~4.7; M850H012, ~3.0; M850H035, ~4.4; M850H001, ~4.3

Table 6. Characteristics for the Analytical Method Used for the Quantitation of Residues of Trifludimoxazin in Water

Analyte	Residues of trifludimoxazin (BAS 850 H) and its metabolites M850H001, M850H002, M850H003, M850H004, M850H012 and M850H035 in drinking and surface water
Equipment ID	Waters Aquity UPLC system equipped with Sciex Instruments API 6500
Limit of quantitation (LOQ)	30 ng/L (30 ppt), for each analyte
Limit of detection (LOD)	6 ng/L (6 ppt), for each analyte
Reliability of the Method/ [ILV]	A successful independent laboratory validation [ILV] has been conducted for BASF Analytical Method No. D1724/01 for the determination of residues of trifludimoxazin in water. The values obtained are indicative of the reliability of Method No. D1724/01.
Linearity	The method-detector response was linear over the 0.005 to 0.125 ng/mL range ($r \geq 0.9899$), or for M850H001, over the 0.025 to 0.5 ng/mL range ($r \geq 0.9979$), for the method validation sets.
Specificity/ Selectivity	The control chromatograms generally have no peaks above the chromatographic background and the spiked sample chromatograms contain only the analyte peak of interest. Peaks were well-defined and symmetrical. There appeared to be no carryover to the following chromatograms.
	An experiment to evaluate any potential matrix effects showed that the matrix load in the samples from each water matrix had no significant influence on analysis (matrix effects <20%),
Confirmatory technique	Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary, except for parent trifludimoxazin, for which a confirmatory technique is available using a different LC column and gradient.
Time required	A set of 13 samples (one reagent blank, two controls, and 10 fortified samples for recovery experiments) requires about 6 hours of work (calculation of the results included).

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

BASF Method Number D1724/01

Draft

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

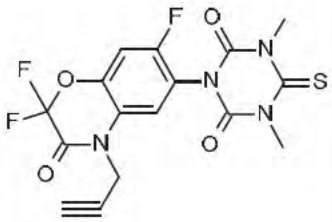
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Ensure that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

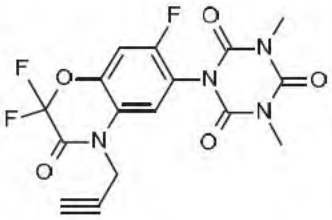
Standard substances are stored in a freezer (≤-5°C) until use.

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.

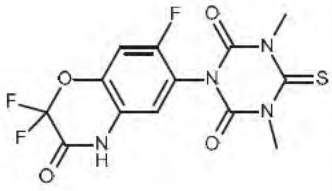
BAS-Code	BAS 850 H
IUPAC Name	1,5-dimethyl-6-thioxo-3-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5654329
CAS-No.	1258836-72-4
Molecular Formula	$C_{16}H_{11}F_3N_4O_4S$
Molecular Weight	412.3



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

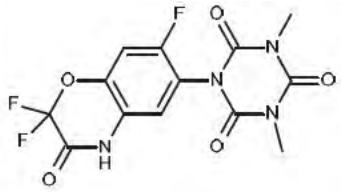


BAS-Code	M850H002
IUPAC Name	1,5-dimethyl-6-thioxo-3- (2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5757725
CAS-No.	N/A
Molecular Formula	$C_{13}H_9F_3N_4O_4S$
Molecular Weight	374.3

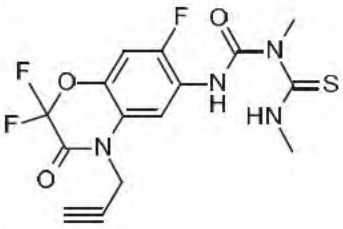


2.2 Test and Reference Items (Cont.)

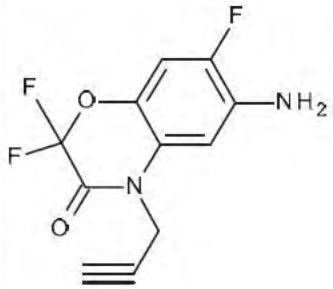
BAS-Code	M850H003
IUPAC Name	1,3-dimethyl-5-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4,6-trione
BASF Reg. No.	5757726
CAS-No.	N/A
Molecular Formula	$C_{13}H_9F_3N_4O_5$
Molecular Weight	358.2



