FINAL REPORT

Study Title

Independent Laboratory Validation of BASF Analytical Method R0048/01, "Method for the Determination of M850H040 (Reg. No. 6095223) in Surface and Drinking Water by 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 R0048/01 was developed and validated at to determine the residues of M850H040 (Reg. No. 6095223) in surface and drinking water using LC-MS/MS at BASF Agricultural Solutions, Research Triangle Park, NC (Reference 1). The method was independently validated at OMIC USA, Inc., Portland, OR.

The independent lab validation was conducted using two fortification levels, at the LOQ (30 ng/L) and ten times the LOQ (300 ng/L), for surface and drinking water. For each fortification level and matrix, five replicates were analysed. Additionally, two replicates of unfortified samples and one reagent blank were examined.

1.2 Principle of the Method

Surface and drinking water samples were prepared for determination of the residues of M850H040 by filtering an aliquot through a 0.45 µm pore size PTFE filter, after discarding the initial 0.1-0.2 mL which passed through the filter.

The final determination was conducted using LC-MS/MS in negative electrospray mode. For M850H040, the transition at *m/z* 429→335 was monitored for primary quantitation and the transition at *m/z* 429→296 was monitored for confirmation.

1.3 Specificity

Residues of M850H040 were identified and quantified as an individual analyte, not converted to the equivalent parent concentrations of BAS 850 H. The LC-MS/MS method, utilizing reverse phase (C18) chromatography and negative mode electrospray ionization (ESI), was used for the determination of M850H040, monitoring two transitions (primary quantitation and confirmation).

No interfering peaks were found at the retention time of the analyte in either surface or drinking water matrices. No significant matrix effects (<20% suppression or enhancement) were observed for surface or drinking water, therefore calibration standards, prepared with purified water, were used for analysis.

2 REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test Systems

The following test systems were considered in this study:

Test System 1: **Drinking Water (Well), Bahama, NC** (BASF Sample Name CM17-053)
OMIC sample ID: 18CB03_DW, sample aliquots designated with prefix of: 18DW

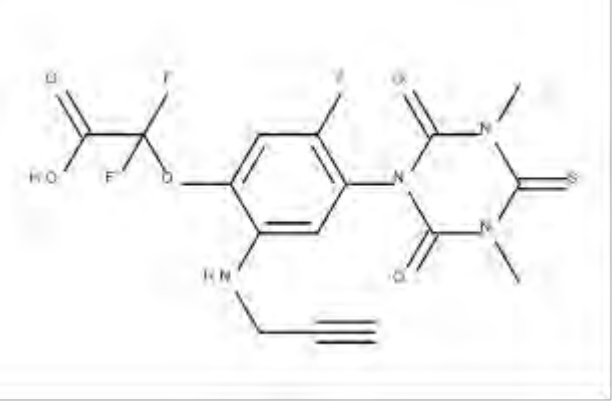
Test System 2: **Surface Water, Golden Lake, ND** (BASF Sample Name CM17-052)
OMIC sample ID: 18CB03_SW, sample aliquots designated with prefix of: 18SW

The sponsor provided containers of frozen samples for each of the test systems. One sample number was assigned for each test system upon receipt. All test systems were stored frozen (-30°C) prior to use. Each test system was allowed to thaw at room temperature prior to aliquotation of the sample for analysis. The description and characterization of the test systems used is given in the respective attached certificates (Appendix 10.7).

2.2 Test Substance

Test substance was provided by the sponsor, BASF, and stored in a freezer (-18°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 Agricultural Solutions 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 10.6.

