MRID: 50406038

FINAL REPORT

Title

Independent Laboratory Validation of 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”

BASF Study Code

BASF Study Number: 412300
CRO Study Number: 137G1602

Guidelines

US Environmental Protection Agency (EPA) Ecological Effects Test Guideline: OCSPP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation,
SANCO/825/00 rev.8.1 (Nov. 16, 2010)

1. INTRODUCTION

1.1 Scope of the Method

BASF Analytical Method No. D1401/02 was developed to determine the residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in soil using LC-MS/MS at BASF Crop Protection in Research Triangle Park, North Carolina (Reference 1). This report represents the validation by an independent laboratory, EPL Bio Analytical Services, in Niantic, Illinois.

The ILV was conducted using two fortification levels, the LOQ (0.001 mg/kg) and ten times the LOQ (0.01 mg/kg) for soil. Five replicates were analysed for each fortification level. Additionally, one reagent blank and two replicates of unfortified samples were examined.

1.2 Principle of the Method

The residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 were extracted from 5 grams of soil twice using 25 mL of methanol-water mixture with 0.1% Formic Acid (70:30, v/v). After each 25 ml addition, the samples were shaken for approximately 45 minutes on a mechanical shaker and were centrifuged. The supernatants of the two extractions were combined and vortexed.

For BAS 850 H and M850H001, 15 mL of the combined extract was transferred to a glass culture tube and was dried to at least the 4.2 mL mark under nitrogen at 50°C to remove methanol. Twelve mL of cyclohexane-ethyl acetate solution (90:10, v/v) was added and samples were vortexed and centrifuged. Ten mL of the top organic layer was removed and evaporated to dryness under nitrogen at 50°C and reconstituted using 0.2 mL of methanol with 0.1% formic acid followed by 0.8 mL of water with 0.1% formic acid. In both reconstitution steps, samples were vortexed and sonicated to fully dissolve the residues and to ensure a homogenous solution. Samples known to exceed the highest calibration level were diluted using methanol-water solution with 0.1% formic acid (20:80, v/v).

Final determination on BAS 850 H and the M85H001 metabolite was conducted using LC-MS/MS in the positive ion mode. For BAS 850 H, the transition at m/z 413 → 74 was monitored for primary quantification; the transition at m/z 413 → 134 was monitored for confirmation. For the M850H001 metabolite, the transition m/z 397 → 114 was monitored for primary quantification; the transition at m/z 397 → 141 was monitored for confirmation.

For M850H002, M850H003 and M850H004, 1.25 mL of the combined extract was transferred to a graduated glass culture tube and was dried to approximately 0.3 mL under nitrogen at 50°C. The sample was reconstituted by adding water with 0.1% formic acid to the 0.8 mL mark followed by adding methanol with 0.1% formic acid to the 1 mL mark. In both reconstitution steps, samples were vortexed and sonicated to fully dissolve the residues and to ensure a homogenous solution.

Final determination on the M850H002, M850H003, and M850H004 metabolites was conducted using LC-MS/MS in the negative ion mode. For M850H002, the transition at m/z 373 → 193 was monitored primary quantification; the transition at m/z 373 → 323 was monitored for confirmation. For M850H003, the transition m/z 357 → 307 was monitored for primary quantification; the transition at m/z 357 → 137 was monitored for confirmation. For M850H004, the transition m/z 385 → 103 is monitored for primary quantification; the transition at m/z 385 → 255 is monitored for confirmation.

1.3 Specificity

To demonstrate the specificity of the analytical method, one additional mass transition for each BAS 850 H, M850H001, M850H002, M850H003 and M850H004 was monitored simultaneous to the primary quantitation transition. The method was able to accurately determine residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 and no interference was observed at the retention time of the analyte peaks.

2. REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test Systems

The test system in this study was the soil from BASF Study 411690 [trial R130128 (North Dakota, clay loam), Reference 2].

