MRID: 51207302

FINAL REPORT

Study Title

Independent Laboratory Validation of BASF Method R0067/01: Method for the Determination of BAS 850 H (Reg. No. 5654329) and its Metabolite M850H001 (Reg. No. 5749359), in Soil with LOQ of 0.1 µg/kg using LC-MS/MS

Guidelines Covered

OCSPP 850.6100
SANCO/825/00 rev 8.1 (Nov. 16, 2010)

1 INTRODUCTION

1.1 Scope of the Method

BASF method R0067/01 was developed to determine the residues of BAS 850 H (Reg. No. 5654329) and M850H001 (Reg. No. 5749359) in soil with LOQ 0.1 µg/kg using LC-MS/MS. The method was developed and validated at ADPEN Laboratories (Reference 1). The method was independently validated at JRF America, Audubon, PA.

The independent lab validation was conducted using two fortification levels, at the LOQ (0.1 µg/kg) and ten times the LOQ (1.0 µg/kg), for soil. Five replicates were analyzed at each fortification level. Additionally, two unfortified sample replicates and one reagent blank were analyzed.

1.2 Principle of the Method

Using BASF Analytical Method No. R0067/01, residues of BAS 850 H and M850H001 in 5-g soil samples are extracted twice by shaking with methanol:water with 0.1% formic acid (70:30 v/v). The sample extracts are decanted each time and combined into a 50-mL centrifuge tube. An aliquot (50%) from the extract is concentrated to the aqueous layer under nitrogen at 50ºC. The extract is then partitioned with a mixture of cyclohexane-ethyl acetate (90:10 v/v). An aliquot of the organic layer (95%) is evaporated to dryness at 50 ºC under nitrogen. Residues are re-dissolved in methanol-water with 0.1% formic acid (20:80 v/v) for the LC-MS/MS determination.

The final determination is conducted using LC-MS/MS in positive electrospray ionization mode. The mass transitions monitored were *m/z* 413→74 (primary, quantitative) and *m/z* 413→134 (secondary, confirmatory) for BAS 850 H and *m/z* 397→114 (primary, quantitative) and *m/z* 397→114 (secondary on different column, confirmatory) for M850H001. The results are calculated by direct comparison of sample peak areas to that of external standards. The method procedure used for this independent laboratory validation is provided in Appendix 10.1.

The method has a limit of quantitation of 0.1 µg/kg in soil for BAS 850 H and its metabolite, M850H001 with the limit of detection of 0.026 µg/kg (26% of LOQ). All analytes are determined individually.

1.3 Specificity

The method determines residues of BAS 850 H and M850H001 in soil by LC-MS/MS, which is a highly selective and sensitive LC-MS/MS method. No interfering peaks were found at the retention times for these analytes. The multiple reaction monitoring (MRM) transitions identifying each analyte used in this subject study were determined by product ion spectra in the method validation study.

Matrix effects were determined to be significant during method validation. Therefore, matrixes matched calibration standards, prepared alongside samples, and were used for all analysis in this Independent Laboratory Validation (ILV) study.

For BAS 850 H, 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. For M850H001, only one transition ion provided sufficient sensitivity; therefore, a secondary HPLC column and instrument conditions were used for confirmation.

2 REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test Systems

The following test system was used in this study:

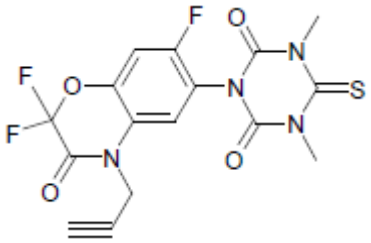
Test System 1: ***Clay Loam Soil*** (BASF Sample ID R1801810004R02); JRFA sample ID: 205205
The description and characterization of the test system used is provided in the respective certificates (Appendix 10.2).

The sponsor provided a container of frozen sample. One sample number was assigned to this test system upon receipt. The test system was stored frozen (-20°C) prior to use.

