Title

Method of Analysis of BAS 850 H and its Relevant Metabolites in Soil with Limit of Determination (LOD) Calculation (Method D1401/02)

Guidelines Covered

U.S. EPA Ecological Effects Test Guidelines, OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation; SANCO/3029/99 rev 4. (11/07/2000)

1.0 INTRODUCTION

1.1 Background and Purpose of Study

BAS 850 H is an herbicide used in several crops. A residue analytical method (D1401/02), for the analysis of BAS 850 H and metabolites, M850H001, M850H002, M850H003 and M850H004, in soil was validated at ADPEN Laboratories, Inc., Jacksonville, Florida.

The purpose of this study is to validate BASF Analytical Method Number. D1401/02 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) and M850H004 (Reg. No. 5833884) in soil by LC-MS/MS.

1.2 Principle of the Method

A soil sample aliquot (5 g) is extracted by shaking twice with methanol-water with 0.1% formic acid (70:30, v/v).

For the analysis of BAS 850 H and its metabolite, M850H001, an aliquot (30%) from the extract is concentrated to the aqueous layer under nitrogen at 50ºC and partitioned with a mixture of cyclohexane:ethyl acetate (90:10, v/v). An aliquot of the organic layer of the resulting extract is then evaporated to dryness under nitrogen at 50 °C and reconstituted in methanol-water with 0.1% formic acid (20:80, v/v) for the LC-MS/MS determination.

For the analysis of M850H002, M850H003 and M850H004, a 1.25-mL aliquot (2.5%) from the original extract is concentrated to the aqueous layer under nitrogen at 50 ºC. The sample is diluted with water with 0.1% formic acid (0.8 mL), and followed by methanol with 0.1% formic acid (0.2 mL) for the LC-MS/MS determination.

Transitions for BAS 850 H and M850H001 were monitored in positive ion mode for primary and confirmation quantification, and transitions for M850H002, M850H003 and M850H004 were monitored in negative mode for primary and confirmation quantification. The results are calculated by direct comparison of the sample peak responses to those of external standards.

1.3 Specificity

To demonstrate the specificity of the analytical method, one additional mass transition (confirmatory) were monitored for each of the analytes simultaneous to the primary quantitation transitions as specified below.

	Mode	Quantitation (*m/z*)	Confirmation (*m/z*)
BAS 850 H	Positive	413→74	413→134
M850H001		397→114	397→141
M850H002	Negative	373→193	373→323
M850H003		357→307	357→137
M850H004		385→103	385→255

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 peak. No matrix suppression or enhancement was found to affect the analyte.

2.0 MATERIALS AND METHODS

2.1 Test Systems

The test systems considered in this study were the top 3 inches of soil from Study 411690 [Trial ID R130128 (North Dakota, clay loam), Reference 1] and Study 698742 [Trial ID R140774 (North Carolina, loamy sand), Reference 2].

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

Each analysis set was uniquely identified with an analytical set number, which consisted of a unique number (e.g., WO-16060603R). The test system samples were assigned unique lab code numbers according to ADPEN SOP 3.2 and these were recorded in each analytical set (e.g., Sample matrix: North Dakota soil, 160526001-003, from analytical set number WO-16060603R). The actual sample numbers used for the analysis were identified in the raw data and in this final report.

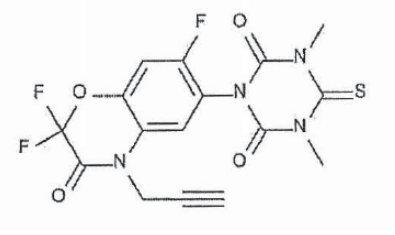
2.2 Test and Reference Substances

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

The certificates of analysis are presented in **Appendix B**. A detailed summary of the reference substances are presented below:

BASF Code Name:	BAS 850 H
BASF Registry Number:	5654329
IUPAC Name:	1,5-dimethyl-6-thloxo-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 Number:	1258836-72-4
Molecular Formula:	$C_{18}H_{11}F_3N_4O_4S$
Molecular Weight:	412.3 g/mol
Batch No.:	L84-130
Purity:	99.2%
Expiry date:	February 1, 2020

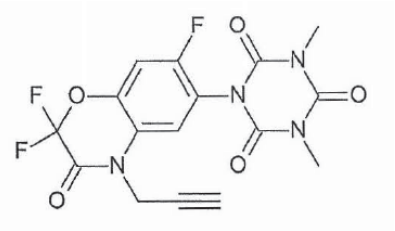
Chemical structure:



Test and Reference Substance (continued)

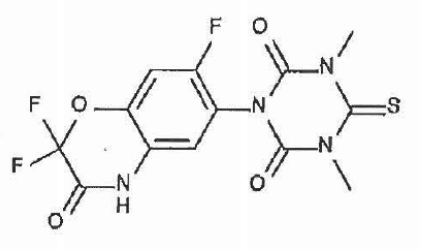
BASF Code Name:	M850H001
BASF Registry Number:	5749359
IUPAC Name:	1,3-dimethyl-5-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4,6-trione
Molecular Formula:	$C_{16}H_{11}F_3N_4O_5$
Molecular Weight:	396.3 g/mol
Batch No.:	L80-52
Purity:	98.7%
Expiry date:	April 1, 2018

Chemical structure:



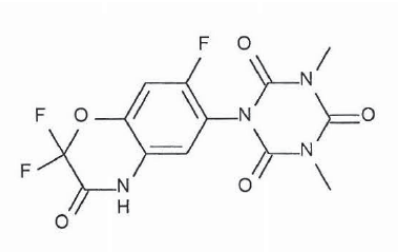
BASF Code Name:	M850H002
BASF Registry Number:	5757725
IUPAC Name:	1,5-dimelhy1,6-thioxo-3-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4-dione
Molecular Formula:	$C_{13}H_9F_3N_4O_4S$
Molecular Weight:	374.3 g/mol
Batch No.:	L84-162
Purity:	96.8%
Expiry date:	February 1, 2020

Chemical structure:



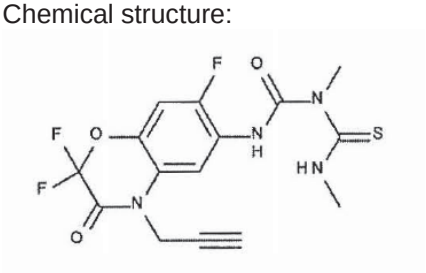
BASF Code Name:	M850H003
BASF Registry Number:	5757726
Chemistry Name:	1,3-dimethyl-5-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4,6-trione
Molecular Formula:	$C_{13}H_9F_3N_4O_5$
Molecular Weight:	358.2 g/mol
Batch No.:	L84-160; L85-70
Purity:	99.2%; 99.4%
Expiry date:	August 1, 2017; April 1, 2018

Chemical structure:



BASF Code Name:	M850H004
BASF Registry Number:	5833884
IUPAC Name:	N,N-dimethyl-N'-(2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dlhydro-2H-1,4-benzoxazin-6-yl) dicarbonimidothioicdiamlde
Molecular Formula:	$C_{15}H_{13}F_3N_4O_3S$
Molecular Weight:	386.4 g/mol
Batch No.:	L85-50
Purity:	99.5%
Expiry date:	April 1, 2018

Chemical structure:



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

2.3 Route of Administration

In this method validation study, the test items were applied to the test system as analytical standard solutions (BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in methanol with 0.1% formic acid) by micropipette to ensure precise delivery of a small amount of the test items.

3.0 ANALYTICAL METHOD

3.1 Principle of the Method

Using BASF Method D1401/02, residues of BAS 850 H and its metabolites, M850H001, M850H002, M850H003 and M850H004, in soil matrices are determined using LC-MS/MS. The working method validated in this study is provided in **Appendix C**. A brief description of the methodology as follows:

A 5 g soil sample aliquot is extracted by shaking twice with methanol:water with 0.1% formic acid (70:30, v/v). For analysis of BAS 850 H and M850H001, an aliquot (30%) from the extract is concentrated to the aqueous layer under nitrogen at 50ºC and partitioned with a mixture of cyclohexane-ethyl acetate (90:10, v/v). An aliquot of the organic layer of the resulting extract is then evaporated to dryness under nitrogen at 50 °C and reconstituted in methanol-water with 0.1% formic acid (20:80, v/v) for the LC-MS/MS determination.

For the analysis of M850H002, M850H003 and M850H004, a 1.25-mL aliquot (2.5%) from the original extract is concentrated to the aqueous layer under nitrogen at 50 ºC. The sample is diluted with water with 0.1% formic acid (0.8 mL), and followed by methanol with 0.1% formic acid (0.2 mL) for the LC-MS/MS determination.

The MRM transitions were monitored in positive mode for primary and confirmation quantification, and are shown below

Analyte	Mode	Quantitation (*m/z*)	Confirmation (*m/z*)
BAS 850 H	Positive ion	413→74	413→134
M850H001		397→114	397→141
M850H002	Negative ion	373→193	373→323
M850H003		357→307	357→137
M850H004		385→103	385→255

The results are calculated by direct comparison of the sample peak responses to those of external standards

3.2 Specificity/Selectivity

The residues of BAS 850 H are determined by LC-MS/MS, monitoring (in the positive ion mode) ion transitions at *m/z* 413 → *m/z* 74 (proposed as the primary transition for quantitation) and *m/z* 413 → *m/z* 114 (typically for confirmatory purposes).