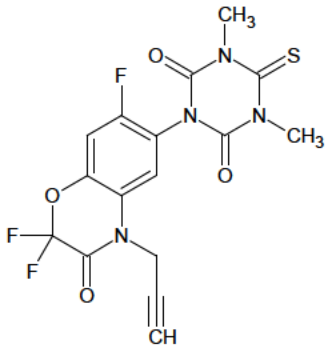
The soil was sent from ADPEN Laboratories Inc. to EPL on August 1, 2017. It was received by EPL on August 2, 2017. Upon arrival at the laboratory, the sample was opened, inspected, and checked against enclosed shipping forms. The test system was received frozen and was stored under frozen conditions at all times, unless removed from the freezer for laboratory analysis. The control sample was logged in to EPL records and given a unique sample number (1602-S001).

2.2 Test and Reference Substances

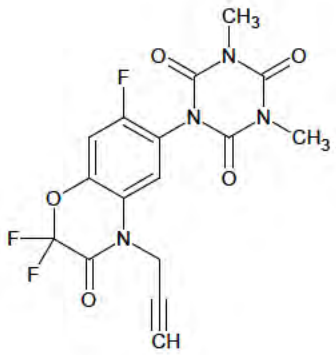
The standard substances were stored refrigerated until use. BASF has retained reserve samples of these chemicals and has documentation specifying the location of the synthesis and characterization information available at BASF Crop Protection, Research Triangle Park, North Carolina.

The BAS 850 H, M850H001, M850H002, M850H003 and M850H004 reference substances were provided by the sponsor and received on August 2, 2017.

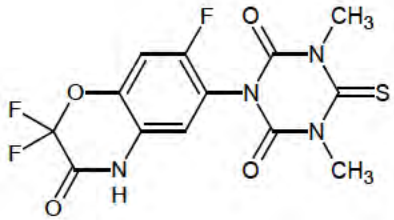
BAS Code	BAS 850 H
Chemical Name	1,5-dimethyl-6-thioxo-3-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5654329
CAS No.	1258836-72-4
Lot No.	L84-130
Purity (Certificate)	99.2%
Expiry	February 01, 2020
Molecular Formula	$C_{16}H_{11}F_3N_4O_4S$
Molecular Weight	412.3



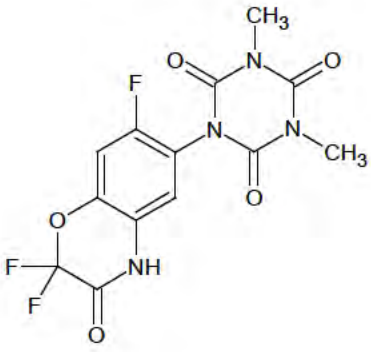
Common Name	M850H001
Chemical Name	1,3-dimethyl-5-[2,2,7-trifluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-1,3,5-triazinane-2,4,6-trione
BASF Reg. No.	5749359
CAS No.	None
Lot No.	L85-52
Purity (Certificate)	98.7%
Expiry	April 01, 2018
Molecular Formula	$C_{16}H_{11}F_3N_4O_5$
Molecular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



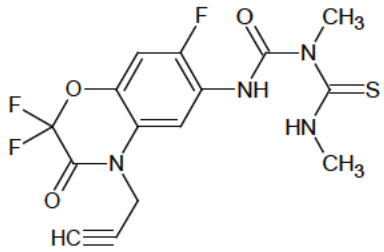
Common Name	M850H002
Chemical Name	1,5-dimethyl-6-thioxo-3-(2,2,7-trifluoro-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-1,3,5-triazinane-2,4-dione
BASF Reg. No.	5757725
CAS No.	None
Lot No.	L84-162
Purity (Certificate)	96.8%
Expiry	February 01, 2020
Molecular Formula	$C_{13}H_9F_3N_4O_4S$
Molecular Weight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



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



Common Name	M850H004
Chemical 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
BASF Reg. No.	5833884
CAS No.	None
Lot No.	L85-50
Purity (Certificate)	99.5%
Expiry	April 01, 2018
Molecular Formula	$C_{15}H_{13}F_3N_4O_3S$
Molecular Weight	386.4



3. ANALYTICAL METHOD

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 used for the analysis of the samples.

The residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 were extracted from 5 grams of soil twice using 25 mL of methanol-water mixture with 0.1% Formic Acid (70:30, v/v). After each 25 ml addition, the samples were shaken for approximately 45 minutes on a mechanical shaker and were centrifuged. The supernatants of the two extractions were combined and vortexed.

For BAS 850 H and M850H001, 15 mL of the combined extract was transferred to a glass culture tube and was dried to at least the 4.2 mL mark under nitrogen at 50°C to remove methanol. Twelve mL of cyclohexane-ethyl acetate solution (90:10, v/v) was added and samples were vortexed and centrifuged. Ten mL of the top organic layer was removed and evaporated to dryness under nitrogen at 50°C and reconstituted using 0.2 mL of methanol with 0.1% formic acid followed by 0.8 mL of water with 0.1% formic acid. In both reconstitution steps, samples were vortexed and sonicated to fully dissolve the residues and to ensure a homogenous solution. Samples known to exceed the highest calibration level were diluted using methanol-water solution with 0.1% formic acid (20:80, v/v).

Final determination on BAS 850 H and the M85H001 metabolite was conducted using LC-MS/MS in the positive ion mode. For BAS 850 H, the transition at m/z 413 → 74 was monitored for primary quantification; the transition at m/z 413 → 134 was monitored for confirmation. For the M850H001 metabolite, the transition m/z 397 → 114 was monitored for primary quantification; the transition at m/z 397 → 141 was monitored for confirmation.

For M850H002, M850H003 and M850H004, 1.25 mL of the combined extract was transferred to a graduated glass culture tube and was dried to approximately 0.3 mL under nitrogen at 50°C. The sample was reconstituted by adding water with 0.1% formic acid to the 0.8 mL mark followed by adding methanol with 0.1% formic acid to the 1 mL mark. In both reconstitution steps, samples were vortexed and sonicated to fully dissolve the residues and to ensure a homogenous solution.

Final determination on the M850H002, M850H003, and M850H004 metabolites was conducted using LC-MS/MS in the negative ion mode. For M850H002, the transition at m/z 373 → 193 was monitored primary quantification; the transition at m/z 373 → 323 was monitored for confirmation. For M850H003, the transition m/z 357 → 307 was monitored for primary quantification; the transition at m/z 357 → 137 was monitored for confirmation. For M850H004, the transition m/z 385 → 103 is monitored for primary quantification; the transition at m/z 385 → 255 is monitored for confirmation.

3.1 Valiation 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 ILV 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, 0.01 mg/kg. The example of recovery calculation is provided in Figure 1.

3.2 Influence of Matrix Effects on Analysis

Matrix effect was tested in this ILV study. This involved comparing calibration standards prepared in control matrix against calibration standard solutions prepared with only solvent. No matrix effects were noticed during the study for any analyte/matrix/ion transition; therefore solvent-based analysis were conducted.

4. RESULTS AND DISCUSSION

The results of this ILV study demonstrate that BASF Analytical Method No. D1401/02 was successfully validated in the first trial. All analytes and mass transitions were successfully validated in the first trial when the increased injection volume (40 µL) was used for the determination of M850H002, M850H003 and M850H004.

5. SUMMARY OF METHOD

Summary of Method

Type of Method	LC-MS/MS
Test System	Clay Loam Soil
Selected mass transitions (*m/z*)	**BAS 850 H** *m/z* 413→*m/z* 74* *m/z* 413→*m/z* 134 **M850H001** *m/z* 397→*m/z* 114* *m/z* 397→*m/z* 141 **M850H002** *m/z* 373→*m/z* 193* *m/z* 373→*m/z* 323 **M850H003** *m/z* 357→*m/z* 307* *m/z* 357→*m/z* 137 **M850H004** *m/z* 385→*m/z* 103* *m/z* 385→*m/z* 255 *Primary quantification transition
Analytical Procedure	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”
Confirmatory Technique	A secondary MRM transition was used for confirmation.
Method of Quantitation	The quantitation is based on the monitoring of two mass transitions for BAS 850 H, M850H001, M850H002, M850H003 and M850H004. Recovery data were reported for each mass transition considered.
LOD	0.0002 mg/kg
LOQ	0.001 mg/kg (lowest fortification level)

Levels of Fortification	0.001 mg/kg and 0.01 mg/kg
Time Required	A set of 13 samples requires approximately 8 hours of work (calculation of the results excluded).