2.2 Test Substance

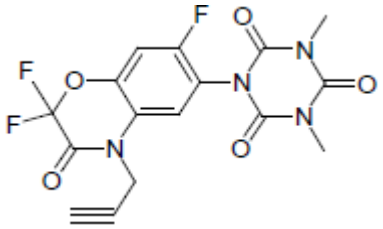
Reference substance was provided by the sponsor, BASF, and stored in a refrigerator (~4°C) until use. A reserved portion of the reference material, as well as documentation specifying the location of the synthesis and characterization information is retained by BASF Corporation at Research Triangle Park, North Carolina. All analysis was completed before the expiration of the reference material. The Certificate of Analysis is included with this report in Appendix 10.3.

BAS 850 H

BASF Reg. No.	5654329
Molecular Formula	$C_{16}H_{11}F_3N_4O_4S$
Molecular Weight	412.3 g/mol
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
Lot No.	L84-130
Purity (%)	99.2%
Storage Advice	Room temperature (25°C or cooler)
GLP	Yes
Expiration Date	January 01, 2028
Chemical structure	

M850H001

BASF Reg. No.	5749359
Molecular Formula	$C_{16}H_{11}F_3N_4O_5$
Molecular Weight	396.3 g/mol
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
Lot No.	L85-52
Purity (%)	98.7%
Storage Advice	Room temperature (25°C or cooler)

GLP	Yes
Expiration Date	March 01, 2022
Chemical structure	

Standard Stability

The stability of the analytes in standard solutions has been determined in related studies on BAS 850 H and its metabolites in soil (Reference 2).

Standard stability was not established in this study and was determined in related studies on BAS 850 H and its metabolites in soil (Reference 2), in which analyte stability for stocks and intermediate (fortification) standard solutions in methanol with 0.1% formic acid was demonstrated for up to a period of 235 Days for both BAS 850 H and M850H001 when stored refrigerated. Calibration solutions of BAS 850 H and M850H001 in methanol-water with 0.1% formic acid (20:80, v/v) exhibited stability for up to 83 days when stored refrigerated.

During the course of this ILV study, the test/reference substance solutions were stored under refrigerated conditions and all solutions were used within the demonstrated time period of stability. An example of preparation and dilution scheme is presented in Appendix 10.4.

3 ANALYTICAL PROCEDURE

3.1 Validation

BASF method R0067/01 was used during independent laboratory validation in which 13 samples were used in the sample set: one reagent blank, two unfortified control samples, five samples fortified at the LOQ (0.1 µg/kg), and five samples fortified at ten times the LOQ (1.0 µg/kg). The method procedure used for this independent laboratory validation is provided in Appendix 10.1

3.2 Route of Administration

For each sample, 5g (± 0.1 g) of matrix was measured using a calibrated balance into Teflon 50mL-centrifuge tube and fortified with the appropriate fortification solution using a calibrated micropipette (20-100 µL size).

The following scheme was used for fortification of the samples:

Sample Type	Sample Weight (g)	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [µg/g]
Control	5.00±0.1	-	-	-
Fortification (LOQ)	5.00±0.1	0.01	0.050	0.0001*
Fortification (10×LOQ)	5.00±0.1	0.10	0.050	0.001

* Limit of quantitation

3.3 Extraction

5g ± 0.1 g samples of control soil were weighed out then fortified as described above. Samples were extracted twice by shaking with 25 mL of methanol:water with 0.1% formic acid (70:30 v/v) for 45 minutes. The sample extracts were centrifuged for 10 minutes at approximately 3500 rpm and then the supernatant was decanted each time and combined into a 50-mL volumetric flask. The combined extracts were brought to volume using methanol:water with 0.1% formic acid (70:30, v/v). Samples were vortexed then transferred to a glass container.

3.4 Sample Clean-up

10 mL of extract was transferred to a glass tube and concentrated to the aqueous layer (~5 mL) under nitrogen at 50ºC. An additional 5 mL of extract was added to the glass tube and concentrated to the aqueous layer (~5 mL). This was repeated two more times (concentrating an additional 5 mL of extract each time), for a total extract volume of 25mL. The final extract was concentrated to the aqueous layer (7.4 mL mark) under nitrogen at 50ºC.

The aqueous extract was then transferred to a separatory funnel and partitioned with a mixture of cyclohexane-ethyl acetate (90:10 v/v). 9.5 mL of the organic layer (95%) was evaporated to dryness at 50 ºC under nitrogen.