The residues of M850H001 are determined by LC-MS/MS, monitoring (in the positive mode) ion transitions at *m/z* 397 → *m/z* 114 (proposed as the primary transition for quantitation) and *m/z* 397 → *m/z* 141 (typically for confirmatory purposes).

The residues of M850H002 are determined by LC-MS/MS, monitoring (in the negative mode) ion transitions at *m/z* 372 → *m/z* 193 (proposed as the primary transition for quantitation) and *m/z* 372 → *m/z* 323 (typically for confirmatory purposes).

The residues of M850H003 are determined by LC-MS/MS, monitoring (in the negative mode) ion transitions at *m/z* 357 → *m/z* 307 (proposed as the primary transition for quantitation) and *m/z* 357 → *m/z* 137 (typically for confirmatory purposes).

The residues of M850H004 are determined by LC-MS/MS, monitoring (in the negative mode) ion transitions at *m/z* 385 → *m/z* 103 (proposed as the primary transition for quantitation) and *m/z* 385 → *m/z* 255 (typically for confirmatory purposes).

The results are calculated by direct comparison of the sample peak responses to those of external standards. The MRM transitions used to identify BAS 850 H and its metabolites were determined by product ion spectra (**Appendix K**). As LC-MS/MS is regarded as a highly-specific detection method when two ion transitions have been validated, an additional confirmatory method or technique is not necessary.

3.3 Validation of Method

For validation, untreated soil samples were fortified with residues of BAS 850 H and metabolites, M850H001, M850H002, M850H003 and M850H004 then analyzed according to the established method validation 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 analyte at the method limit of quantitation, 0.001 mg/kg, and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 0.1 mg/kg. The example of recovery calculation is provided in **Appendix D**. The validation data including the detail analytical data for each matrix types are provided in **Appendix E**.

3.4 Influence of Matrix Effects on Analysis

In conjunction with this study, matrix-matched standards and solvent-based standards were analyzed in a separate experiment to evaluate any potential matrix effects on LC-MS/MS analysis. This involved comparing calibration standards prepared in control matrix against calibration standard solutions prepared with only solvent. The matrix-matched standards were made by adding an aliquot each of three solvent-based standards with control soil matrix to the desired matrix-matched standards concentration. Each set of matrix-matched standards (for each soil matrix) was bracketed by a block of calibration standards and was to have an additional single injection of each of the tested standard levels occur during the run. Only the

standards which immediately bracket a matrix set, and all standard injections within that matrix set, were used in calculations involving matrix effects.

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

No matrix effects were noticed during these experiments for any analyte/matrix/ion transition; therefore, validation samples were quantitated against calibration standard solutions prepared in solvent (methanol-water with 0.1% formic acid (20:80, v/v)).

Control samples were treated in exactly the same way as fortified samples. All results obtained from measurements of control samples were below LOD (0.0002 ppm).

To test the repeatability of the method, the sample replicates were divided into the following sets of analysis: one reagent blank, two control samples, five fortifications at both the LOQ and 10× LOQ for soil samples from sites in North Dakota (clay loam; Trial ID R130128) and North Carolina (loamy sand Trial ID R140774).

Primary and confirmatory mass transitions (m/z) for BAS 850 H and its metabolites, M850H001, M850H002, M850H003 and M850H004, were analyzed using LC-MS/MS.

4.2 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 all matrices (for both transitions) demonstrated that the matrix load in the samples from soil 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 two standard concentration levels, were less than 20%.

4.3 Storage Stability of Standard Solutions

Storage stability of stock, fortification standard and calibration solutions of BAS 850 H and M850H001 was not evaluated during the method validation study and was generated within the BASF freezer storage stability study (Reference 3).

Stock and intermediate (fortification) standards solutions of BAS 850 H and its metabolites were prepared in methanol with 0.1% formic acid and exhibited stability up to 235 days for BAS 850 H and M850H001 (Reference 3), and 81 days for M850H002, M850H003 and M850H004.

The calibration solutions of BAS 850 H and its metabolites were prepared every month by serial dilution of the fortification standards solutions of BAS 850 H and its metabolites with methanol-water with 0.1% formic acid (20:80, v/v). The calibration solutions showed stability up to 83 days for BAS 850 H and M850H001 (Reference 3), 81 days for M850H002 and M850H003, and 61 days for M850H004.

During the course of this study, the test/reference substance solutions were stored in a refrigerator at an average temperature of 3°C and all solutions were used within the demonstrated time period of stability.

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

Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

4.4 Storage Stability of Extracts

Extract stability was not evaluated during method validation study. Extract stability data was generated within the BASF freezer storage stability study (Reference 4) using various soil types from terrestrial dissipation studies (References 1, 2 and 5), including the soil types used in the validation study, except the loamy sand soil (Washington site, Study 698741, Trial ID R130352; Reference 5), was not from the same location (North Carolina site, Trial ID R140774, Study 698742, Reference 2) that was used in the validation study. However, the soil types in both Washington and North Carolina were characterized as loamy sand soil type.

To establish stability, reserved initial extracts and final volume extracts from a control sample and two recovery samples spiked at 0.01 ppm that had been stored in refrigerator were prepared at the final volume stage and analyzed according to the method. Quantification of each analyte in the stored samples was performed using only primary ion transition for quantitation. The results showed that the five analytes are stable in initial and final extracts for at least the time period tested, which was 22-23 days.

Extract stability in the combined extract solution (methanol-water with 0.1% formic acid, 70:30, v/v) and final volume solution (methanol-water with 0.1% formic acid, 20:80, v/v) was established in soil (clay loam and sandy loam) for BAS 850 H and its metabolites and proven to be stable for 22-23 days. This is sufficient to support the storage intervals and conditions incurred by sample extracts in the subject study.

4.5 Solution Verification

The concentrations of the fortification standards used to spike the method validation recovery samples were verified by LC/MS/MS prior to their use in this study. This was achieved by analyzing the fortification standard solutions against calibration standards prepared from the same stock solution in separate dilution series. The results - analyte concentrations in the tested standard solutions were within ±20% of the targeted concentration - together with the results from the successful method validation, indicate that the fortification standards used for the definitive method validation experiment were accurately prepared.

6.0 STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included simple descriptive statistics, such as determinations of averages, standard deviation and/or relative standard deviation (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 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 secured (i.e. locked) storage with limited access. Freezer temperatures were continuously monitored by electronic means.

7.0 SUMMARY OF METHOD

Summaries of the method parameters and characteristics are provided in Section 9 on pages 24-25.

8.0 COMMENTS FROM INDEPENDENT LABORATORY VALIDATION

This ILV was successfully completed in the first trial at EPL Bio Analytical Services (Reference 6). Recovery results and statistical data demonstrate BASF Analytical Method D1401/02 can be performed successfully for quantitation of BAS 850 H (Reg. No. 5654329) and its four metabolites M850H001 (Reg. No. 5749359), M850H002 (Reg. No. 5757725), M850H003 (Reg. No. 5757726), and M850H004 (Reg. No. 5833884) in soil.

Due to the low signals of the confirmatory transitions for M850H003 m/z 357 → 137) and M850H004 (m/z 385 → 255), the 10 µL injection volume was not suitable for quantitation.

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

9.0 DISCUSSION

Linearity	Good linearity (r > 0.99) was observed in the range of 0.25 to 10 ng/mL (nominal) for BAS 850 H and M850H001 and in the range of 0.025 to 1.25 ng/mL (nominal) for M850H002, M850H003 and M850H004.

Specificity: The method determines residues of BAS 850 H, and its metabolites, M850H001, M850H002, M850H003 and M850H004.

Analytes and selected mass transitions (m/z)

Analyte	Mode	Quantitation (*m/z*)	Confirmation (*m/z*)
BAS 850 H	Positive ion	413→74	413→134
M850H001		397→114	397→141
M850H002	Negative ion	373→193	373→323
M850H003		357→307	357→137
M850H004		385→103	385→255

Limit of Quantification and Limit of Detection: The limit of quantification (LOQ) was defined by the lowest fortification level successfully tested. The LOQ for all five analytes (BAS 850 H, M850H001, M850H002, M850H003 and M850 H004) in soil was 0.001 mg/kg (ppm). The LOD for each analyte in soil was set at 0.0002 mg/kg, which was 20% of the defined LOQ. The LOD for each analyte in soil was shown to be detectable as the absolute amount of analyte injected (0.025 ng for positive and 0.0025 ng for negative) into the LC-MS/MS when the lowest calibration standard was analyze (0.25 ng/mL for both positive and negative) with acceptable signal to noise ratio (S/N is >3:1)

Repeatability: The overall relative standard deviations (RSD,%) for all fortification levels were below 20.

Reproducibility: Reproducibility of the method was not determined within this validation study.

Levels of Fortification: 0.001 mg/kg (ppm) and 0.01 mg/kg

Time Required: A set of 13 samples requires about 1.5 working days (12 hours) of work to complete the procedure.