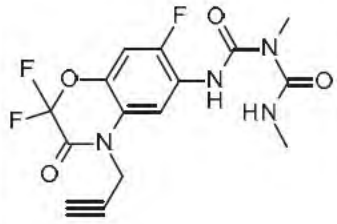
BAS-Code	M850H004
IUPAC Name	N,N-dimethyl-N'-[2,2,7-trifluroro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl] dicarbonimidothioicdiamide
BASF Reg. No.	5833884
CAS-No.	N/A
Molecular Formula	$C_{15}H_{13}F_3N_4O_3S$
Molecular Weight	386.4



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	85	15
	6.25	40	60
	6.50	5	95
	7.50	5	95
	7.75	85	15
	8.00	85	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]** $$\frac{\text{Response} - \mathit{Intercept}}{\mathit{Slope}} \qquad C_A$$

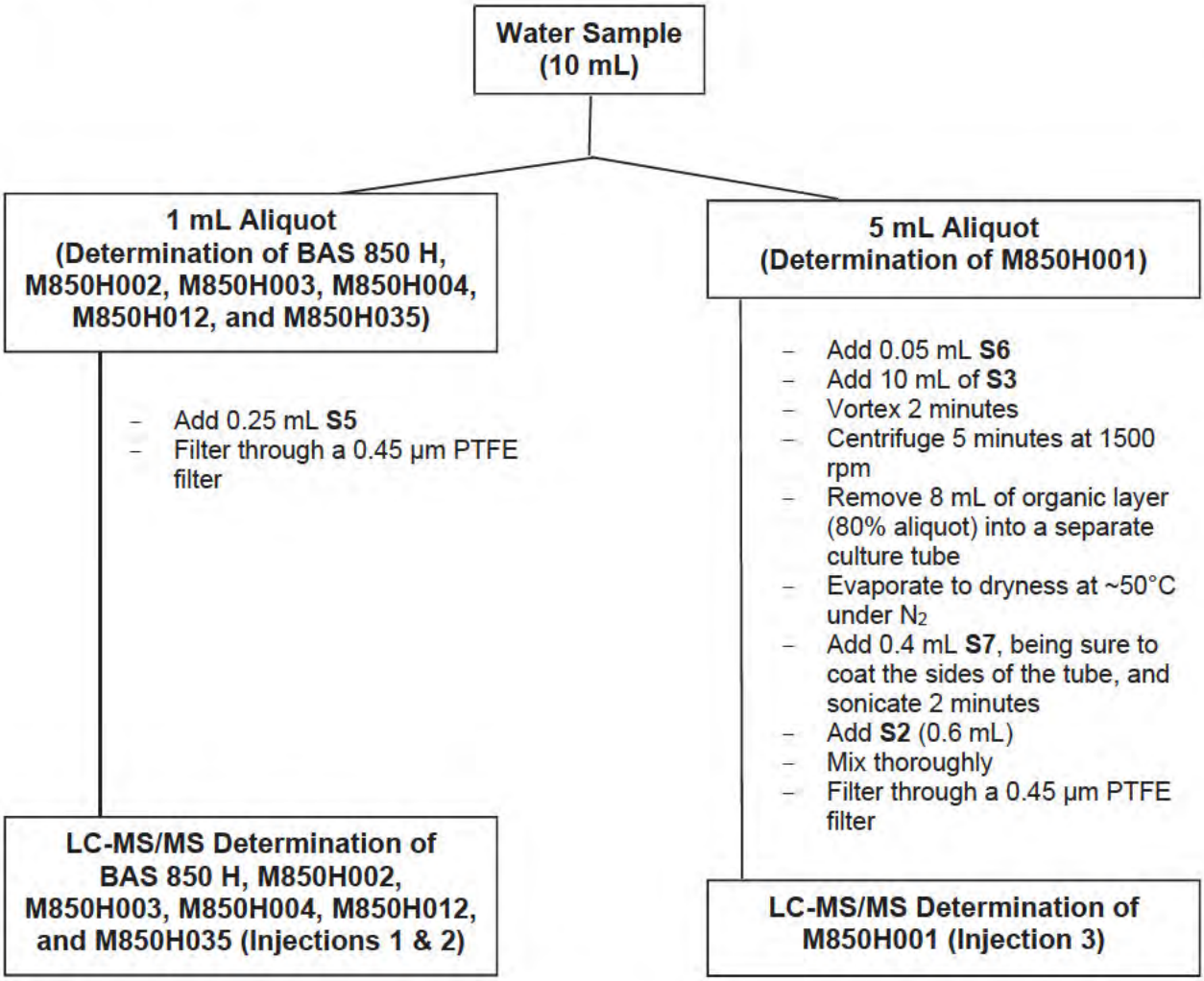
II. **Residue [ng/L]** $$\frac{V_{end} \times C_A \times 1000}{G \times A_F}$$