3.5 Preparation for Measurement

0.5 mL of methanol with 0.1% formic acid was added; samples were vortexed and then sonicated for 2 minutes. 2.0 mL of water with 0.1% formic acid was added and samples were vortexed. Samples were filtered using a 0.45 µm PTFE filters, diluted using control matrix extract (for 10xLOQ samples), then vialed for the LC-MS/MS determination

3.6 Stability of Extracts

Extract stability was not established in this study and was determined during validation of the method. During ILV study, sample extracts were analyzed within 1 day of extraction.

3.7 Influence of Matrix Effects on Analysis

For BAS 850 H and M850H001, significant matrix effects were observed during the method validation study (Reference 1). Therefore, matrix matched standards were used in this ILV. Matrix matched standards should be prepared alongside samples improving similar matrix load between samples and matrix matched standards.

Analytical procedure: BASF Method R0067/01: Method for the determination of BAS 850 H (Reg. No. 5654329), and its metabolite M850H001 (Reg. No. 5749359), in Soil with LOQ of 0.1 µg/kg using LC-MS/MS

Confirmatory technique: For BAS 850 H, a secondary MRM transition was used for confirmation. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique was not necessary. For M850H001, only one transition provided enough sensitivity, so a second HPLC column and instrument method using the same transition was used for confirmation.

LOQ: 0.1 µg/kg (lowest fortification level)

LOD: The LOD was determined during the method validation and was not conducted during this independent laboratory validation. The limit of detection (LOD) for each analyte was set at 0.026 µg/kg or 26% of the LOQ during method validation. In addition, the LOD for each analyte in each matrix was calculated using the standard deviation of the LOQ fortifications in each matrix.

Levels of fortification: LOQ and 10×LOQ

Time required: A set of 13 samples requires approximately 8 hours of work (This time includes the evaluation of the results, the preparation of the equipment, and reporting of all raw data under GLP)

6 DISCUSSION

Linearity

For BAS 850 H and M850H001, five mixed matrix matched calibration standards were prepared by fortifying untreated control extracts. Acceptable linearity was observed for the standard range and mass transitions/instrumental methods tested for each analyte and matrix. The method-detector response was linear over the 0.025 to 0.5 ng/mL calibration range. Good linearity ($r2 \geq 0.99$) was observed over this range.

Standard Stability

Standard stability was not established in this study and was determined in a separate study, in which analyte stability for stocks and intermediate (fortification) standard solutions in methanol with 0.1% formic acid was demonstrated for up to a period of 235 Days for both BAS 850 H and M850H001 when stored refrigerated. Calibration solutions of BAS 850 H and M850H001 in methanol-water with 0.1% formic acid (20:80, v/v) exhibited stability for up to 83 days when stored refrigerated.

During the course of this ILV, the test/reference substance solutions were stored under refrigerated conditions and all solutions were used within the demonstrated time period of stability.

Extract Stability

Extract stability was not established in this study and was determined during validation of the method. During ILV study, sample extracts were analyzed within 1 day of extraction.

Specificity/Selectivity

The method determines residues of BAS 850 H and M850H001 in soil by LC-MS/MS, which is a highly selective and sensitive LC-MS/MS method. No interfering peaks were found at the retention times for these analytes. The multiple reaction monitoring (MRM) transitions identifying each analyte, used in this subject study, were determined by product ion spectra in the method validation study.

Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) was defined by the lowest fortification level successfully tested. The LOQ is 0.1 µg/kg for each analyte, corresponding to a concentration in the final extract of 0.1 ng/mL.

Limit of Detection (LOD)

The LOD was determined during the method validation and was not conducted during this independent laboratory validation. The limit of detection (LOD) for each analyte was set at 0.026 µg/kg or 26% of the LOQ during method validation. In addition, the LOD for each analyte in each matrix was calculated using the standard deviation of the LOQ fortifications in each matrix.

Repeatability

The overall relative standard deviations (RSD, %) for all fortification levels were below 20%. It was demonstrated that BASF method R0067/01 fulfils acceptability requirements with regards to specificity, repeatability, limit of quantitation, and recoveries. This method fulfils the ability to detect and quantify the material.

Method – (continued)

BASF
We create chemistry

Technical Procedure:
Method for the Determination of Residues of BAS 850 H (Reg. No. 5654329) and its metabolite M850H001 (Reg. No. 5749359), in Soil with LOQ of 0.1 µg/kg using LC-MS/MS

BASF Method Number:
R0067/01

Method – (continued)

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 of 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. 26% of LOQ). At this concentration, the analyte must be qualitatively detectable in sample matrix (analyte peak height at least 3-5 x baseline noise).