Confirmatory Technique: For BAS 850 H and its metabolites, M850H001, M850H002, M850H003 and M850H004, secondary MRM transitions were used for confirmation in addition to the primary MRM transitions as identified below.

BAS 850 H: *m/z* 413 → *m/z* 134
M850H001: *m/z* 397 → *m/z* 141
M850H002: *m/z* 373 → *m/z* 323
M850H003: *m/z* 357 → *m/z* 137
M850H004: *m/z* 385 → *m/z* 255

It could be demonstrated that the method D1401/02 fulfills the requirements with regard to specificity, repeatability, limit of quantification, and recoveries and is; therefore, applicable to correctly determine residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in soil.

BASF
The Chemical Company

Technical Procedure:

Analytical Method for the Determination of Residues of BAS 850 H and its four metabolites, M850H001, M850H002, M850H003 and M850H004 in Soil by LC-MS/MS

BASF Method Number:

D1401/02

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).

1.0 INTRODUCTION

BAS 850 H is a PPO herbicide, and is developed by BASF to be used for broad a spectrum of crops in US. For registration of this herbicide and for establishing the DT50/90 values from field dissipation studies for these use patterns, a residue analytical method D1401/02 for the active ingredient and its metabolites in soil is developed by BASF.

BASF Method Number D1401/02 was successfully tested during method development in various soil types.

The method has a limit of quantitation of 0.001 mg/kg in soil for BAS 850 H and its metabolites, M850H001, M850H002, M850H003 and M850H004 with the limit of detection of 0.0002 mg/kg. All analytes are determined individually.

2.0 MATERIALS

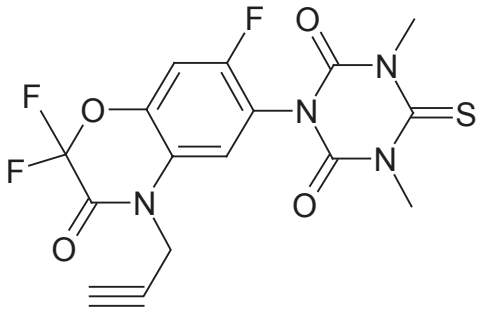
2.1 Safety

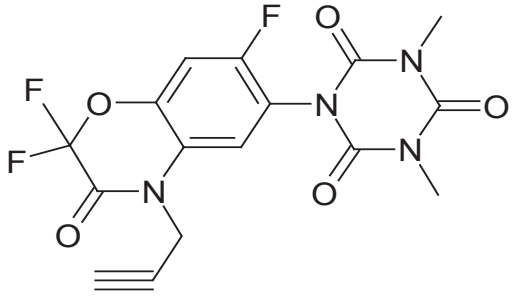
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Ensure that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

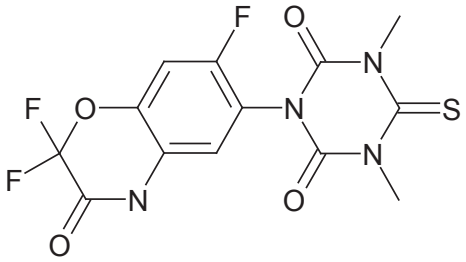
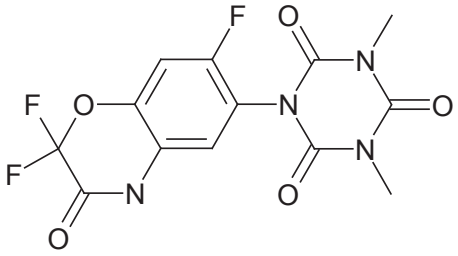
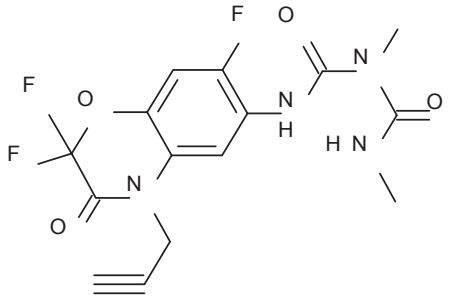
Standard substances are stored in a freezer (≤-5 °C) until use.

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.

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
Code No.:	BAS 850 H
BASF Reg. No.:	5654329
Molecular Formula:	$C_{16}H_{11}F_3N_4O_4S$
Molecular Weight:	412.3
Molecular Structure:	

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
Code No.:	M850H001
BASF Reg. No.:	5749359
Molecular Formula:	$C_{16}H_{11}F_3N_4O_5$
Molecular Weight:	396.3
Molecular Structure:	

2.2 Test and Reference Items continued)

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
Code No.:	M850H002
BASF Reg. No.:	5757725
Molecular Formula:	$C_{13}H_9F_3N_4O_4S$
Molecular Weight:	374.3
Molecular Structure:	
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
Code No.:	M850H003
BASF Reg. No.:	5757726
Molecular Formula:	$C_{13}H_9F_3N_4O_5$
Molecular Weight:	358.2
Molecular Structure:	
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
Code No.:	M850H004
BASF Reg. No.:	5833884
Molecular Formula:	$C_{15}H_{13}F_3N_4O_3S$
Molecular Weight:	386.4
Molecular Structure:	

2.2.1 Reference Standards (used for calibration

Same as fortification compounds.

2.3 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Top Loader	Model PJ3600	Mettler DeltaRange	----
Balance, Analytical	Model AT100	Mettler	----
Beakers	Various sizes	PYREX Brand, VWR Scientific Products	13922-029
Bottle, Amber glass	Qorpak , 2 oz and 4 oz with Teflon®-lined screw cap	VWR Scientific Products Boston Round, Amber	89042-908
Centrifuge	Allegra 6	Bechman Coulter	----
Centrifuge Tubes Teflon®)	40 mL	VWR	21009-477
Culture Tube caps	PTFE Lined Cap	Various	----
Culture Tube caps	16 mm	VWR	60828-768
Culture Tube, Graduated	10 mL	Various	----
Culture Tubes	Glass, disposable, 16x100mm size	Fisher	14-961-29
Cylinder, Graduated	Various sizes	Various	----
Flask, Erlenmeyer, 24/40	1000 mL	Various	----
Glass Centrifuge Tubes	50 mL	VWR	8422-50
HPLC Column	Acquity BEH C18 1.7 µm 2.1x100 mm	Waters	186002352
HPLC Column	X Brigde C18 2.5 µm 4.6x50 mm	Waters	186003090
HPLC-MS/MS	AB Sciex 5500 Mass Spectrometer	AB Sciex	--
Mechanical shaker	KS501 Digital	IKA Labortechnik	----
Nitrogen evaporator	N-EVAP 112	Organomation Associates, Inc.	----
Repeater Pipette	1000 µL 250 µL 25 µL	Gilson Microman Fisher Scientific	F148506G
Volumetric, pipettes	0.5 mL, 1 mL, 2.5 mL, 5 mL, 10mL, 20 mL	Fisher Scientific – Class A	13-650-2A
Vortex	Genie 2	VWR Scientific Products	14216-184

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

2.4 Reagents

2.4.1 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Acetonitrile	HPLC Grade	EMD	AX0145-P1
Cyclohexane	GR Grade	EMD	CX2290-3
Ethly Acetate	HPLC Grade	EMD	EX0245-1
Formic acid	98% GR ACS	EMD	FX0440-7
Methanol	HPLC Grade	EMD	MX0475-P1
Water	HPLC Grade	BDH ARISTAR PLUS	87003-652

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

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Solvent	S1	Methanol with 0.1% Formic Acid Add 1 mL of formic acid to 1000 mL of methanol into a, e.g., 1L Erlenmeyer flask 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 a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Extraction Solvent	S3	Methanol-Water mixture with 0.1% Formic Acid 70:30,v/v Add 700 mL of S1 to 300 mL of S2 into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Final Volume Dilution 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 a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Clean up Solvent	S5	Cyclohexane-ethyl acetate mixture 90:10, v/v) Add 450 mL of Cyclohexane and 50 mL of Ethyl Acetate into a 500 mL flask and mix well to ensure complete homogenous solution.
Mobile Phase A	LC1	1% Formic Acid in Water Add 990 mL of water to 10 mL of concentrated formic acid into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Mobile Phase B Method A)	LC2	0.1% Formic Acid in Methanol Add 999 mL of methanol, to 1 mL of concentrated formic acid into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Mobile Phase B Method B)	LC2	0.1% Formic Acid in Acetonitrile Add 999 mL of acetonitrile, to 1 mL of concentrated formic acid into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

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

2.4.2.1 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 and M850H004)

Prepare a stock solution containing 1 mg/mL by weighing an appropriate amount of each reference item or standard into a volumetric flask and adding the required volume of **S1**.

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 .