M850H040

BASF Reg. No.	6095223
Molecular Formula	$C_{16}H_{13}F_3N_4O_5S$
Molecular Weight	430.4 g/mol
IUPAC Name	{4-(3,5-dimethyl-2,6-dioxo-4-sulfanylidene-1,3,5-triazinan-1-yl)-5-fluoro-2-[(prop-2-yn-1-yl)amino]phenoxy}(difluoro)acetic acid
Batch No.	L2017-109
Purity (%)	87.2
Storage Advice	Refrigerator or Freezer
GLP	Yes
Expiration Date	December 1, 2019
Chemical structure	

Standard Stability

Standard stability was not established in this study and was determined during validation of the method (Reference 1). M850H040 indicated sufficient stability in stock (methanol) and fortification solutions (methanol) for 32 days, when stored in the refrigerator. Stability of intermediate and calibration standard solutions (purified water) demonstrated sufficient for 32 days, when stored in the refrigerator.

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. An example of preparation and dilution scheme is presented in Appendix 10.3.

3 ANALYTICAL PROCEDURE

3.1 Validation

BASF Method R0048/01 was used during independent laboratory validation in which 12 samples were used for each matrix (drinking and surface water): Two control samples, five samples treated at the LOQ and five treated at ten times the LOQ. Additionally, one reagent blank (purified water) was included with each set.

3.2 Route of Administration

For each sample, 5 mL (± 0.1 mL) of matrix was measured using a calibrated digital pipette (1-10 mL size) into a glass culture tube and fortified with the appropriate fortification solution using a calibrated digital pipette (10-100 µL size).

The following scheme was used for fortification of the samples:

Sample Type	Sample Volume	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [µL]	Level of Fortification [ng/L]
Control	5 mL	-	-	0.00
Fortification (LOQ)	5 mL	0.003	50	30*
Fortification (10×LOQ)	5 mL	0.03	50	300

* Limit of quantitation

3.3 Preparation for Measurement

The samples were capped and vortexed at high speed to achieve a homogeneous solution. The solution was filtered through a 0.45 µm PTFE syringe filter into an glass autosampler vial, after discarding the first 0.1-0.2 mL that passed through the filter.

For control and LOQ fortification levels, samples are ready for injection. For 10×LOQ fortification levels, samples were diluted 10 times with solvent (purified water) prior to injection**.** The final concentration of each sample, as prepared, is presented below:

Sample Type	Level of Fortification [ng/L]	Dilution Factor	M850H040 Final Concentration (ng/mL)
Control	0.00	1	0.0
Fortification (LOQ)	30*	1	0.03
Fortification (10×LOQ)	300	10	0.03

* Limit of quantitation

The example of recovery calculation is provided in Appendix 10.1. The validation data including the detail analytical data for each matrix types are provided in Appendix 10.2. The working procedure of the method BASF Method R0048/01 is provided as Attachment A.

Stability of Extracts

Extract stability was not established in this study and was determined during validation of the method (Reference 1). M850H040 indicated sufficient stability in water extracts for approximately 1 week, when stored in the refrigerator. All sample analysis was completed within 1 day of initial preparation.

3.4 Influence of Matrix Effects on Analysis

Due to an increase of injection volume from the method proposed 10 µL, to 50 µL for this study, the degree of matrix effects on the response of M850H040 was assessed. Matrix matched standards and solvent standards were analyzed in a separate experiment to evaluate any potential matrix effects on LC-MS/MS analysis. The matrix-matched standards were prepared by adding an aliquot of each of the control matrices (99%) to each of three solvent standards (1%) at the desired standard concentrations of half the LOQ, LOQ, and two times the LOQ or 0.015, 0.03 and 0.06 ng/mL, respectively. The solvent standards, used for assessment, were prepared in the same manner, adding purified water (99%) to achieve the equivalent standard concentration levels.

The data generated was evaluated by comparing the average area response of the standards for three injections without matrix and three injections with matrix, for each of the three standard concentration levels. For each test system (drinking and surface water), the difference was not shown to be significant (<20% difference). Results are provided in Appendix 10.2, Tables 5-6.

4 RESULTS

Control samples were treated in exactly the same way as fortified samples. All results obtained from measurements of control samples were below LOD (6 ng/L) for M850H040. No interfering peaks were found at the retention time of M850H040 and no blank correction in the recovery data was needed. No significant matrix effects (<20% suppression or enhancement) were observed for surface or drinking water test systems, therefore calibration standards prepared with purified water were used for analysis.