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

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]

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

Residue in the sample [ng/L]

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

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

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

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

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

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

Typical Recovery Calculation for LC-MS/MS Quantitation

Sample No. 784160-05-04-A. Control surface water sample fortified at the LOQ with trifludimoxazin (and other analytes), Master Sheet No. 784160-5.

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

	Trifludimoxazin
Peak Area =	13380
Intercept =	357
Slope =	6.35E+05
Conc. (ng/mL) =	0.0205

The concentration of analyte in µg/kg (ppb) is calculated as shown in equation:

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

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 [mL]
A_F = Aliquotation factor

	Trifludimoxazin
V_{end} =	1.25 mL
A_F =	10%
G =	10.00
Conc. (ng/mL) =	0.0205
Residue (ppb) =	0.0256

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

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

	Trifludimoxazin
Amount fortified (ppb) =	0.03
Residue (ppb) =	0.0256
Residue in control =	<LOD (<0.006 ppb)
%Recovery	85%

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

Protocol Amendments and Deviations

There was one deviation which documented the following:

1. Mixed calibration “precursor solutions” were not prepared according to the technical procedure (TP) attached to the protocol. The TP was not intended to be restrictive in this respect, nevertheless, the procedure was updated to reflect the dilution scheme used in the method validation and allow for flexibility in the preparation of these solutions. In addition, typographical errors in the gradient for method sections 4.2.2 and 4.2.3 were corrected. The updated TP as corrected (and as validated in this study) is shown in Appendix B.

None of the amendments / deviations noted above affect the validity of the study.

Appendix B:

Evaluation of the Limit of Detection (LOD) for 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"

BASF Study Number:
784160_1

BASF Registration Document Number:
2017/7018069

Page Count:

Contains 60 pages

1. INTRODUCTION

1.1 Background

BAS 850 H is an herbicide used in multiple crops. To analyze for BAS 850 H and its metabolites, 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" was validated (**Reference 1**). The purpose of this study is to evaluate the LOD for this validated method.

1.2 Definitions

Method Detection Limit (MDL):	The method detection limit (MDL) is the lowest level that the instrument can reliably differentiate from a blank or non-detect sample.
Limit of Detection (LOD):	The limit of detection (LOD) is the lowest level that can be reliably brought through the method and quantitated.
Limit of Quantitation (LOQ):	The limit of quantitation (LOQ) is the lowest level of fortification tested of an analyte in the matrix, before extraction, and is determined by the proposed tolerance.

2. MATERIALS AND METHODS

2.1 Test Systems

The test system considered in this study was surface water and surface water extract. The surface water was characterized at AGVISE Laboratories (604 Highway 15 West, Northwood, ND 58267). A copy of the characterization data is provided in the Appendix A.

The matrix effects were evaluated in the validation of method D1724/01 (**Reference 1**). No significant (≥20%) matrix effects were shown for either water matrix. Surface water was chosen as the matrix to use when conducting the MDL and LOD evaluation.

Each analysis set was uniquely identified with a Master Sheet Number, which consisted of the study number plus a unique number (e.g., 784160_1-01). The test system samples were assigned unique numbers according to SOP 10.04.05 and these were recorded in each analytical set or “Master Sheet” [e.g. water sample 784160_1-02-01, from Master Sheet No. 784160_1-02]. The actual sample numbers used for the analysis were identified in the raw data and in this final report.