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:

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

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

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

Fortification Solutions Preparation

Note:	Reference standard M850H004 contains an impurity of BAS 850 H. The amount may vary in different batches. Therefore, two separate sets of calibration and fortification solutions are prepared for these analytes

Mixed Fortification Solutions BAS 850 H, M850H001)

Prepare a 10, 1 and 0.1 μg/mL fortification solution using the scheme in table below. Combine all stock solutions together to make fortifications with all analytes. Dilute volumetrically with appropriate solvents as described in the table below and ensure a complete homogeneous solution e.g. 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	0.50	50	10.0
10.0	5	50	1.0
1	5	50	0.10

Note:	A different concentration scheme may be used, if other fortification levels are needed for the analysis. If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

Mixed Fortification Solutions M850H002, M850H003 and M850H004)

Prepare a 10, 1 and 0.1 μg/mL fortification solution using the scheme in table below. Combine all stock solutions together to make fortifications with all analytes. Dilute volumetrically with appropriate solvents as described in the table below and ensure a complete homogeneous solution e.g. 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	0.50	50	10.0
10.0	5	50	1.0
1	5	50	0.10

Note: A different concentration scheme may be used, if other fortification levels are needed for the analysis.

If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

Calibration Standard Solutions BAS 850 H, M850H001)

Prepare mixed standard calibration solutions for LC-MS/MS analysis, in flasks, by using the solutions that were prepared in Section "Mixed Fortification Solutions BAS 850 H, M850H001)". Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution e.g. by vortexing .

Preparation of Mixed Standard Solutions for Calibration

Take solution ng/mL)	Volume mL)	Dilute with S4 to a final volume of mL)	Concentration ng/mL)
100	2.5	25	10.0
100	1.25	25	5.0
100	0.625	25	2.5
10	3.125	25	1.25
5	2.5	25	0.5
5	1.25	25	0.25

Note: A different concentration scheme may be used, if other fortification levels are needed for the analysis.

If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

Calibration Standard Solutions (M850H002, M850H003 and M850H004

Prepare mixed standard calibration solutions for LC-MS/MS analysis, in flasks, by using the solutions that were prepared in Section "Mixed Fortification Solutions M850H002, M850H003 and M850H004)". Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution e.g. by vortexing).

Preparation of Mixed Standard Solutions for Calibration

Take solution ng/mL)	Volume mL)	Dilute with S4 to a final volume of mL)	Concentration ng/mL)
100	2.5	25	10.0
100	1.25	25	5.0
100	0.625	25	2.5
10	3.125	25	1.25
5	2.5	25	0.5
5	1.25	25	0.25
2.5	0.8	20	0.1
1.25	0.8	20	0.05
0.5	1.0	20	0.025

Note: A different concentration scheme may be used, if other fortification levels are needed for the analysis.

If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

Additional Information:

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

2.4.3 Stability of Standard Solutions

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

Results during method development demonstrated that stock solutions of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in methanol with 0.1% formic acid were stable less than 10% decline) for **90** days when stored refrigerated.

Results during method development demonstrated that calibration solutions of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in methanol-water with 0.1 formic acid 20:80 v/v were stable less than 10% decline) for 30 days when stored refrigerated.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

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

3.2 Sample Storage

Soil samples are to be kept frozen until analysis. Freezer storage stability on soil for the BAS 850 H and metabolites will be determined in another study.

3.3 Weighing and Fortification

For control and treated samples, weigh 5 ± 0.1 g of soil sample into a 50 mL Teflon centrifuge tube.

For fortified samples, weigh 5 ± 0.1 g of control sample into a 50 mL Teflon centrifuge tube and add fortification solutions according to the table below and then proceed to section 3.4.

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	5 g	-	-	0.00 µg/g
Fortification (LOQ)*	5 g	0.1 µg/mL	0.05 mL	0.001 µg/g
Fortification (10xLOQ)	5 g	1.0 µg/mL	0.05 mL	0.01 µg/g
Fortification (100xLOQ)	5 g	10 µg/mL	0.05 mL	0.1 µg/g
Treated	5 g	-	-	-

Limit of quantification

3.4 Extraction BAS 850 H, M850H001, M850H002, M850H003 and M850H004)

Add exactly 25 mL of **S3** to the pre-weighed sample from Section 3.3. Shake the sample using a mechanical shaker, at about 300 rpm, for approximately 45 minutes and centrifuge for about 5 min at approximately 4000 rpm. Decant the supernatant into a 50 mL glass or Teflon centrifuge tube.

Add exactly another 25 mL of extraction solvent **S3** to the soil marc, vortex to dislodge the soil and shake the sample using a mechanical shaker, at about 300 rpm, for approximately 45 minutes followed by centrifuging for 5 minutes at approximately 4000 rpm. Decant the supernatant into the 50 mL centrifuge tube that already contains the first extract. Vortex the combined extract thoroughly and proceed to Section 3.5 for sample clean-up.

Note: Only Teflon or glass equipment should be used for analysis of BAS 850 H and its metabolites. Plastics have been confirmed to cause interference and suppression on LC-MS/MS.

3.5 Sample Clean-up BAS 850 H, M850H001)

a) Transfer exactly 15 mL of extract from Section 3.4 30% aliquot) into a glass culture tube.

NOTE: First aliquot 4.2 mL of the extract into the tube and mark the level, then proceed with the remaining 10.8 mL.

b) Dry it down to at least the 4.2 mL mark under nitrogen at 50 °C removing all methanol.
c Add 12 mL of **S5** to the tube.
d Secure sample with green Teflon-lined screw cap and then vortex 2 min. and centrifuge (if necessary at ~2000 rpm for 5 min.
e) Remove exactly 10 mL of the top organic layer into a clean culture and go to dryness under nitrogen at 50°C.
f Proceed to section **3.7** to prepare the sample for measurement on the LC-MS/MS.

3.6 Sample Clean-up M850H002, M850H003 and M850H004)

a Transfer exactly 1.25 mL of extract from Section 3.4 2.5% aliquot) into a glass culture tube.
b Dry down to ~0.3 mL water layer only under nitrogen at 50°C.
c) Proceed to section **3.8** to prepare the sample for measurement on the LC-MS/MS.

3.7 Preparation for Measurement BAS 850 H, M850H001)

Add exactly 0.2 mL of **S1** to the samples from section **3.5**. Vortex and then sonicate for 2 minutes to dissolve the residue at the bottom and from the side of the glass culture tube. Add exactly 0.8 mL of **S2.** Vortex thoroughly to ensure a homogenous solution. Transfer the sample to an LC-MS/MS vial for analysis.

3.8 Preparation for Measurement M850H002, M850H003 and M850H004)

Add **S2** to the 0.8 mL mark to the samples from Section 3.6. Vortex and then sonicate to dissolve the residue at the bottom and from the side of the glass culture tube. Add **S1** to the 1 mL mark. Vortex and sonicate thoroughly to ensure a homogenous solution. Transfer the sample to an LC-MS/MS vial for analysis.

Note:	Do not sonicate samples for more than 1 minute at a time without resting 1 minute to avoid heat in the samples after addition of methanol with 0.1% Formic Acid S1). Heat produced from over sonication can cause evaporation of methanol and consequently can affect recoveries

For control, untreated and LOQ fortifications, samples are ready for the analysis on LC-MS/MS.

In case of residues higher than the LOQ level, dilute the samples with **S4** as needed to fit into the calibration curve.

See Section 4.2 for LC-MS/MS conditions.

Note:	Method could be interrupted at this point

3.9 Influence of Matrix Effects on Analysis

Depending on the soil type, 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.

3.10 Stability of Extracts and Final Volumes

No stability for extracts and final volume samples has yet been investigated. Procedural recoveries can be used to prove the stability over a longer time interval, if necessary. Stabilities will be determined during the method validation.

3.11 Moisture Determination

The procedural recoveries will not be corrected for moisture content of the sample. Results of soil analysis are reported on a “dry weight” basis for residue determination. Therefore field treated soil sample weights must be corrected for moisture content by any method the laboratory customarily uses. The moisture determination will be conducted for the treated samples with residue value above LOD.

An example of a moisture determination procedure is provided below:

The percent moisture is determined using automated moisture determination equipment e.g. Mettler Toledo HR83) or any other standard process using the formula below:

$$\text{Moisture content [\%]} \quad \frac{\text{Weight moist soil - Weight dry soil}}{\text{Weight moist soil}} \times 100$$

The dry residue ppm is then calculated in an excel sheet using the following formula:

$$\text{Dry Residue ppm} \quad \frac{\text{Wet Sample Residue ppm}}{\text{100 – Percent Moisture) / 100}}$$

4.0 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 M850H001)

Method A HPLC Mode): Used for Method Validation

	Parameter		
Chromatographic System	Agilent 1290 system		
Analytical-Column	Waters Xbridge C18 2.5 µm 4.6x50mm		
Column Temperature	50 °C		
Injection Volume	100 µL can be lower if sensitivity allows		
Mobile Phase A Mobile Phase B	Water 1.0% formic acid Acetonitrile 0.1% formic acid		
Flow Rate	800 µL/min		
Gradient including wash and equilibration)	Time min)	Phase A	Phase B
	0.00 0.05 6.00 8.00 8.45 8.50 10.00	70 70 50 1 1 70 70	30 30 50 99 99 30 30
Detection System	AB Sciex 5500 Mass Spectrometer		
Ionization	Electrospray ESI)		
Ionization Temperature	550 °C		
Analyte	**Transitions *(m/z)***	**Polarity**	**Expected Retention Time**
BAS 850 H	413 → 74 413 → 134	positive	~ 7.1 min
M850H001	397 → 114* 397 → 141	positive	~ 4.48 min