6. DISCUSSION

Linearity
Good linearity ($r^2 \geq 0.99$) was observed in the range of 0.25 ng/mL to 10 ng/mL in Trial 1 for BAS 850 H and M850H001. Good linearity ($r^2 \geq 0.99$) was observed in the range of 0.025 ng/mL to 10 ng/mL in Trial 1 for M850H002, M850H003 and M850H004.

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

All analytes have been shown to be stable in stock and fortifcation solutions prepared in methanol with 0.1% formic acid for at least 90 days when stored under refrigeration. Each analyte has been shown to be stable in calibration standard solutions prepared by serial dilution of the intermediate standard solutions with methanol-water with 0.1% formic acid (20:80 v/v) and held under refrigeration for at least 30 days.

Extract Stability
Extract stability was not established in this study. Extract stability data was generated within the BASF freezer storage stability study (Reference 3). Extract stability in the combined extract solution (70:30 methanol-water with 0.1% formic acid, v/v) and final volume solution (20:80 methanol-water with 0.1% formic acid, v/v) was established in soil (clay loam and loamy sand) for BAS 850 H and its metabolites and proven to be stable for 22-23 days.

Specificity
Method D1401/02 determines residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in soil. No interfering peaks were found at the retention time for any of the analytes.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)
The LOQ of the method was set at 0.001 mg/kg in soil for BAS 850 H, M850H001, M850H002, M850H003 and M850H004, which was lower than the lowest relevant endpoint in soil ecotoxicology (LC50 > 1000 mg/kg of active ingredient in dry soil). The LOQ is also defined as the lowest fortification level for each analyte. The LOD for BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in soil was set at 0.0002 mg/kg, which was 20% of the defined LOQ. The LOD for each analyte was shown to be detectable as the absolute amount of analyte injected

(0.01 ng of BAS 850 H and M850H001 on-column, 0.001 ng of M850H002, M850H003 and M850H004 on-column) with acceptable signal to noise ratio (S/N > 3:1).

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

It was demonstrated that the method D1401/02 fulfills the requirements with regards to specificity, repeatability, LOQ, LOD, linearity, and recoveries and is therefore applicable to correctly determine residues of BAS 850 H, M850H001, M850H002, M850H003 and M850H004 in soil.

7. RECOMMENDATIONS/CONCLUSIONS FROM ILV

This ILV was successfully completed in the first trial at EPL Bio Analytical Services. 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 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 recommended 10 µL injection volume was not suitable for quantitation during the ILV study. Therefore the 40 µL injection volume was used to obtain the acceptable sensitivity.

The method is well-written and contains enough 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.

8. PROTOCOL, AMENDMENTS, AND DEVIATIONS

No protocol amendment was issued.

No deviation was found during the study.

Table 11 Example Standard Solutions Preparation and Dilution Data

Stock Standard Solutions

Standard ID#	Analyte	Parent Standard ID#	Net Weight (g)	Dilution Volume (mL)[a]	Final Conc. (µg/mL)	Prep. Date
2017-1511	BAS 850 H	17-108	0.01012	10	1012.000	8/4/2017
2017-1512	M850H001	17-105	0.01012	10	1012.000	8/4/2017
2017-1513	M850H002	17-104	0.00999	10	999.000	8/4/2017
2017-1514	M850H003	17-107	0.01007	10	1007.000	8/4/2017
2017-1515	M850H004	17-106	0.01006	10	1006.000	8/4/2017