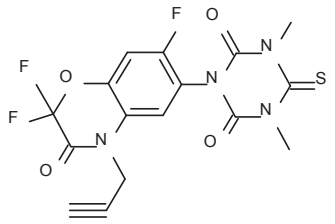
2.2 Test and Reference Substances

The test/reference standards, shown below, were synthesized by BASF Aktiengesellschaft (Limburgerhof, Germany) and were maintained at room temperature (for M850H004, refrigerator or freezer) until use in this study. BASF Aktiengesellschaft determined characterization and purity prior to the substances being used in this study. Details of these determinations are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany. BASF has retained a reserve sample of each chemical and has documentation at BASF

Corporation, BASF Crop Protection (Research Triangle Park, North Carolina, USA). The certificate of analysis for each test substance is provided in Appendix B. A detailed summary of the reference substances is presented below.

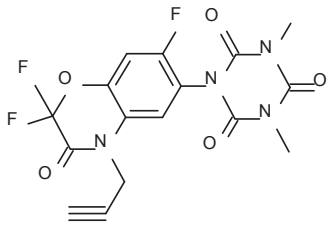
Common Name	Trifludimoxazin
BAS Code Name	BAS 850 H
IUPAC Name	1,5-dimethyl-6-thioxo-3-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5654329
Molecular Formula	$C_{16}H_{11}F_3N_4O_4S$
Molecular Weight	412.3
Lot No.	L84-130
Purity:	99.2%
Expiration Date	February 01, 2020

Chemical structure:



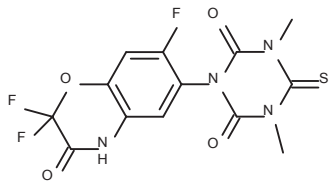
Common Name	None
BAS Code Name	M850H001
IUPAC Name	1,3-dimethyl-5-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane 2,4,6-trione
BASF Reg. No.	5749359
Molecular Formula	$C_{16}H_{11}F_3N_4O_5$
Molecular Weight	396.3
Lot No.	L85-52
Purity:	98.7%
Expiration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

Chemical structure:



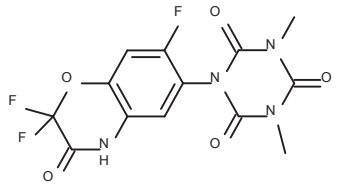
Common Name	None
BAS Code Name	M850H002
IUPAC Name	1,5-dimethyl-6-thioxo-3- (2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5757725
Molecular Formula	$C_{13}H_9F_3N_4O_4S$
Molecular Weight	374.3
Lot No.	L84-162
Purity:	96.8%
Expiration Date	February 01, 2020

Chemical structure:



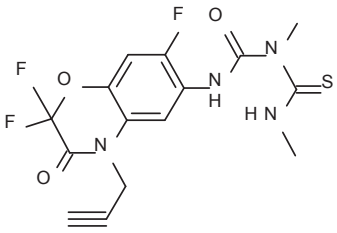
Common Name	None
BAS Code Name	M850H003
IUPAC Name	1,3-dimethyl-5-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4,6-trione
BASF Reg. No.	5757726
Molecular Formula	$C_{13}H_9F_3N_4O_5$
Molecular Weight	358.2
Lot No.	L85-70
Purity:	99.4%
Expiration Date	April 01, 2018

Chemical structure:



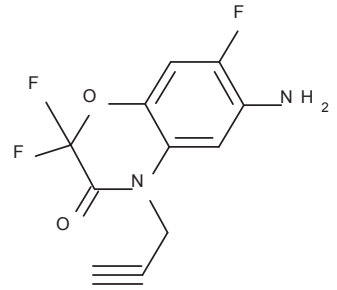
Common Name	None
BAS Code Name	M850H004
IUPAC Name	N,N-dimethyl-N'-[2,2,7-trifluroro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl] dicarbonimidothioicdiamide
BASF Reg. No.	5833884
Molecular Formula	$C_{15}H_{13}F_3N_4O_3S$
Molecular Weight	386.4
Lot No.	L85-50
Purity:	99.5%
Expiration Date	April 01, 2018

Chemical structure:



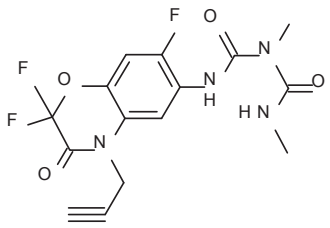
Common Name	None
BAS Code Name	M850H012
IUPAC Name	6-amino-2,2,7-trifluoro-4-(prop-2-yn-1-yl)-2H-1,4-benzoxazin-3(4H)-one
BASF Reg. No.	5797901
Molecular Formula	$C_{11}H_7F_3N_2O_2$
Molecular Weight	256.2
Lot No.	L85-66
Purity:	98.9%
Expiration Date	September 01, 2018

Chemical structure:



Common Name	None
BAS Code Name	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
Molecular Formula	$C_{15}H_{13}F_3N_4O_4$
Molecular Weight	370.3
Lot No.	L2017-007
Purity:	100.0%
Expiration Date	February 01, 2019

Chemical structure:



The test/reference items in solution were used in the study to generate data for both instrument and method performance.