Quantitation of M850H040 was done by LC-MS/MS according to method R0048/01, Section 4.2.1, with the exception of a different detector system (QTrap 6500). To obtain sensitivity of the analyte, the injection volume was raised to 50 µL from the method proposed 10 µL. Furthermore, the chromatographic column rinse and re-equilibration steps were extended by one minute each, for a total run time of five minutes. Two transitions were monitored (*m/z* 429 → 335, primary quantitation and *m/z* 429 → 296, confirmation), in negative electrospray ionization mode (ESI). Instrument parameters used during this independent validation are presented in Appendix 10.4.

The validation of surface and drinking water was completed after two trials for each matrix. The trials were repeated due to insufficient instrument performance during initial analysis. Reinjection was not possible due to lapse of extract storage time (greater than one week). The recoveries obtained from the second trial in surface and drinking water were acceptable,

Statistics and Data Integrity

Statistical treatment of the data included simple descriptive statistics, such as determinations of averages, standard deviation and/or relative standard deviation (RSD) for the procedural recoveries and area counts and calculation of the calibration curve and correlation coefficient (r) by linear regression of the instrument responses for the reference standards. The statistical calculations throughout this report were performed using an automated computer spreadsheet (Microsoft Excel®) and were rounded for presentation purposes. Slight differences may be noted in hand calculations using the recoveries presented in the tables. These are due to rounding and have no effect on the scientific conclusions presented in this report. The detailed analytical data may be consulted for confirmation of the calculated results.

Several measures were taken to ensure the quality of the study results. The quality assurance unit at OMIC USA inspected the analytical procedures for compliance with Good Laboratory Practices that included adherence to the protocol. The dates inspected are detailed in the quality assurance unit statement. Study samples and test/reference items were maintained in secured (i.e. locked) storage with limited access. Freezer and refrigerator temperatures were continuously monitored, and records are included in the raw data.

5 SUMMARY OF THE METHOD

Type of method: Filtration, LC-MS/MS

Test systems: Drinking Water (well)
Surface Water (lake)

Analyte and selected mass transitions (*m/z*):

M850H040	429 → 335*
	429 → 296

* Primary quantitation transition

Analytical procedure: BASF method R0048/01: Method for the Determination of M850H040 (Reg. No. 6095223) in Surface and Drinking Water by LC-MS/MS"

Confirmatory technique: 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.

LOD: 6 ng/L (ppt)

LOQ: 30 ng/L (ppt) (lowest fortification level)

Levels of fortification: LOQ and 10×LOQ

Time required: A set of 13 samples requires about half a day (4-6 hours) of work (This time included the evaluation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP)

Appendix 10.1: Example Calculations of Residues and Recoveries

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

For the calculation of recoveries, the nominal sample volume was considered 5 mL in the final calculation of residues in ng/L (ppt).

The residues of M850H040 in ng/L (ppt) are calculated as shown in equations **I** and **II**:

I. Concentration in Final Volume (ng/mL) $= \frac{\text{Response} - Intercept}{Slope} \times DF = C_{End}$

II. Residues in the Sample Matrix (ng/L) $= \frac{V_f \times C_{End} \times 1000}{G \times A_F}$

V_f	=	Final volume (mL)
C_{End}	=	Concentration of analyte obtained from the calibration curve (ng/mL)
DF	=	Dilution Factor, included in calculations of C_{End}
G	=	Volume of sample extracted (mL)
A_F	=	Aliquotation factor
1000	=	Unit Conversion (mL/L)

Note: Results are expressed as analyte concentration, not converted to parent equivalent.