System pressure is approximately 2000 PSI using this method
*Proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time

4.2.1 Instrumentation and Conditions (BAS 850 H M850H001) continued

Method B UPLC Mode) [1]:

	Parameter		
Chromatographic System	Agilent 1290 system		
Analytical-Column	Waters Acquity UPLC BEH C18 1.7 µm 2.1x50mm		
Column Temperature	50 °C		
Injection Volume	100 µL can be lower if sensitivity allows		
Mobile Phase A Mobile Phase B	Water 1.0% formic acid MeOH 0.1% formic acid		
Flow Rate	600 µL/min		
Gradient including wash and equilibration)	Time min)	Phase A	Phase B
	0.00 0.05 5.00 6.00 7.45 7.50 8.00	70 70 50 1 1 70 70	30 30 50 99 99 30 30
Detection System	AB Sciex 5500 Mass Spectrometer		
Ionization	Electrospray ESI)		
Ionization Temperature	450 °C		
Analyte	**Transitions *(m/z)***	**Polarity**	**Expected Retention Time**
BAS 850 H	413 → 74 413 → 134	positive	~ 5.1 min
M850H001	397 → 114* 397 → 134	positive	~ 2.55 min

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

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

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

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

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

[1] Alternative method using different instrumentation parameters and conditions.

4.2.2 Instrumentation and Conditions M850H002, M850H003, M850H004)

Method C HPLC Mode): Used for Method Validation

	Parameter		
Chromatographic System	Agilent 1290 system		
Analytical-column	Waters Xbridge C18 2.5 µm 4.6x50mm		
Column Temperature	50 °C		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	Water 1.0% formic acid Acetonitrile 0.1% formic acid		
Flow Rate	800 µL/min		
Gradient including wash and equilibration)	Time min)	Phase A	Phase B
	0.00 0.05 1.90 2.50 3.45 3.50 5.00	70 70 40 1 1 70 70	30 30 60 99 99 30 30
Detection System	AB Sciex 5500 Mass Spectrometer		
Ionization	Electrospray ESI)		
Ionization Temperature	650 °C		
Analyte	**Transitions m/z)**	**Polarity**	**Expected Retention Time**
M850H002	373 → 193* 373 → 323	negative	~ 2.90 min
M850H003	357 → 307* 357 → 137	negative	~ 2.23 min
M850H004	385 → 103* 385 → 255	negative	~ 3.04 min

System pressure is approximately 2000 PSI using this method

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

4.2.2 Instrumentation and Conditions M850H002, M850H003, M850H004) continued

Method D UPLC Mode) [1]:

	Parameter		
Chromatographic System	Agilent 1290 system		
Analytical-column	Waters Acquity UPLC BEH C18 1.7 µm 2.1x50mm		
Column Temperature	50 ºC		
Injection Volume	10 µL		
Mobile Phase A Mobile Phase B	Water 1.0% formic acid MeOH 0.1% formic acid		
Flow Rate	600 µL/min		
Gradient including wash and equilibration)	Time min)	Phase A	Phase B
	0.00 0.05 0.90 1.50 2.45 2.50 3.00	70 70 40 1 1 70 70	30 30 60 99 99 30 30
Detection System	AB Sciex 5500 Mass Spectrometer		
Ionization	Electrospray ESI)		
Ionization Temperature	650 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
M850H002	373 → 323* 373 → 193	negative	~ 1.45 min
M850H003	357 → 307* 357 → 137	negative	~ 1.15 min
M850H004	385 → 103* 385 → 255	negative	~ 1.55 min

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

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

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

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

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

[1] Alternative method using different instrumentation parameters and conditions.

4.2.3 Calibration Procedures

Calculation of results is based on peak area measurements using a calibration curve. The calibration curve is obtained by direct injection of BAS 850 H and its metabolites, M850H001, M850H002, M850H003 and M850H004 standards containing a known amount of analytes in the range of 0.025 ng/mL to 10 ng/mL.

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.

4.2.4 Calculation of Residues and Recoveries

Calculation of results is based on area measurements.

For the procedural recoveries, the sample weight will be considered 5 g in the final calculation of residues [mg/kg]. The method requires that the sample weight to be 5 ± 0.1 g for fortification samples. The recovery is the percentage of the fortified amount µg or ng , which is recovered through the method and the weights cancels out, as shown in the equation below, during the final calculation step.

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

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

b) Residue [mg/kg] $\frac{V_{end} \times C_A}{G \times A_F \times 1000}$

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	Weight of the sample extracted [g]
A_F	Aliquotation factor
1000	Factor remaining after all unit conversions

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

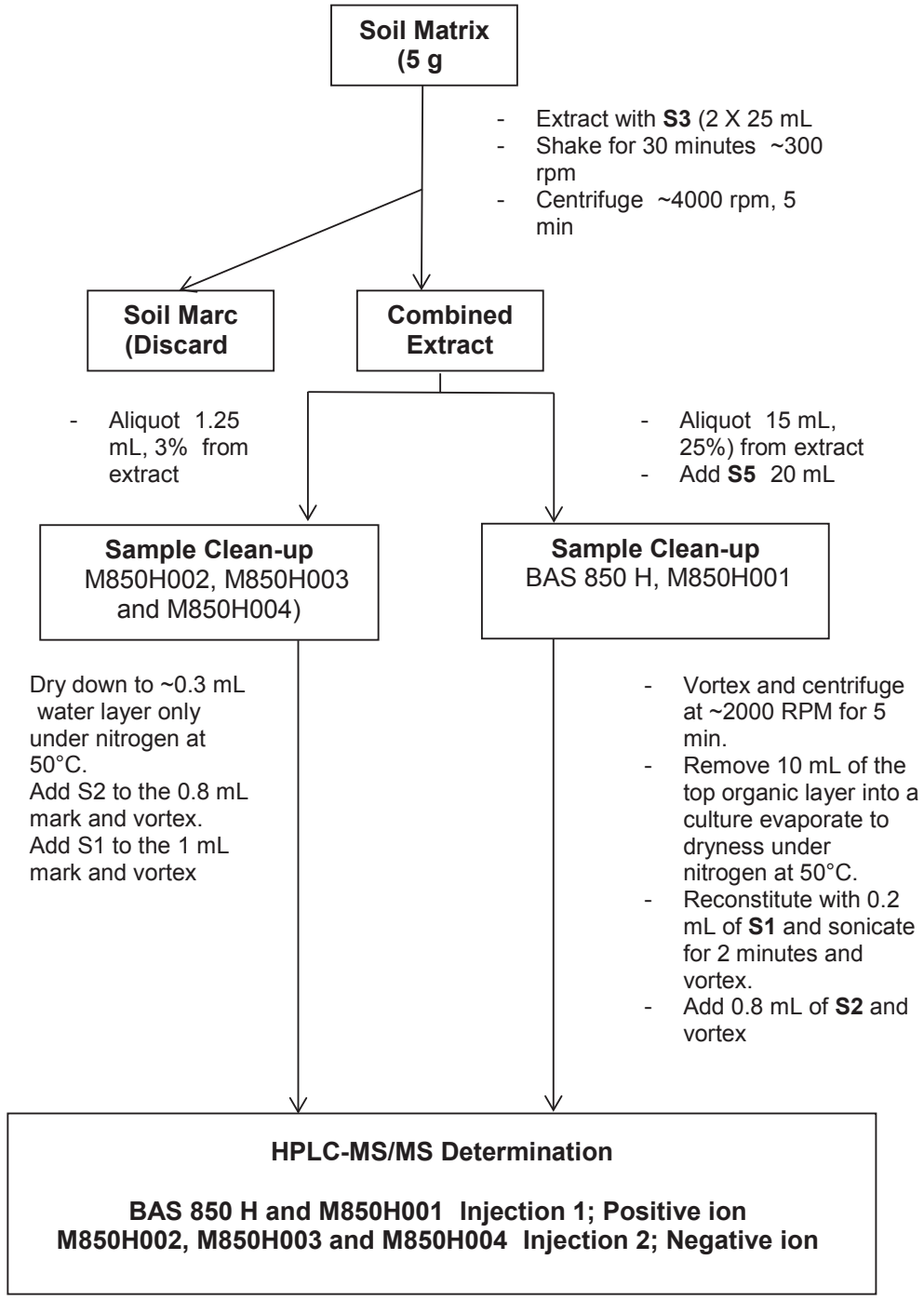
c) Recovery % $\frac{\text{Residue in fortified sample} - \text{Residue in contol}}{\text{Amount of analyte fortified}} \times 100$

Soil residues based on soil dry weight

d) Residue [mg/kg] Dry residue) $\frac{\text{Wet Sample Residue ppm}}{100 - \text{Percent Moisture}} \times 100$

5.0 FLOWCHART

5.1 Analysis of BAS 850 H and its metabolites, M850H001, M850H002, M850H003 and M850H004 in Soil



S1 Methanol with 0.1% formic acid
S2 Water with 0.1% formic acid
S3 70:30, v/v S1:S2
S5 90:10, v/v Cyclohexane: Ethyl Acetate

6.0 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 1.5 working days 12 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.