Method – (continued)

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 relevant metabolites in soil was developed by BASF (Reference 1). The current method, R0067, is developed in soil with the required LOQ of 0.1 µg/kg, which is below the ecotox end points from a seedling emergence study (References 2 and 3)

BASF Method Number R0067 was successfully tested during method development in clay and sandy loam soil types.

History of the method

TP Version	Release Date	Change
01	11 March 2020	New method

The method has a limit of quantitation of 0.1 µg/kg in soil for BAS 850 H and its metabolite, M850H001 with the limit of detection of 0.026 µg/kg (26% of LOQ). All analytes are determined individually.

2.0 MATERIALS

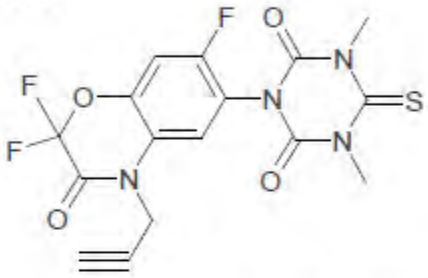
2.1 Safety

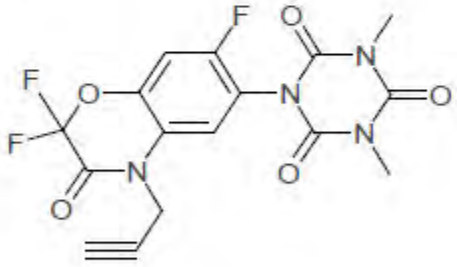
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Ensure that 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 Safety Data Sheets (SDS) of the individual substances. All procedures involving organic solvents should be performed in a well-ventilated hood. Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

Method – (continued)

2.2 Test and Reference Items

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

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:	

Method – (continued)

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	Kinetex EVO C18 3.0×50 mm, 2.6 µm	Phenomenex	00b-4725-y0
HPLC Column	X-Bridge BEH C18 XP Column, 4.6×50 mm, 2.5 µm	Waters	186006037
HPLC	1290	Agilent	–
Mass Spectrometer	AB Sciex 6500	AB Sciex	–
Mechanical shaker	KS501 Digital	IKA Labortechnik	–
Nitrogen evaporator	TurboVap	Biotage	–
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, 25 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.

Method – (continued)

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
Ethyl 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
Solution	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.
Solution	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 Solution	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	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.

Method – (continued)

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 and M850H001)

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

Mixed Fortification Solutions (BAS 850 H and M850H001)

Prepare 10, 1 , 0.1, and 0.01 µg/mL fortification solutions in **S1** from the 1 mg/mL (1000 µg/mL) stock solutions prepared in **S1** from section "Stock Solutions (BAS 850 H and M850H001)" 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
0.10	5	50	0.010

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.

Method – (continued)

Precursor Calibration Standard Solutions (BAS 850 H and M850H001)

Prepare mixed standard calibration solutions in **S4** for LC-MS/MS analysis, in flasks, by using the 1.0 µg/mL (1000 ng/mL) and 0.1 µg/mL (100 ng/mL) solutions prepared in **S1** from section "Mixed Fortification Solutions (BAS 850 H and M850H001)". Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by vortexing). These standard solutions will be used to prepare matrix calibration standard solutions.

Preparation of Mixed Standard Solutions

Take solution (ng/mL)	Volume (mL)	Dilute with S4 to a final volume of (mL)	Concentration (ng/mL)
1000 (in S1)	1.25	25	50.0
1000 (in S1)	0.625	25	25.0
100 (in S1)	2.5	25	10.0
100 (in S1)	1.25	25	5.0
100 (in S1)	0.625	25	2.5

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.

2.4.2.2 Matrix Matched Calibration Standards (BAS 850 H and M850H001)

In case matrix-matched standards (and instrument recovery samples) are needed, calibration standard solutions are prepared in matrix solution, i.e., using a final volume mixture from multiple control samples or using a large batch of sample, carried through the analytical procedure. Matrix-matched standards should be prepared in a way that the matrix load is at least 90% of the matrix load in the unknown samples.