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:

M850H040, primary quantitation (*m/z* 429 → 335), in surface water, fortified at 30 ng/L (LOQ):

The following values were used in this calculation:

OMIC Sample ID (extracted 07/18/2018)	**18SW2-08**
Analytical Report	20180718_SurfaceWater_SW2.pdf
Peak area of fortified sample (18SW2-08)	23,517
Peak area of control sample (18SW2-02)	N/A, no peak integrated
Calibration Slope	8.51955e5 or 851,955
Intercept	929.48995
Dilution Factor (DF)	1 (included in calculation of C_{End})
Sample Volume (G)	5 mL (nominal volume)
Final Volume (V_f)	5 mL (nominal volume)
Aliquotation Factor (A_F)	1 (100%)

Concentration of fortified sample (C_{End}) = $\frac{23517 - (929.48995)}{851955} \times 1 =$ 0.02651 ng/mL

Residue (fortified sample) = $\frac{0.02651 \text{ ng/mL} \times 5\text{mL} \times 1000}{5\text{mL} \times 1}$ = 26.510 ng/L

Recovery (%) = $\frac{(26.510 \text{ ng/L} - 0 \text{ ng/L})}{30 \text{ ng/L}} \times 100$ = **88%**

*reported value is 88.4% due to full precision of area counts used for reported results.

MultiQuant Software version 3.0.2 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 10.3: Standard and Fortification Solutions Preparation

Stock Solutions

A stock solution of M850H040 was prepared at a concentration of approximately 1 mg/mL in methanol by accurately weighing 12.24 mg 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. The relative purity 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 (mg)	Concentration (mg/mL)
M850H040	87.2 %	12.24	1.067

Fortifications Solutions

A fortification solution (10 µg/mL) was prepared by diluting the stock solution of M850H040 into a 50 mL volumetric flask and diluting to volume with methanol. Fortification solutions were prepared using a dilution series as exemplified in the tables below.

Solution Name	Take solution concentration (µg/mL)	Volume (mL)	Dilute with methanol to a final volume of (mL)	Concentration (µg/mL)
Spike A	1067 (Stock)	0.469	50	10
Spike B	10	5.0	50	1
10x LOQ Fortification	1	1.5	50	0.03
LOQ Fortification	0.03	5.0	50	0.003

Calibration Standard Solutions

Intermediate standard solutions were prepared in a separate dilution series using HPLC grade water, as exemplified in the tables above. Calibration solutions were prepared by dilution of an intermediate solution using HPLC grade water. Each set of standards used for the calibration of the LCMS/MS was aliquoted fresh for each analytical run. An example dilution scheme is shown in the following table.

Solution Name	Take solution concentration (ng/mL)	Volume (mL)	Dilute with water to a final volume of (mL)	Concentration of each analyte (ng/mL)
Calibration 1	30	1.67	50	1.0 [a]
Calibration 2	1.0	7.5	50	0.15
Calibration 3	1.0	3.0	50	0.06
Calibration 4	1.0	1.5	50	0.03 [b]
Calibration 5	1.0	0.75	50	0.015
Calibration 6	1.0	0.30	50	0.006 [c]

[a]This solution concentration was not used in the calibration curve.
[b]This concentration is equivalent to LOQ
[c]This concentration is equivalent to LOD (20% of LOQ)

Appendix 10.4: Instrument Conditions and Parameters

LC-MS/MS Conditions (Primary and Confirmatory Transitions)

	Parameter
Chromatographic System	Waters Acquity I Class UPLC (CM/SM-FTN/BSM)
Analytical-column	Waters Acquity BEH C18 (2.1x50mm, 1.7µm)
Column Temperature	50°C
Injection Volume	50 µL
Mobile Phase A	Water with 0.1% Formic Acid
Mobile Phase B	Acetonitrile with 0.1% Formic Acid
Flow Rate	0.6 mL/min
Gradient	Time (min) / % Phase A / % Phase B: 0.0 / 85 / 15; 1.0 / 50 / 50; 1.25 / 5 / 95; 3.25 / 5 / 95; 3.5 / 85 / 15; 5.0 / 85 / 15
Detection System	AB Sciex QTrap6500 Mass Spectrometer
Source Ionization	Turbo Spray IonDrive Electrospray (ESI)
Polarity	Negative
Ionization Temperature	450 °C
IonSpray Voltage	-4500
Curtain Gas	40
Ion Source Gas 1	60
Ion Source Gas 2	50