7.0 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 limit of quantification is 0.001 mg/kg for BAS 850 H and its metabolites, M850H001, M850H002, M850H003 and M850H004 with the limit of detection of 0.0002 mg/kg. All analytes are determined individually. The limit of detection was estimated at 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
Will be added later.

Confirmatory Techniques
The HPLC-MS/MS final determination is a highly selective detection technique. For every compound the quantitation is possible at two different transitions. Therefore, no additional confirmatory technique is required.

Potential Problems
The glassware used for the method should be thoroughly rinsed with acetonitrile to prevent contamination.

Plastic containers should not be used for this method. Only Teflon® or glass can be used.

It is highly recommended to perform an instrument check routinely during LC-MS/MS analysis for analyte peak enhancement or suppression. For analysis of BAS 850 H and M850H001, the instrument check sample is prepared by reconstituting the control matrix in section 3.7 with 200 µL of a 1.25 ng/mL standard prepared in methanol with 0.1% formic acid, vortex, then add 800 µL of a 1.25 ng/mL standard prepared in water with 0.1% formic acid the standard prepared in water should be made fresh the day of use), then vortex and sonicate thoroughly. For analysis of M850H002, M850H003 and M850H004, the instrument check sample is prepared by adding 12.5 µl of a 10 ng/mL calibration standard to the 0.3 mL water layer from section 3.6. Add water with 0.1% formic acid to the 0.8 mL mark, vortex, then add methanol with 0.1% formic acid to the 1 mL mark.

The compounds are also sensitive to matrix build up in the instrument. Pay close attention to the analyte response and signal to noise ratios over time. It is recommended to clean the orifice plate regularly, and more thorough cleaning of the hardware as needed, as well as a gradient system to flush the column on a routine basis.

Typical Recovery Calculation for LC-MS/MS Quantitation

Calculation of results is based on area measurements. The recoveries and residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in ppm are calculated with the following formulas:

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

Where, m = slope
b = y intercept
x = Amount found (ng)
y = Peak Area

The following equations were used within LIMS for residue and recovery calculations:

b) $\text{Amount injected (mg)} \quad \frac{\text{injection size } (\mu\text{L})}{\text{final sample vol. (mL)}} \times \text{sample wt. (g)} \times \frac{1\,\text{mL}}{1000\ \mu\text{L}} \times \frac{1000\,\text{mg}}{1\,\text{g}}$

Where,

$\text{Final sample volume (mL)} \quad \frac{\text{extract volume (mL)}}{\text{aliquot factor}} \times \text{Final Extract Volume} \times \text{Dilution Factor (DF)}$

c) $\text{Amount found (ppm)} \quad \frac{\text{ng found}}{\text{mg injected}}$

d) $\text{Percent recovery (\%)} \quad \frac{\text{Amount found (ppm)} - \text{Amount found in control (ppm)}}{\text{Amount fortified (ppm)}} \times 100$

Example: Recovery calculations of BAS 850 H (m/z 413 → 74) in Soil (R130128) fortified at LOQ level, 0.001 ppm. See WO-16060602RE.

a) Calibration curve:

$$y = (96532)x - 175.5$$

Solving for x:

$$10632 = (96532)x - 175.5$$

$$x = (10632 + 175.5)/(96532) = 0.111960 \text{ ng}$$

b) $\text{Amount of sample injected (mg)} = \frac{100\ \mu\text{L}}{4.00\ \text{mL}} \times 5\ \text{g} \times \frac{1\,\text{mL}}{1000\,\mu\text{L}} \times \frac{1000\,\text{mg}}{1\,\text{g}} = 125.00\ \text{mg}$

c) $\text{Amount found (ppm)} = \frac{0.111960\ \text{ng}}{125.00\ \text{mg}} = 0.00090\ \text{ppm}$

d) $\text{Recovery (\%)} = \frac{0.00090\ \text{ppm}}{0.001\ \text{ppm}} \times 100 = 90\%$

Standard solutions were refrigerated during use in this study. Stock solutions were prepared in methanol acidified with 0.1% formic acid. The subsequent dilutions of the stock standards were prepared in methanol: water (20:80, v/v) acidified with 0.1% formic acid. Fortification solutions and calibration standards were prepared from separate weighings of the stock solutions or from separate dilution series of the same stock solution.

Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

Typical analytical standards dilution and use records

Standard Number	Analyte	Standard ID	Adjusted Net Weight	Final Dilution Vol. (mL)	Final Conc.	Solvent [1]	Prep. Date	Expiry Date
Stock solutions								
C8517	BAS 850 H	P5424	5.16 mg	5.0	1031.70 ng/µL	S1	12/01/16	03/01/17
C8518	M850H001	P5619	4.92 mg	5.0	985.03 ng/µL	S1	12/01/16	03/01/17
C8519	M850H002	P5100	4.94 mg	5.0	987.4 ng/µL	S1	12/01/16	03/01/17
C8520	M850H003	P5426	4.99 mg	5.0	1000.0 ng/µL	S1	12/01/16	03/01/17
C8521	M850H004	P5374	5.04 mg	5.0	1009 ng/µL	S1	12/01/16	03/01/17
Serial dilutions – Intermediates								
Stock Mix [2]-I9129	BAS 850 H	C8517	1031.7 ng/µL	25	10 µg/mL	S1	12/02/16	03/01/17
	M850H001	C8518	985.0 ng/µL					
Stock Mix [2]-I7959	M850H002	C8519	987.4 ng/µL	25	10 µg/mL	S1	12/02/16	03/01/17
	M850H003	C8520	1000 ng/µL					
	M850H004	C8521	1009 ng/µL					

[1] S1 = MeOH with 0.10% Formic Acid
[2] Stock standard preparations prepared in a mix based upon positive or negative analytes.

Standard Number	Analyte [1]	Standard ID	Concentration (ng/ µL)	Aliquot Volume (mL)	Final Dilution Vol. (mL)	Final Conc. (ng/mL)	Solvent [2]	Prep. Date	Expiry Date
Calibration Standards (BAS 850 H and M850H001) [3]									
W13555	2-Mix	I9129	10.0	0.25	25	100.0	S4	12/05/16	01/05/17
		W13555-1	0.100	2.5		10.0			
		W13555-1	0.100	1.25		5.0			
		W13555-1	0.100	0.625		2.5			
		W13555-1	0.100	0.3125		1.25			
		W13555-2	0.010	1.25		0.50			
		W13555-2	0.010	0.625		0.25			
Calibration Standards (M850H002, M850H003 and M850H004) [3]									
W13535	3-Mix	I9130	10.0	0.25	25	100.0	S4	12/02/16	01/02/17
		W13535-1	0.100	2.5		10.0			
		W13535-2	0.010	3.125		1.25			
		W13535-2	0.010	1.25		0.50			
		W13535-2	0.010	0.625		0.25			
		W13535-2	0.010	0.25		0.010			
		W13535-3	0.00125	1.0		0.050			
		W13535-3	0.00125	0.5		0.025			

[1] BAS 850 H, M850H001, M850H002, M850H003, and M850H004
[2] S4 = (20:80) MeOH: water with 0.1% Formic Acid
[3] Two analyses sets were required for all five analytes: BAS 850 H and M850H001 (positive mode analysis) and M850H002, M850H003 and M850H004 (negative mode analysis).

The summary of the extract stability data in days of storage is presented in Table 14 and shown below:

Analytes	Matrix	Solution Tested	Limit of Demonstrated Storage Stability (days)[1]
BAS 850 H, M850H001, M850H02, M850H03 and M850H04	Clay Loam Soil, 0-6" North Dakota (Trial R130128) [2]	From extract stored in 70/30 MeOH/H_2O + 0.1% FA	23
	Loamy Sand Soil, 0-3" Washington (Trial R130352) [3]		22
	Sandy Loam Soil, 0-3" California (Trial R130353) [4]		23
BAS 850 H, M850H001, M850H02, M850H03 and M850H04	Clay Loam Soil, 0-6" North Dakota (Trial R130128) [2]	Stored in final volume solution 20/80 MeOH/H_2O + 0.1% FA	23
	Loamy Sand Soil, 0-3" Washington (Trial R130352) [3]		22
	Sandy Loam Soil, 0-3" California (Trial R130353) [4]		23

[1] Extract stability data was generated under freezer storage stability study 411640 (Reference 4).
[2] Clay loam soil from BASF study 411690, Trial R130128 (Reference 1)
[3] Same soil type as North Carolina site used in validation study; soils are from BASF study 698742, Trial R140774 (Reference 2)
[4] Sandy loam soil from BASF study 698741, Trial R130353 (Reference 5)

Appendix B:

Evaluation of the Limit of Detection (LOD) for Method D1401/02, "Analytical Method for the Determination of Residues of BAS 850 H and its Four Metabolites, M850H001, M850H002, M850H003 and M850H004 in Soil by LC-MS/MS"

BASF Study Number:
411638_01

BASF Registration Document Number:
2017/7016641

Contains 56 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 D1401/02, “Analytical Method for the Determination of Residues of BAS 850 H and its four metabolites, M850H001, M850H002, M850H003 and M850H004 in Soil 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 loamy sand soil from test site (NC, Trial R140774) of BASF study 698742 (Reference 2). The test system was characterized at AGVISE Laboratories (604 Highway 15 West, Northwood, ND 58267).