Preparation of Mixed Matrix Matched Standard Solutions for Calibration

Take solution (ng/mL)	Volume (mL)	Dilute with Control Matrix to a final volume of (mL)	Concentration (ng/mL)
50	0.010	1.0	0.5
25	0.010	1.0	0.25
10	0.010	1.0	0.10
5	0.010	1.0	0.05
2.5	0.010	1.0	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.

Method – (continued)

2.4.3 Stability of Standard Solutions

BASF recommends that stock solutions (1 mg/mL) of all analytes be made fresh every three months. Pre-calibration solutions should be prepared every month.

The stability of BAS 850 H and M850H001 in standard solutions stored at an average temperature of 3°C has been determined in a separate study as noted below.

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 4).

Calibration solutions of BAS 850 H and M850H001 are prepared monthly by serial dilution of the fortification standards solutions of BAS 850 H and M850H001 with methanol/water with 0.1% formic acid (20:80, v/v). Calibration solutions exhibited stability up to 83 days for BAS 850 H and M850H001 (Reference 4).

If solutions are stored at different conditions or/and for a longer time, the stability of the reference items must be confirmed.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples must be sufficiently homogenized prior to analysis in order to ensure that the aliquot taken for residue analysis is representative for the whole sample.
In case of small sample sizes (micro-extraction), special emphasis must be put on sample homogenization, e.g. cryo-milling or additional homogenization steps.

3.2 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 volumetrically according to the table below and then proceed to Section 3.3. Extraction BAS 850 H, M850H001.

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	5 g	--	--	0.00 µg/kg
Fortification (LOQ)*	5 g	0.01 µg/mL	0.05 mL	0.1 µg/kg
Fortification (10xLOQ)	5 g	0.10 µg/mL	0.05 mL	1.0 µg/kg
Fortification (100xLOQ)	5 g	1.0 µg/mL	0.05 mL	10.0 µg/kg
Treated	5 g	--	--	--

* Limit of quantification

Method – (continued)

3.3 Extraction (BAS 850 H and M850H001)

Add exactly 25 mL of **S3** to the pre-weighed sample from Section **3.2**. 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 volumetric flask.

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 volumetric flask that already contains the first extract. Bring the extract to 50 mL final volume, then vortex the combined extract thoroughly and proceed to Section **3.4** 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.

Extractability
The exact ^{14}C-metabolism extraction solvents are used to demonstrate extractability in this method (Reference 5).

3.4 Sample Clean-up (BAS 850 H, M850H001)

a) Transfer exactly 25 mL of extract from Section 3.3 (50% aliquot) into a graduated glass tube.
b) Concentrate to just below the 7.5 mL mark (~7.4 mL) under nitrogen at 50 °C. All methanol must be removed.
c) Add 10 mL of **S5** to the aqueous extracts in tube (Step **3.4[b]**).
d) Secure sample with green Teflon-lined screw cap and then mix thoroughly using shaking gently by hand followed by vertexing. Allow layers to separate.
e) Remove exactly 9.5 mL of the top organic layer into a clean culture and evaporate to dryness under nitrogen at 50 °C.
f) Proceed to Section 3.5 to prepare the sample for measurement on the LC-MS/MS.

3.5 Preparation for Measurement (BAS 850 H, M850H001)

Add exactly 0.5 mL of **S1** to the samples from Step **3.4[f]**. 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 2.0 mL of **S2.** Vortex thoroughly to ensure a homogenous solution.

Filter all samples using 0.45µm PTFE syringe filters directly into HPLC injection vials, sending the first approximately 0.1 – 0.2 mL to waste.

In case of high residues, an appropriate dilution (e.g. 50 µL + 950 µL) with control extract is necessary to remain in the range of the calibration curve.

See Section **4.2** for LC-MS/MS conditions.

Note:	Method could be interrupted at this point

Method – (continued)

3.6 Influence of Matrix Effects on Analysis

For the analysis of BAS 850 H and M850H01, significant matrix effects were observed during method development in all soil matrices tested. Therefore, the use of matrix matched standards is recommended (see Section **2.4.2.2**).

3.7 Stability of Extracts and Final Volumes

Stability of extracts/final volumes will be conducted as part of the method validation study.

Procedural recoveries can also be used to prove the stability over a longer time interval, if necessary.