[a] Prepared in 0.1% formic acid in methanol

Fortification Standard Solutions

Standard ID#	Analytes	Parent Standard ID#	Parent Conc. (µg/mL)	Aliquot Volume (mL)	Dilution Volume (mL)[b]	Final Conc. (µg/mL)	Prep. Date
2017-1516	BAS 850 H	2017-1511	1012.000	0.5	50	10.120	8/4/2017
	M850H001	2017-1512	1012.000			10.120	
2017-1517	BAS 850 H	2017-1516	10.120	5.0	50	1.012	8/4/2017
	M850H001		10.120			1.012	
2017-1518	BAS 850 H	2017-1517	1.012	5.0	50	0.101	8/4/2017
	M850H001		1.012			0.101	

[b] Prepared in 0.1% formic acid in methanol

Standard ID#	Analytes	Parent Standard ID#	Parent Conc. (µg/mL)	Aliquot Volume (mL)	Dilution Volume (mL)[c]	Final Conc. (µg/mL)	Prep. Date
2017-1519	M850H002	2017-1513	999.000	0.5	50	9.990	8/4/2017
	M850H003	2017-1514	1007.000			10.070	
	M850H004	2017-1515	1006.000			10.060	
2017-1520	M850H002	2017-1519	9.990	5.0	50	0.990	8/4/2017
	M850H003		10.070			1.007	
	M850H004		10.060			1.006	
2017-1521	M850H002	2017-1520	0.999	5.0	50	0.0999	8/4/2017
	M850H003		1.007			0.101	
	M850H004		1.006			0.101	

[c] Prepared in 0.1% formic acid in methanol

Calibration Standard Solutions

Standard ID#	Analytes	Parent Standard ID#	Parent Conc. (ng/mL)	Aliquot Volume (mL)	Dilution Volume (mL)[a]	Final Conc. (ng/mL)	Prep. Date
2017-1750	BAS 850 H	2017-1524	101.000	2.5	25	10.100	9/7/2017
	M850H001		101.000			10.100	
2017-1749	BAS 850 H	2017-1524	101.000	1.25	25	5.050	9/7/2017
	M850H001		101.000			5.050	
2017-1748	BAS 850 H	2017-1524	101.000	0.625	25	2.525	9/7/2017
	M850H001		101.000			2.525	
2017-1747	BAS 850 H	2017-1750	10.100	3.125	25	1.263	9/7/2017
	M850H001		10.100			1.263	
2017-1746	BAS 850 H	2017-1749	5.050	2.5	25	0.505	9/7/2017
	M850H001		5.050			0.505	
2017-1745	BAS 850 H	2017-1749	5.050	1.25	25	0.253	9/7/2017
	M850H001		5.050			0.253	

[a] Prepared in methanol-water with 0.1% formic acid (20:80, v/v)

Table 12 Instrument Conditions and Parameters

UPLC Conditions (BAS 850 H & M850H001)			
Chromatographic System:	Agilent 1290 system		
Column:	Waters Xbridge C18 2.5 µm 4.6x50mm		
Temperature:	50 °C		
Flow rate (µL/min):	800		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	70	30
	0.05	70	30
	6.00	50	50
	8.00	1	99
	8.45	1	99
	8.50	70	30
	10.00	70	30
Mobile Phase A:	1% formic acid in DI water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	40 µL		

MS/MS Conditions							
Detection System:	Sciex 6500 Triple Quad						
Ionization:	ESI						
Polarity:	Positive						
Curtain Gas (CUR, psi):	15						
Collision Gas (CAD):	11						
Temperature (TEM, °C):	650						
Ion Source Gas 1 (GS1, psi):	70						
Ion Source Gas 2 (GS2, psi):	70						
Ion Source Voltage (IS, V):	5000						
MRM Conditions	Transition (*m/z*)	Dwell (msec)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
BAS 850 H (Reg. No. 5654329)	413 → 74	50	100	10	83	12	7.3
	413 → 134		100	10	78	12	
M850H001 (Reg. No. 5749359)	397 → 114	50	70	10	88	16	4.7
	397 → 141		70	10	61	16	

UPLC Conditions ((M850H002, M850H003, & M850H004)			
Chromatographic System:	Agilent 1290 system		
Column:	Waters Xbridge C18 2.5 µm 4.6x50mm		
Temperature:	50 °C		
Flow rate (µL/min):	800		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	70	30
	0.05	70	30
	1.90	40	60
	2.50	1	99
	3.45	1	99
	3.50	70	30
	5.00	70	30
Mobile Phase A:	1% formic acid in DI water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	40 µL		