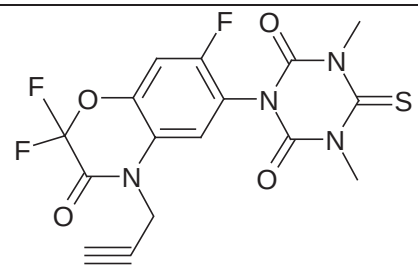
The matrix effects were evaluated in the validation of method D1401/02 (Reference 1). No significant matrix effects were shown (≥20%) for any soil matrix. A loamy sand soil from test site R140774 of BASF study 698742 (Reference 2) was chosen as the soil to use when conducting the MDL and LOD evaluation.

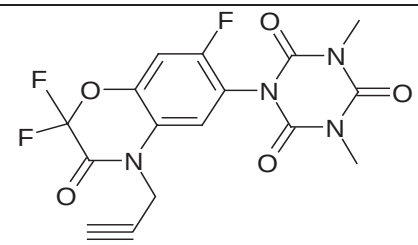
Each analysis set was uniquely identified with a Work Order Number, which consisted of the date the set was created and a unique number (e.g., WO-17072402). The test system sample was assigned unique numbers according to ADPEN SOPs and these were recorded in each analytical set or “Work Order” [e.g. soil sample WO-17072402]. The actual sample numbers used for the analysis were identified in the raw data and in this final report.

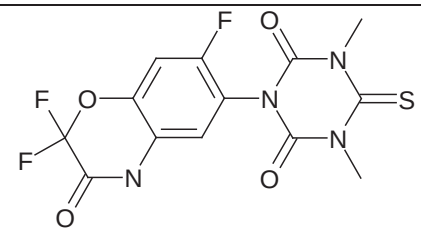
2.2 Test and Reference Substances

The test/reference standards shown below were synthesized by BASF SE (Limburgerhof, Germany) and used during the analytical portion of this study. The test/reference items were maintained frozen until use in this study. BASF SE 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 BASF Agricultural Center, Limburgerhof, Germany.

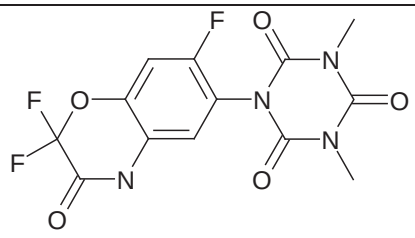
Test and Reference Substances (continued)

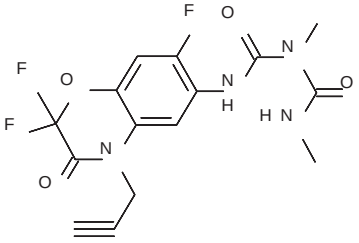
Code No.:	**BAS 850 H**	Molecular Structure:
IUPAC Name:	1,5-dimethyl-6-thioxo-3-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4-dione	
CAS Registry No.:	1258836-72-4	
BASF Reg. No.:	5654329	
Molecular Formula:	$C_{16}H_{11}F_3N_4O_4S$	
Molecular Weight: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

Code No.:	**M850H001**	Molecular Structure:
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	
CAS Registry No.:	None	
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 1, 2018	
Storage:	Freezer	

Code No.:	**M850H002**	Molecular Structure:
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	
CAS Registry No.:	None	
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	

Test and Reference Substances (continued)

Code No.:	**M850H003**	Molecular Structure:
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	
CAS Registry No.:	None	
BASF Reg. No.:	5757726	
Molecular Formula:	$C_{13}H_9F_3N_4O_5$	
Molecular Weight:	358.2 g/mol	
Batch No.:	L84-160	
Purity:	99.2 %	
Expiration Date:	August 01, 2017	
Storage:	Freezer	

Code No.:	**M850H004**	Molecular Structure:
IUPAC Name:	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	
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	

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

A soil sample aliquot (5 g) is extracted by shaking twice with methanol-water with 0.1 % formic acid (70:30, v/v).

For the analysis of BAS 850 H and metabolite M850H001, an aliquot (30%) from the extract is concentrated to the aqueous layer under nitrogen at 50 °C. The exact is then partitioned with a mixture of cyclohexane-ethyl acetate (90:10 v/v). An aliquot of the organic layer (83%) 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 the LC-MS/MS determination.

For the analysis of M850H002, M850H003 and M850H004, an aliquot (2.5%) from the original extract is concentrated to the aqueous layer under nitrogen at 50 °C. The sample is then

brought to 0.8 mL with water with 0.1% formic acid, then 0.2 mL of methanol with 0.1% formic acid is added for the LC-MS/MS determination.

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. D1401/02 required the experimental determination of MDL as defined by 40 CFR Ch.1 Part 136 Appendix B (Reference 3). Method D1401/02 has one limit of quantitation (LOQ); however, it has two injections; one for BAS 850 H (Reg. No. 5654329) and metabolite, M850H001 (Reg. No. 5749359), which is positive ion mode injection, and the other is for the metabolites, M850H002 (Reg. No. 5757725), M850H003 (Reg. No. 5757726) and M850H004 (Reg. No. 5833884)], which is for negative ion mode injection. 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 D1401/02. All transitions were monitored according to the method. The least sensitive transition of the least sensitive analyte for each method injection 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. D1401/02, seven (7) control samples (5 g) were extracted twice with methanol-water with 0.1% formic acid (70:30, v/v) and taken to the final volumes for both injections.

   An aliquot (0.99 mL) from the extract was diluted with a calibration standard (methanol-water with 0.1% formic acid (20:80, v/v, 0.01 mL) to an appropriate concentration level to make the post-extraction fortified control samples for LOD determination.

   The sample is then syringe filtered using a 0.45µm PTFE syringe filter directly into HPLC injection vials, passing the first approximately 0.2 - 0.3 mL to waste.

   These seven matrix spiked samples were injected twice on the LC-MS/MS with bracketing calibration standards 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

   The acceptance criteria for the MDL calculation was:
   a. The calculated 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 calculated 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 as it changes the amount (ng) injected on the column the same.

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

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 Method Results

BASF analytical method D1401/02 has two injection modes (positive ion mode for BAS 850 H (Reg. No. 5654329) and M850H001 (Reg. No. 5749359), and negative mode for metabolites M850H002 (Reg. No. 5757725), M850H003 (Reg. No. 5757726) and M850H004 (Reg. No. 5833884). An MDL calculation and subsequent LOD evaluation were conducted for both modes of injection. 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.

<u>BAS 850 H and M850H001 (Positive ion mode)</u>

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 D1401/02. It was determined qualitatively that the MS/MS ion transition for BAS 850 H (*m/z* 413 → *m/z* 134) was the least sensitive transition and therefore the best candidate to conduct the LOD evaluation for the positive mode.

The MDL was determined to be 0.000008 ng on-column for BAS 850 H. 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 0.0002 ng/mL, and 0.10 mL (100 µL, 0.02 ng on-column) of each sample was injected on the LC-MS/MS according to method D1401/02. Calculation of MDL for BAS 850 H was conducted according to the table provide in *40 CFR Ch. 1 Part 136 appendix B* (**Reference 3**).

Based on this calculated MDL, the LOD for BAS 850 H was set at 0.025 ng on-column (i.e. 0.25 ng/mL injected at 0.10 mL [100 µL]). 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. . Below is a table that details all the results for this injection.

Method Results (continued)

Calculation of MDL and LOD for BAS 850 H

0.0002 ng/mL Matrix-Spike Replicate	Calculated Concentration (ng/mL)
1	0.00019
2	0.00017
3	0.00023
4	0.00020
5	0.00024
6	0.00023
7	0.00019
Standard Deviation (S) =	**0.000026**
N-1 =	**6**
Critical t value (t) =	**3.143**
Injection Volume (µL)	**100**
MDL (ng/mL) =	**0.00008**
MDL (ng on-column) =	**0.000008**
LOD (ng on-column) =	**0.025**
LOD Equivalent to incurred sample at =	**0.0002 mg/kg**

M850H002, M850H003 and M850H004 (Negative ion mode)

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 D1401/02. It was determined qualitatively that the MS/MS ion transition for M850H004 (m/z 385 → m/z 255) was the least sensitive transition and therefore the best candidate to conduct the LOD evaluation for the negative mode.

The MDL was determined to be 0.000002 ng on-column for M850H004. 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 0.0002 ng/mL and 0.02 mL (20 µL, 0.004 ng on-column) of each sample was injected on the LC-MS/MS according to method D1401/02. Calculation of MDL for M850H004 was conducted according to the table provide in *40 CFR Ch. 1 Part 136 appendix B* (**Reference 3**).

Based on this calculated MDL, the LOD for M850H004 was set at 0.0005 ng on-column (i.e. 0.025 ng/mL injected at 0.02 mL [20 µL]). 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. Below is a table that details all the results for this injection.