3.8 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 at or 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:

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

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

$$\text{Dry Residue (ppm)} = \frac{\text{Wet Sample Residue (ppm)}}{(100 - \text{Percent Moisture}) / 100}$$

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.

Method – (continued)

4.2 Instrumental Analysis

4.2.1 Instrumentation and Conditions (BAS 850 H & M850H001)

Method A (HPLC Mode): BAS 850 H and M850H001

	Parameter		
Chromatographic System	Agilent 1290 system		
Analytical-Column	Phenomenex Kinetex EVO C18, 3.0×50 mm, 2.6 µm		
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*	500 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	80	20
	0.05	80	20
	6.00	50	50
	7.00	1	99
	7.90	1	99
	7.91	80	20
	8.00	80	20
Detection System	AB Sciex 6500 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	200 °C		
Analyte	**Transitions *(m/z)***	**Polarity**	**Expected Retention Time**
BAS 850 H	413 → 74** 413 → 134	positive	~ 6.53 min
M850H001	397 → 114	positive	~ 4.50 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

Method – (continued)

4.2.1 Instrumentation and Conditions (BAS 850 H & M850H001) continued

Method B (HPLC Mode): M850H001 Confirmatory method)

	Parameter		
Chromatographic System	Agilent 1290 system		
Analytical-Column	Waters Xbridge BEH C18 XP 2.5 µm 4.6×50mm		
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	80	20
	0.05	80	20
	6.00	50	50
	8.00	1	99
	8.90	1	99
	9.00	80	20
	10.00	80	20
Detection System	AB Sciex 6500 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	200 °C		
Analyte	**Transitions *(m/z)***	**Polarity**	**Expected Retention Time**
M850H001	397 → 114	positive	~ 5.90 min

*System pressure is approximately 2000 PSI using this method

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.

Method – (continued)

4.2.2 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 M850H001 standards containing a known amount of analytes in the range of 0.025 ng/mL to 0.50 ng/mL.

Linear calibration functions with 1/x weighting are recommended for evaluation.

4.2.3 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 **a)** and **b)**:

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

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

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 **c)**:

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

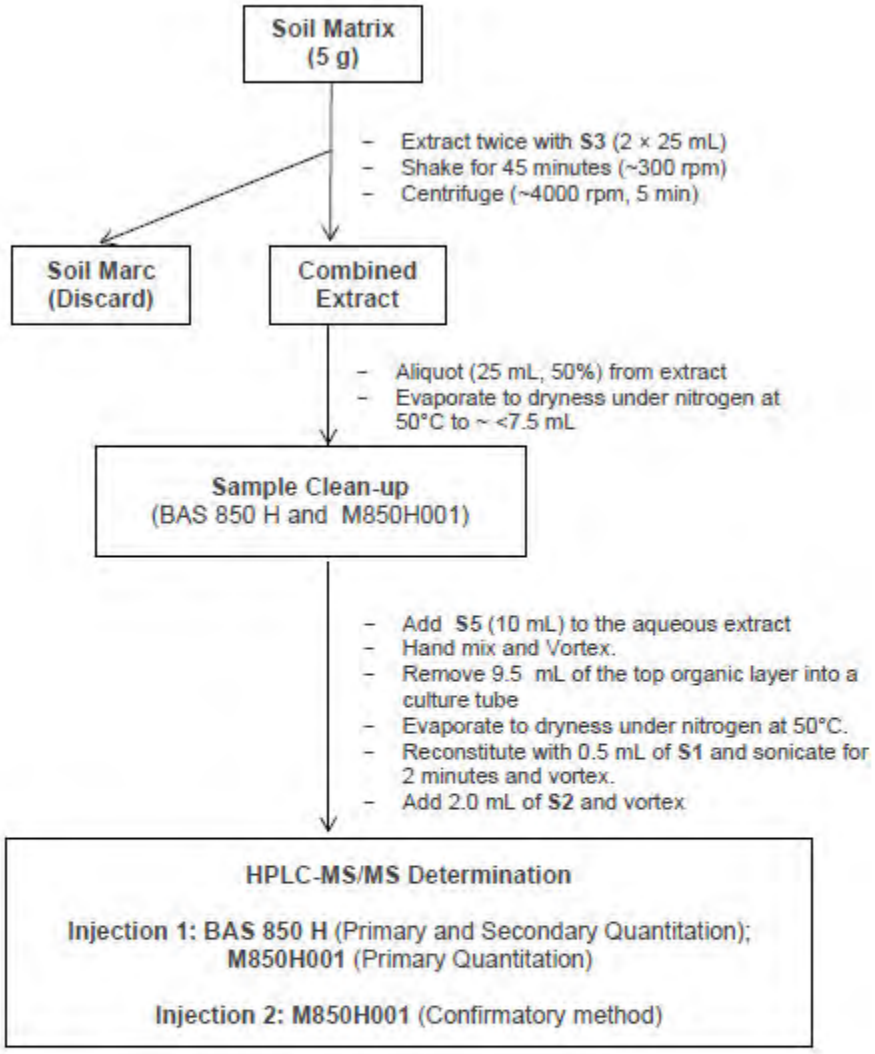
Soil residues based on soil dry weight

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

Method – (continued)

5.0 FLOWCHART

5.1 Analysis of BAS 850 H and M850H001 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

Appendix 10.4: Standard and Fortification Solutions Preparation

Stock Solutions

A stock solution of BAS 850 H was prepared at a concentration of approximately 1000 µg/mL in methanol with 0.1% formic acid by accurately weighing 0.0122 g of the reference standard, using an analytical balance, into a 10 mL grade A volumetric flask. The volume was diluted to 10 mL using methanol with 0.1% formic acid. The relative purity (99.2%) of the analyte was taken into account when calculating the final solution concentration. Fortification and calibration standard solutions were prepared in separate dilution series from the same stock solution.

Compound Name	Purity	Weight (g)	Concentration (µg/mL)
BAS 850 H	99.2%	0.0122	1210

A stock solution of M850H001 was prepared at a concentration of approximately 1000 µg/mL in methanol with 0.1% formic acid by accurately weighing 0.0104 g of the reference standard, using an analytical balance, into a 10 mL grade A volumetric flask. The volume was diluted to 10 mL using methanol with 0.1% formic acid. The relative purity (98.7%) of the analyte was taken into account when calculating the final solution concentration. Fortification and calibration standard solutions were prepared in separate dilution series from the same stock solution.

Compound Name	Purity	Weight (g)	Concentration (µg/mL)
M850H001	98.7%	0.0104	1030

Fortifications Solutions

Intermediate stock solutions (1 µg/mL) were prepared by combining then diluting the stock solutions of BAS 850 H and M850H001 with 0.1% formic acid in methanol. Fortification solutions were prepared using a dilution series as exemplified in the tables below.

Primary Solution	Primary Concentration (µg/mL)	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (µg/mL)	New Solution ID
JRFA-630/1-6	1210	0.413	50.0	10.0	JRFA-630/1-8
JRFA-630/1-7	1030	0.485	50.0	10.0	JRFA-630/1-8
JRFA-630/1-8	10.0	5.00	50.0	1.00	JRFA-630/1-9
JRFA-630/1-9	1.00	5.00	50.0	0.100	JRFA-630/1-10
JRFA-630/1-10	0.100	5.00	50.0	0.0100	JRFA-630/1-11

Appendix 10.5: Example Calculations of Residues and Recoveries

Calculation of results was based on area measurements. The recoveries of all analytes were calculated relative to the linearity curve generated with each set.

The recovery is a percentage of the fortified amount of the analyte (ng), which was recovered after the entire sample work-up steps.

The residues of BAS 850 H in µg/L (ppb) are calculated as shown in equations I and II:

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

II. Residues in the Sample Matrix (µg/g) $= \frac{V_{end} \times C_A \times DF}{G \times A_F \times 1000}$

$\mathbf{V_{end}}$	=	Final volume of the extract after all steps (mL)
$\mathbf{C_A}$	=	Concentration of analyte as read from the calibration curve (ng/mL)
G	=	Volume of sample extracted (mL)
$\mathbf{A_F}$	=	Aliquotation factor
DF	=	Dilution factor

Recovery is the percentage of the fortified amount (µg), which is recovered through the method. The recoveries of spiked compounds are calculated according to equation III:

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

Example of Calculation:

BAS 850 H, primary quantitation (*m/z* 413 → 74), in soil, fortified at 0.10 µg/kg (LOQ):