2.3 Route of Administration

In this study, the test substances were applied to the test system as analytical standard solutions by micropipette to ensure precise delivery of a small amount of the test items.

3. METHODOLOGY TO EVALUATE MDL and LOD

3.1 Method Synopsis

For the analysis of all analytes except M850H001, a 1 mL aliquot of the water sample is diluted with 0.25 mL of methanol containing 0.5% formic acid, filtered (0.45 μm PTFE) and analyzed by LC-MS/MS.

For M850H001, a 5 mL aliquot of the water sample is acidified, partitioned with a mixture of cyclohexane-ethyl acetate (90:10 v/v). An aliquot of the organic layer (80%) is evaporated to dryness at 50 °C under nitrogen. Residues are re-dissolved in methanol-water with 0.1% formic acid (20:80 v/v) for analysis by LC-MS/MS.

The storage stabilities of the extracts and final volume were established in the method validation (**Reference 1**).

3.2 Methodology to Determine MDL

Evaluation of LOD of BASF Analytical Method No. D1724/01 required the experimental determination of MDL as defined by 40 CFR Ch.1 Part 136 Appendix B (**Reference 2**). Method D1724/01 has one limit of quantitation (LOQ); however, it has two preparations / cleanups: one for BAS 850 H and metabolites M850H002, M850H003, M850H004, M850H012, and M850H035, and one for M850H001. Consequently, two independent LOD determinations were conducted within this study. A brief description of the methodology to determine MDL is as follows:

1. Injections of standards containing all analytes were injected using LC-MS/MS parameters from D1724/01. All transitions were monitored according to the method. The least sensitive transition of the least sensitive analyte for each preparation/cleanup was determined qualitatively through visual inspection of factors such as peak height, relative

background level, area count, etc. Once the appropriate analytes and transitions were selected, an estimation was made to what level a sample in matrix would produce a S/N of 2-10.

2. Using BASF Analytical Method No. D1724/01, for analysis of BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035, seven (7) control aliquots (1 mL) were diluted with a standard (0.25 mL, methanol with 0.1% formic acid at a concentration 5 times the desired final concentration, determined in step 1) to make the post-extraction fortified control samples for LOD determination.

   For the analysis of M850H001, seven (7) control aliquots (5 mL) were acidified (0.05 mL water with 10% formic acid). 10 mL cyclohexane-ethyl acetate (90:10, v/v) was added to each sample, mixed and centrifuged. An aliquot (8 mL) of the organic layer was evaporated to dryness and reconstituted with 0.2 mL of a standard (methanol with 0.1% formic acid at a concentration 5 times the desired final concentration, determined in step 1), 0.2 mL methanol with 0.1% formic acid, and 0.6 mL water with 0.1% formic acid to make the post-extraction fortified control samples for LOD determination.

   All samples were then filtered using a 0.45µm PTFE syringe filter directly into HPLC injection vials, passing the first approximately 0.1 - 0.2 mL to waste.

   These 14 matrix spiked samples were injected with appropriate bracketing calibration standards on the LC-MS/MS system for quantitation.

3. Using the standard curve to calculate the concentrations of the seven matrix-spiked samples, the results are put into the equation below:

$$MDL = S \times t_{(N-1,1-\infty=.99} $$

   MDL = Method detection limit
   S = Standard deviation of the matrix-spiked sample set concentrations
   $t_{(N-1,1-\infty=.99)}$ = Critical t value from a student t-test table at 99% confidence

   Acceptance criteria for MDL:
   a. The determined MDL must be seen on the instrument with S/N of ≥ 2.
   b. The concentration of the matrix-spiked samples must be no greater than 10X the determined MDL.