Method Results (continued)

Calculation of MDL and LOD for M850H004

0.0002 ng/mL Matrix-Spike Replicate	Calculated Concentration (ng/mL)
1	0.00024
2	0.00017
3	0.00017
4	0.00023
5	0.00015
6	0.00023
7	0.00024
Standard Deviation (S) =	**0.000039**
N-1 =	**6**
Critical t value (t) =	**3.143**
Injection Volume (µL)	**20**
MDL (ng/mL)=	**0.00012**
MDL (ng on-column) =	**0.000002**
LOD (ng on-column) =	**0.0005**
LOD Equivalent to incurred sample at =	**0.0002 mg/kg**

4.3 Specificity/Selectivity

Quantitation of BAS 850 H and its metabolites was accomplished by LC-MS/MS, monitoring in positive mode. The ion transitions for all analytes are as follows: BAS 850 H was monitored at *m/z* 413 → *m/z* 74 (proposed as the primary transition for quantitation) and *m/z* 413 → *m/z* 134 (typically for confirmatory purposes), M850H001 was monitored at *m/z* 397 → *m/z* 114 (proposed as the primary transition for quantitation) and *m/z* 397 → *m/z* 141 (typically for confirmatory purposes), M850H002 was monitored at *m/z* 372 → *m/z* 193 (proposed as the primary transition for quantitation) and *m/z* 372 → *m/z* 323 (typically for confirmatory purposes), M850H003 was monitored at *m/z* 357 → *m/z* 307 (proposed as the primary transition for quantitation) and *m/z* 357 → *m/z* 137 (typically for confirmatory purposes), and M850H004 was monitored at *m/z* 375 → *m/z* 103 (proposed as the primary transition for quantitation) and *m/z* 385 → *m/z* 255 (typically for confirmatory purposes). 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.

7. CONCLUSIONS

Within the scope of BASF Analytical Method No. D1401/02, the MDL was successfully determined to be 0.000008 ng on-column for BAS 850 H and metabolite, M850H001, and 0.000002 ng on-column for the metabolites, M850H002, M850H003 and M850H004. After an evaluation of the analytes of interest to instrument variability, LC-MS/MS drift, unexpected contamination, and untested matrix effects, the LOD was determined to be 0.025 ng on-column for BAS 850 H and metabolite, M850H001, and 0.0005 ng on-column for the metabolites, M850H002, M850H003 and M850H004. The results of this LOD evaluation study demonstrate that BASF Analytical Method No D1401/02, fulfils the requirements with regard to the method detection limit (MDL) and limit of detection (LOD) and is; therefore, applicable to correctly determine residues of BAS 850 H and its metabolites in soil.

8. PROTOCOL, AMENDMENTS, AND DEVIATIONS

The study was conducted according to a study protocol. No changes were made to the study.

TABLE A-2. CRITICAL VALUES OF STUDENT'S-*t* DISTRIBUTION

$t_{1-\alpha}$

Degrees of Freedom	1 - α								
	0.70	0.75	0.80	0.85	0.90	0.95	0.975	0.99	0.995
1	0.727	1.000	1.376	1.963	3.078	6.314	12.706	31.821	63.657
2	0.617	0.816	1.061	1.386	1.886	2.920	4.303	6.965	9.925
3	0.584	0.765	0.978	1.250	1.638	2.353	3.182	4.541	5.841
4	0.569	0.741	0.941	1.190	1.533	2.132	2.776	3.747	4.604
5	0.559	0.727	0.920	1.156	1.476	2.015	2.571	3.365	4.032
6	0.553	0.718	0.906	1.134	1.440	1.943	2.447	3.143	3.707
7	0.549	0.711	0.896	1.119	1.415	1.895	2.365	2.998	3.499
8	0.546	0.706	0.889	1.108	1.397	1.860	2.306	2.896	3.355
9	0.543	0.703	0.883	1.100	1.383	1.833	2.262	2.821	3.250
10	0.542	0.700	0.879	1.093	1.372	1.812	2.228	2.764	3.169
11	0.540	0.697	0.876	1.088	1.363	1.796	2.201	2.718	3.106
12	0.539	0.695	0.873	1.083	1.356	1.782	2.179	2.681	3.055
13	0.538	0.694	0.870	1.079	1.350	1.771	2.160	2.650	3.012
14	0.537	0.692	0.868	1.076	1.345	1.761	2.145	2.624	2.977
15	0.536	0.691	0.866	1.074	1.34	1.753	2.131	2.602	2.947
16	0.535	0.690	0.865	1.071	1.337	1.746	2.120	2.583	2.921
17	0.534	0.689	0.863	1.069	1.333	1.740	2.110	2.567	2.898
18	0.534	0.688	0.862	1.067	1.330	1.734	2.101	2.552	2.878
19	0.533	0.688	0.861	1.066	1.328	1.729	2.093	2.539	2.861
20	0.533	0.687	0.860	1.064	1.325	1.725	2.086	2.528	2.845
21	0.532	0.686	0.859	1.063	1.323	1.721	2.080	2.518	2.831
22	0.532	0.686	0.858	1.061	1.321	1.717	2.074	2.508	2.819
23	0.532	0.685	0.858	1.060	1.319	1.714	2.069	2.500	2.807
24	0.531	0.685	0.857	1.059	1.318	1.711	2.064	2.492	2.797
25	0.531	0.684	0.856	1.058	1.316	1.708	2.060	2.485	2.787
26	0.531	0.684	0.856	1.058	1.315	1.706	2.056	2.479	2.779
27	0.531	0.684	0.855	1.057	1.314	1.703	2.052	2.473	2.771
28	0.530	0.683	0.855	1.056	1.313	1.701	2.048	2.467	2.763
29	0.530	0.683	0.854	1.055	1.311	1.699	2.045	2.462	2.756
30	0.530	0.683	0.854	1.055	1.310	1.697	2.042	2.457	2.750
40	0.529	0.681	0.851	1.050	1.303	1.684	2.021	2.423	2.704
60	0.527	0.679	0.848	1.046	1.296	1.671	2.000	2.390	2.660
120	0.526	0.677	0.845	1.041	1.289	1.658	1.980	2.358	2.617
∞	0.524	0.674	0.842	1.036	1.282	1.645	1.960	2.326	2.576

Note: The last row of the table (∞ degrees of freedom) gives the critical values for a standard normal distribution (Z), e.g., $t_{\infty, 0.95} = z_{0.95} = 1.645$.

Example Calculation (BAS 850 H)

Method Detection Limit (MDL)

The MDL is the lowest limit the instrument can reliably distinguish from a blank of the least sensitive analyte/transition after extraction, preparation for analysis, and injection on LC-MS/MS.

The calculation is based on the methodology described in 40 CFR Ch 1, part 136 appendix B (Reference 2). Seven (7) unique control samples were brought through the method and fortified at the end of the procedure to have a concentration of 0.0002 ng/mL. These samples have a representative amount of matrix, indicative of an incurred residue sample at the same concentration. The samples were injected along with a bracketing standard curve at an injection volume of 100 µL for the positive injection and 10 µL for the negative injection.

The MDL was calculated to be 0.000008 ng on-column using the following equations:

Equation 1: $\mathrm{MDL\ (ng/mL)} = \mathrm{S} \times \mathrm{t}_{(\mathrm{N-1,1-\infty=.99})}$

S = standard deviation of the 7 matrix spiked samples
$\mathrm{t}_{(\mathrm{N-1,1-\infty=.99})}$ = critical t value from student t-test table (Appendix C)

Equation 2: $\mathrm{MDL\ on\text{-}column\ (ng)} = \mathrm{MDL\ (ng/mL)} \times \mathrm{Injection\ Volume\ (mL)}$

Equation 3: $\mathrm{Concentration\ (ng/mL)} = \frac{\mathrm{Response-Intercept}}{\mathrm{Slope}}$

Below are the calculated concentrations of the 7 samples and the corresponding values for the above formula.

Example Calculation (continued)

Standard Curve Info

Intercept =	-46.3
Slope =	9.97E+03
r =	0.9932

0.002 ng/mL Matrix-Spike Replicate	Calculated Concentration (ng/mL)
1	0.00019
2	0.00017
3	0.00023
4	0.00020
5	0.00024
6	0.00023
7	0.00019
Standard Deviation (S) =	**0.000026**
N-1 =	**6**
Critical t value (t) =	**3.143**
Injection Volume (µL)	**100**
MDL (ng/mL) =	**0.00008**
MDL (ng on-column) =	**0.000008**

Limit of Detection (LOD)

The LOD for BAS 850 H was set at 0.025 ng on-column (i.e. 0.25 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 M850H004 was set at 0.0005 ng on-column (i.e. 0.025 ng/mL injected at 0.02 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 0.0002 mg/kg (ppm) were brought through the analytical method (extracted, aliquoted, cleaned up, and brought to final volume), the resulting concentration of the sample would be 0.25 ng/mL and 0.025 ng/mL, respectively for BAS 850 H and M850H004; and it would be at LOD.

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 soil is 0.001 mg/kg (ppm) for BAS 850 H and its metabolites.

The method LOQ is 5 times greater than the method LOD so that any contaminations or background in the control which are less than 20% and will be negligible and unquantifiable.