MS/MS Conditions							
Detection System:	Sciex 6500 Triple Quad						
Ionization:	ESI						
Polarity:	Negative						
Curtain Gas (CUR, psi):	25						
Collision Gas (CAD):	10						
Temperature (TEM, °C):	400						
Ion Source Gas 1 (GS1, psi):	60						
Ion Source Gas 2 (GS2, psi):	40						
Ion Source Voltage (IS, V):	-3000						
MRM Conditions	Transition (*m/z*)	Dwell (msec)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
M850H002 (Reg. No. 5757725)	373 → 193	50	-80	-10	-53	-20	2.8
	373 → 323		-80	-10	-31	-20	
M850H003 (Reg. No. 5757726)	357 → 307	50	-110	-10	-32	-20	2.1
	357 → 137		-110	-10	-51	-20	
M850H004 (Reg. No. 5833884)	385 → 103	50	-40	-10	-15	-10	2.9
	385 → 255		-40	-10	-16	-10	

Figure 1 Residue Calculations for Soil

Data was acquired with validated Analyst software. The data processing was completed in MultiQuant, which is a companion software program accessed via Analyst.

For the validation recoveries, the exact sample weight was used in calculating the final residues (ppm).

The following equations are used for residue and recovery calculations for BAS 850 H and its metabolites M850H001, M850H002, M850H003 and M850H004 in soil:

a) Residue Found (mg/kg) =

$$\frac{\text{Sample Analytical Result (ng/mL)} \times V_{end}\text{ (mL)}}{\text{Aliquot Factor * Sample Weight (g)} \times 1000\text{ (ng/mg)}}$$

The Aliquot Factor and Final Volume calculations for soil aliquot A samples (BAS 850 H and M850H001 determinations):

1. Without dilution, as the final volume (V_{end}) is 1 mL as listed in the section 3.7 of method 1401/02:

$$A_F = \frac{10\,(\text{mL}) \times 15\,(\text{mL})}{50\,(\text{mL}) \times 12\,(\text{mL})} = 0.25; \qquad V_{end} = 1\text{ mL}$$

2. For the 10×LOQ samples, dilution is needed. The dilution factor is 5 when 200 µL of sample was combined with 800 µL of the dilution solution (methanol-DI water with 0.1% formic acid, 20:80, v/v). The final volume (V_{end}) is 1 mL:

$$A_F = \frac{10\,(\text{mL}) \times 15\,(\text{mL})}{50\,(\text{mL}) \times 12\,(\text{mL}) \times 5} = 0.05; \qquad V_{end} = 1\text{ mL}$$

The Aliquot Factor and Final Volume calculations for soil aliquot B samples (M850H002, M850H003, and M850H004 determinations):

Without dilution, the final volume (V_{end}) is 1 mL as listed in section 3.8 of method 1401/02:

$$A_F = \frac{1.25\,(\text{mL})}{50\,(\text{mL})} = 0.025; \qquad V_{end} = 1\text{ mL}$$

b) Fortification Level (ppm, mg/kg) =

$$\frac{\text{Volume Spiking Solution (mL)} \times \text{Spiking Solution Conc (µg/mL)}}{\text{Sample Weight (g)}}$$

c) Recovery (%) = $\dfrac{\text{Residue Found in Spike (mg/kg)} \times 100}{\text{Fortification Level (mg/kg)}}$

As an example, calculations to obtain BAS 850 H (primary transition) recovery results using 1602-S001-S5A from data set V001A are shown below:

a) Calibration curve: y = (3.39553e4)x - 149.67213

Solving for x: **Error! Bookmark not defined.**$x = \frac{40641 + 149.67213}{3.39553e4} = 1.20130$ ng/mL

b) Residue found (mg/kg) = $\frac{1 \text{ mL} \times 1.20130 \text{ ng/mL}}{4.958 \text{ g} \times 0.25 \times 1000} = 0.000969$ mg/kg

c) Spike Level (mg/kg) = $\frac{0.05 \text{ mL} \times 0.101 \text{ µg/mL}}{4.958 \text{ g}} = 0.00102$ mg/kg

d) Recovery (%) = $\frac{0.000969 \text{ mg/kg}}{0.001102 \text{ mg/kg}} \times 100 = 95.2\%$