The following values were used in this calculation:

JRFA Sample ID (extracted 07/01/2020)	LOQ 1
Analytical Report	07012020 ILV Soil
Peak area of fortified sample (LOQ 1)	34021.313
Peak area of control sample (UTC-1)	66.185
Calibration Slope	283048.7806
Intercept	1961.954257
Sample Weight (G)	4.98
Aliquotation Factor (A_F)	0.5
Final Volume (V_{end})	2.50 mL
Dilution Factor (DF)	1
Unit Conversion Factor	1000 ng/µg

Concentration (ng/mL) = $\frac{34021.313 - 1961.954257}{283048.7806} = 0.113$ ng/mL

Residue (µg/L) = $\frac{2.5 \text{ mL} \times 0.113 \text{ ng/mL} \times 1}{4.98\text{g} \times 0.5 \times 1000 \text{ ng/µg}} = 0.000114$ µg/g

$$\text{Recovery (\%)} = \frac{0.000114\ \mu g/g \times 100\%}{0.0001\ \mu g/g} = \mathbf{114\%}$$

Analyst Software version 1.6.2 was used to generate calibration curves and concentrations in ng/mL. The values reported in the tables are calculated with full precision, using Excel, but displayed with rounding. Minor / insignificant discrepancies may be observed when recalculated.

Appendix 10.7: Instrument Conditions and Parameters

LC-MS/MS Conditions (Primary and Secondary Quantitation)

	Parameter
Chromatographic System	Agilent 1290 Infinity
Analytical-column	Phenomenex Kinetex 2.6 µm, 3mm x 50 mm; SN: H20-180518
Column Temperature	40°C
Injection Volume	20 µL
Mobile Phase A	1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.5 mL/min
Divert Valve	1.0-7.5min to MS; remaining elution time to waste

Gradient

Time (min)	% Phase A	% Phase B
0.0	80.0	20.0
0.05	80.0	20.0
6.00	50.0	50.0
7.00	1.0	99.0
7.90	1.0	99.0
7.91	80.0	20.0
8.00	80.0	20.0

Detection System	AB Sciex QTrap 6500 Mass Spectrometer
Source Ionization	Turbo Spray Ion Drive Electrospray (ESI)
Polarity	Positive
Ionization Temperature	150 °C
Ion Spray Voltage	5500.00
Curtain Gas	20.00
Ion Source Gas 1	60
Ion Source Gas 2	40

Analyte	Transitions (*m/z*)	Dwell Time (msecs)	DP	CE	EP	CXP	Retention Time (min)
BAS 850 H	413 → 74*	150.00	156.00	89.00	10.00	10.00	~6.87
	413 → 134	150.00	151.00	71.00	10.00	14.00	
M850H001	397 → 114*	150.00	116.00	81.00	10.00	12.00	~4.97

* Primary quantitation transition. Either transition could be used for quantitation in case interference is observed at the same retention time.

LC-MS/MS Conditions (Confirmatory Method used for M850H001)

	Parameter
Chromatographic System	Agilent 1290 Infinity
Analytical-column	Waters XBridge BEH C18 XP 2.5 µm, 4.6 mm x 50 mm; SN: 01843010518334
Column Temperature	40°C
Injection Volume	20 µL
Mobile Phase A	1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.8 mL/min
Divert Valve	1.0-9.0 min to MS; remaining elution time to waste

Gradient

Time (min)	% Phase A	% Phase B
0.00	80.0	20.0
0.05	80.0	20.0
6.00	50.0	50.0
8.00	1.0	99.0
8.90	1.0	99.0
9.00	80.0	20.0
10.00	80.0	20.0

Detection System	AB Sciex QTrap 6500 Mass Spectrometer
Source Ionization	Turbo Spray Ion Drive Electrospray (ESI)
Polarity	Positive
Ionization Temperature	150 °C
Ion Spray Voltage	5500.00
Curtain Gas	20.00
Ion Source Gas 1	60
Ion Source Gas 2	40

Analyte	**Transitions (*m/z*)**	**Dwell Time (msecs)**	**DP**	**CE**	**EP**	**CXP**	**Retention Time (min)**
M850H001	397 → 114	150.00	116.00	81.00	10.00	12.00	~6.1