   If either of the above two criteria were not met, the experiment had to be repeated at a higher or lower spiking concentration, respectively, until all criteria are met. Reinjection of the samples with lower or higher injection volumes could be done in place of preparing new matrix-spiked samples.

4. A post-extraction fortified control sample at the MDL was injected on the LC-MSMS (no standard curve required) to verify that the MDL can be seen with a S/N ≥ 2.

3.3 Methodology to Determine LOD

Based on an evaluation of the susceptibility of the analyte of interest to instrument variability, LC-MS/MS drift, unexpected contamination, and untested matrix effects, the MDL was raised to an appropriate value that will mitigate the anticipated issues. This new value will be the LOD.

4. RESULTS AND DISCUSSION

4.1 Results

BASF analytical method D1724/01 has two preparations/cleanups (simple dilution for BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035; and a liquid-liquid partition / concentration for M850H001). An MDL calculation and subsequent LOD evaluation were conducted for each preparation/cleanup. The transitions (both primary and confirmatory) used for quantitation in this method were determined using product ion spectra (**Reference 1**). Results for both LOD determinations are detailed below.

BAS 850 H, M850H002, M850H003, M850H004, M850H012, and M850H035

To determine the least sensitive analyte and transition for the purpose of LOD determination, solvent based standard solutions were injected using the validated analytical LC-MS/MS method (all transitions were evaluated plus the confirmatory chromatographic technique for BAS 850 H) from method D1724/01. It was determined qualitatively that the MS/MS ion transition for M850H012 (*m/z* 257 → *m/z* 116) was the least sensitive transition and therefore the best candidate to conduct the LOD evaluation for this preparation/cleanup.

The MDL was calculated to be 0.00011 ng on-column for M850H012. To determine this value, the seven (7) control samples were run through the extraction procedure and were fortified with standard solution prior to the LC-MS/MS determination step to achieve a concentration of 0.005 ng/mL, and 0.10 mL (100 µL, 0.0005 ng on-column) of each sample was injected on the LC-MS/MS system according to method D1724/01. Calculation of MDL for M850H012 was conducted according to the table provide in *40 CFR Ch. 1 Part 136 appendix B* (**Reference 2**).

To verify this MDL, a matrix spiked control sample was intended to be injected in triplicate at the calculated MDL to verify a signal to noise ratio ≥ 2. This test was actually performed at 0.000175 ng on-column and produced a signal to noise ratio of approximately 3. As the tested concentration yielded a reasonable signal for an MDL and it meets all other acceptance criteria, the more conservative MDL of 0.000175 ng on-column is being reported. An example chromatogram of a matrix-spiked sample at MDL of 0.000175 ng can be found in Figure F.21.

Based on this calculated MDL, the LOD for M850H012 was set at 0.00048 ng on-column (i.e. 0.0048 ng/mL injected at 0.10 mL). The LOD values on-column correspond to a LOD of 6 ng/L (ppt) for M850H012 (based on the workup of the method, e.g. aliquot actor, final volume, etc.). This increase from the MDL to the LOD is to account for variability in the residue method, natural drift of the LC-MS/MS instrumentation, potential contamination issues, untested matrix effects, and potential unseen background interferences. Detailed calculations for MDL determination is shown in the following table.

Calculation of MDL for M850H012

0.005 ng/mL Matrix-Spike Replicate	Calculated Concentration (ng/mL)
1	0.00446
2	0.00526
3	0.00425
4	0.00459
5	0.00439
6	0.00434
7	0.00432
Standard Deviation (S) =	**0.000346**
N-1 =	**6**
Critical t value (t) =	**3.143**
Injection Volume (µL)	**100**
MDL (ng/mL) =	**0.0011**
MDL (ng on-column) =	**0.00011**

M850H001

To determine the least sensitive analyte and transition for the purpose of LOD determination, solvent based standard solutions were injected using the validated analytical LC-MS/MS method (all transitions were evaluated) from method D1724/01. It was determined qualitatively that the MS/MS ion transition for M850H001 (m/z 397 → m/z 141) was the least sensitive transition and therefore the best candidate to conduct the LOD evaluation for this cleanup.