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

Appendix A. Recommendations for BASF Analytical Method D1401/02

The following recommendations should be incorporated into the technical procedure:

Section 4.2.2: Instrumentation and Conditions (M850H002, M850H003, & M850H004)

Method C (HPLC Mode):

> In the ILV study, 10 ul injection volume resulted low signals with unacceptable signal to noise values for analytes, M850H003 [confirmatory transition (m/z 357 → 137)] and M850H004 [confirmatory transition (m/z 385 → 255)] and this was observed during system suitability test. Consequently, the 10 µL injection volume was not suitable for quantitation. Instead, 40 ul injection volume was used to get adequate peak sensitivity (S/N at least >3) and acceptable quantitation

BASF
The Chemical Company

Final 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

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

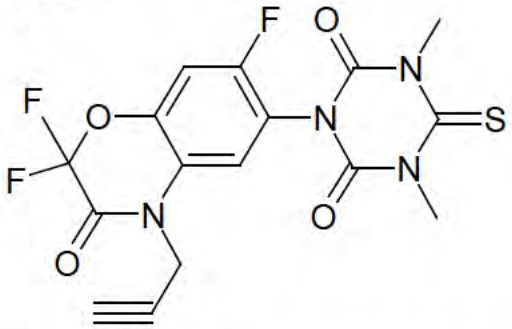
2.1 Safety

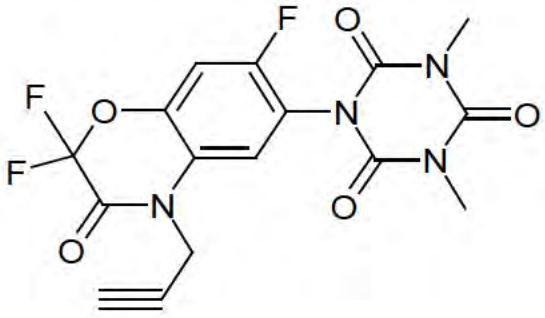
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Ensure that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

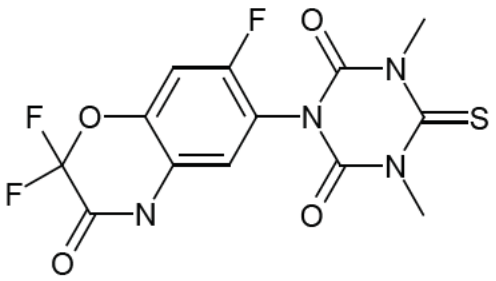
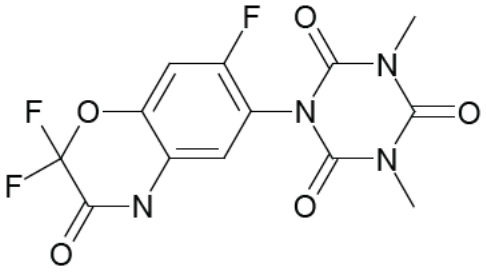
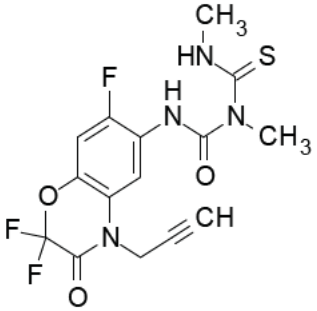
Standard substances are stored in a freezer (≤-5 °C) until use.

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-ethylacetate 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 B and D, used for UPLC method)	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 A and C, used for HPLC mode)	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 <u>(BAS 850 H, M850H001)</u>

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:

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

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.