The MDL was determined to be 0.00099 ng on-column for M850H001. To determine this value, the seven (7) control samples were run through the extraction procedure and were fortified with standard solution prior to the LC-MS/MS determination step to achieve a concentration of 0.025 ng/mL and 0.1 mL (100 µL, 0.0025 ng on-column) of each sample was injected on the LC-MS/MS system according to method D1724/01. Calculation of MDL for M850H001 was conducted according to the table provide in *40 CFR Ch. 1 Part 136 appendix B* (**Reference 2**).

To verify this MDL, one of the seven spiked control samples was injected in triplicate using an injection volume appropriate to inject 0.001 ng on-column. The resultant chromatogram peak had a signal to noise ratio ≥ 2. An example chromatogram of a matrix-spiked sample at MDL can be found in Figure F.22.

Based on this calculated MDL, the LOD for M850H001 was set at 0.0024 ng on-column (i.e. 0.024 ng/mL injected at 0.1 mL). The LOD values on-column correspond to a LOD of 6 ng/L (ppt) for M850H001 (based on the workup of the method, e.g. aliquot actor, final volume, etc.). This increase from the MDL to the LOD is to account for variability in the residue method, natural drift of the LC-MS/MS instrumentation, potential contamination issues, untested matrix effects, and potential unseen background interferences. Detailed calculations for MDL determination is shown in the following table.

Calculation of MDL for M850H001

0.025 ng/mL Matrix-Spike Replicate	Calculated Concentration (ng/mL)
1	0.0205
2	0.0254
3	0.0291
4	0.0290
5	0.0257
6	0.0243
7	0.0227
Standard Deviation (S) =	**0.00314**
N-1 =	**6**
Critical t value (t) =	**3.143**
Injection Volume (µL)	**100**
MDL (ng/mL)=	**0.0099**
MDL (ng on-column) =	**0.00099**

4.3 Limit of Detection (LOD)

The LOD for M850H012 was set at 0.00048 ng on-column (i.e. 0.0048 ng/mL injected at 0.10 mL). This increase from the MDL is to account for variability in the residue method, natural drift of the LC-MS/MS instrumentation, potential contamination issues, untested matrix effects, and potential background interferences.

The LOD for M850H001 was set at 0.0024 ng on-column (i.e. 0.024 ng/mL injected at 0.10 mL). This increase from the MDL is to account for variability in the residue method, natural drift of the LC-MS/MS instrumentation, potential contamination issues, untested matrix effects, and potential background interferences.

If an incurred residue sample at 6 ng/L (ppt) were brought through the analytical method (extracted, aliquoted, cleaned up, and brought to final volume), the resulting concentration of the sample would be 0.0048 ng/mL and 0.024 ng/mL for M850H012 and M850H001, respectively; and it would be at LOD.

4.4 Limit of Quantitation (LOQ)

The method limit of quantitation (LOQ) is the lowest level of fortification tested of the analyte in the matrix before extraction. It is determined by the proposed tolerance. The validated method LOQ for residues in water is 30 ng/L (ppt) for BAS 850 H and its metabolites.

4.5 Specificity/Selectivity

Quantitation of BAS 850 H and its metabolites was accomplished by LC-MS/MS, monitoring in positive and negative mode, depending on the analyte. The ion transitions for all analytes are as follows:

Analyte	Ionization Mode	Transition (*m/z*)	Primary or Confirmatory Quantitation
BAS 850 H	Positive	413 → 74	Primary and Confirmatory[1]
M850H001	Positive	397 → 141	Primary
		397 → 134	Confirmatory
M850H002	Negative	373 → 323	Primary
		373 → 193	Confirmatory
M850H003	Negative	357 → 307	Primary
		357 → 193	Confirmatory
M850H004	Positive	387 → 131	Primary
		387 → 74	Confirmatory
M850H012	Positive	257 → 163	Primary
		257 → 116	Confirmatory
M850H035	Positive	371 → 257	Primary
		371 → 163	Confirmatory

[1]Alternative chromatographic method used for confirmation

All of these transitions were considered when choosing the best candidates for the evaluation of LOD.

5. CALCULATIONS AND RAW DATA

An example calculation is included in Appendix D.

6. STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included simple descriptive statistics, such as determinations of standard deviation for the matrix-spiked samples 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® or Analyst®) 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.

All signal to noise (S/N) calculations were conducted by the following equation in Analyst®:

$$S/N = \frac{\Delta\, Height\ of\ Analyte\ Peak}{\Delta\, Height\ of\ Appropriate\ Noise\ Region}$$

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.