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

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 III:

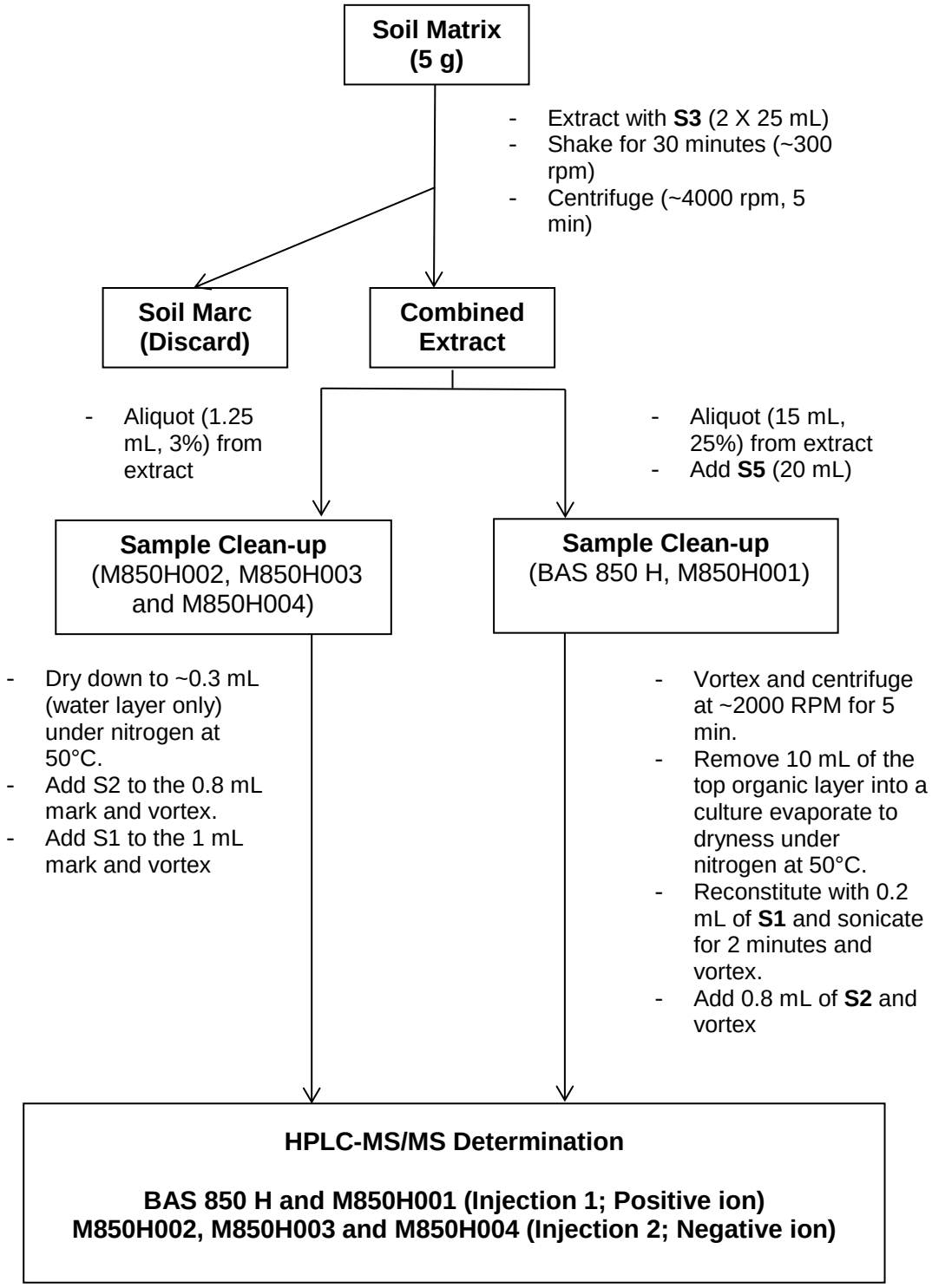
c) $$\text{Recovery \%} = \frac{\text{Residue in fortified sample} - \text{Residue in contol}}{\text{Amount of analyte fortified}} \times 100$$

Soil residues based on soil dry weight

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

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
Added in the validation

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.