Analyte	**Transitions (*m/z*)**	Dwell Time (msecs)	DP	CE	EP	CXP	Retention Time (min)
M850H040	429 → 335*	30	-36	-20	-10	-13	~ 1.35
	429 → 296	50	-36	-37	-10	-13	

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

The injection volume was raised from 10 µL to 50 µL to obtain sensitivity of the analyte at the LOD (greater than 3:1 signal to noise) for both primary and confirmation transitions. Therefore, the chromatographic column rinse, and re-equilibration gradient steps were extended by one minute each, for a total run time of 5 minutes. The divert valve (prior to the detection system) was utilized before and after elution of the analyte from the chromatographic column, to reduce the matrix load on the detection system.

ATTACHMENT A: Method Procedure

BASF Method No. R0048/01:
“Method for the Determination of M850H040 (Reg. No. 6095223) in Surface and Drinking Water by LC-MS/MS”

We create chemistry

Method Procedure:

Method for the determination of M850H040 (Reg.No. 6095223) in Surface and Drinking Water by LC-MS/MS

BASF Method Number R0048/01

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 (it can be acquired from the same study or from a different source e.g. a different study or commercially purchased).
Unknown Sample:	A sample with unknown residues.
Treated Sample:	A sample that has been treated with the test substance.
(Solvent) Blank:	Solvent, solution or mobile phase injected together with a sample set during the analytical run.
Reagent Blank:	A sample consisting of solvents and reagents only in the absence of any sample matrix subjected to complete analysis according to the method. This type of sample is also known as "blank of reagents" or "procedural blank," and is analyzed within the sample set in order to evaluate possible contamination from chemicals/reagents.
Procedural Recovery: **(Fortification Sample)**	A control sample to which a known amount of analyte has been added before sample work up. This sample is then extracted and analyzed according to the method along with, or "concurrently", with the unknown samples in order to determine the performance of the method.
Instrument Recovery:	A control sample carried through the method to which a known amount of analyte has been added directly before injection. This sample is analyzed within the sample set in order to evaluate the matrix effect on the instrument response. The instrument recovery is also known as a "matrix-matched standard."
Analytical Run:	A group of samples that undergo a determinative measurement on an analytical instrument 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 analytical method. This is also known as reporting limit.
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 3x baseline noise).

1 Introduction

BAS 850 H is a PPO herbicide, and is developed by BASF to be used for a broad spectrum of crops in the US. The analytical method R0048/01 allows for the determination of M850H040 residues in surface and drinking water.

BASF Method Number R0048/01 was successfully tested during method development in surface and drinking water.

This method was developed at BASF Agricultural Solutions, Research Triangle Park, NC, USA.

2 Materials

2.1 Safety

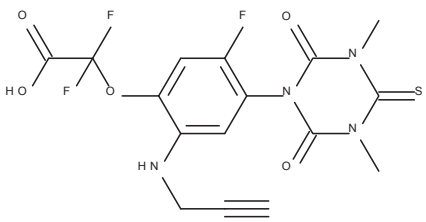
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Store work clothing 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. All procedures involving organic solvents should be performed in a well-ventilated hood.

Safety details are given in the Safety Data Sheets (SDS) of the individual substances. The SDS should be considered before start of a study/handling the substance.

Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

2.2 Test and Reference Items

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

BAS-Code	M850H040	
IUPAC Name	{4-(3,5-dimethyl-2,6-dioxo-4-sulfanylidene-1,3,5-triazinan-1-yl)-5-fluoro-2-[(prop-2-yn-1-yl)amino] phenoxy}(difluoro)acetic acid	
Reg. No.	6095223	
CAS-No.	N/A	
Molecular Formula	$C_{16}H_{13}F_3N_4O_5S$	
Molecular Weight	430.4 g/mol	

2.3 Equipment:

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Analytical	Model AT100	Mettler	----
Balance, Top Loader	Model PJ3600	Mettler DeltaRange	----
Beakers	Various sizes	PYREX Brand, VWR Scientific Products	13922-029
Bottle, Amber glass	Qorpak, 2 oz and 4 oz with Teflon®-lined cap	VWR Scientific Products Boston Round, Amber	89042-908
Culture tube caps	16 mm	VWR	60828-768
Culture Tubes	Glass, disposable, 16x100mm size	Fisher	14-961-29
Cylinder, Graduated	Various sizes	Various	----
Flasks	Various sizes	Various	----
Plastic syringe	1 mL	Various	----
Positive Displacement Pipette and tips	1000 µL, 250 µL, 25 µL	Gilson Microman Fisher Scientific	----
Repeater Pipette and tips	50 mL	BrandTec Scientific	----
Syringe filter	PTFE Acrodisc® 0.45 µm pore size	Pall Gelman	4543
Volumetric, pipettes	0.5 mL, 1 mL, 2.5 mL, 5 mL, 10mL, 20 mL	Various – Class A	----
Volumetric flask	Various sizes	Various – Class A	----
Vortex	Genie 2	VWR Scientific Products	14216-184
LC System	Acquity UPLC I-Class System	Waters	----
Mass Spectrometer	Sciex 5500 Mass Spectrometer	Sciex	----
HPLC Column	Acquity BEH C18, 2.1x50 mm, 1.7 um	Waters	186002350

Note: The equipment and instrumentation listed above represents typical laboratory equipment and can be substituted by equipment of similar technical specifications. Suitability of the entire set of equipment is confirmed if the recoveries of the fortification experiments are in the expected concentration range.

2.4 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Formic acid (LC Mobile Phase Use)	95%	Sigma Aldrich	F0507-100 mL
Methanol	HPLC Grade	MilliporeSigma	MX0475P-1
Acetonitrile	HPLC Grade	MilliporeSigma	AX0156-1
Water	HPLC Grade	BDH Aristar Plus	87003-652

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

2.5 Solvent Mixtures

Description	Code	Composition
Mobile Phase A	**LC1**	0.1% Formic Acid in Water Add 1 mL of formic acid to 1 L of water into an appropriate container and mix well to ensure complete homogeneous solution.
Mobile Phase B	**LC2**	0.1% Formic Acid in Acetonitrile Add 1 mL of formic acid to 1 L of acetonitrile into an appropriate container and mix well to ensure complete homogeneous solution.

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

2.6 Working Solutions

2.6.1 Stock Solutions

Prepare a 1.0 mg/mL stock solution individually by weighing an appropriate amount of analyte into a flask and add the required volume of methanol. To calculate the final true concentration, the actual weight (and if required purity see below) has to be documented and taken into account.

For example, to prepare 10 mL of 1.0 mg/mL stock solution of M850H040 in methanol, weigh 10 mg M850H040 into a 10 mL volumetric flask. Dissolve and dilute to mark with methanol. Ensure a complete homogeneous solution (e.g. by sonication or vortexing).

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

- Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.

- Fortification and calibration standard solutions should be prepared from one stock solution in separate dilution series.

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

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

2.6.2 Fortification Solutions

Prepare standard solutions in a flask. Dilute, e.g. volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by vortexing).

Examples for Preparation of Fortification Solutions

Initial Concentration (µg/mL)	Aliquot Volume (mL)	Dilute with methanol to a final volume of (mL)	Final Concentration (µg/mL)
1000	0.25	25	10
10	1.0	10	1
1	0.75	25	0.03
0.03	2.5	25	0.003

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

2.6.3 Calibration Standard Solutions

Prepare standard calibration solutions for LC-MS/MS analysis by using the solutions that were prepared in Section "stock solutions" or "fortification solutions" in flasks. Dilute e.g. volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by vortexing).

Examples for Preparation of standard solutions for calibration

Initial Concentration (ng/mL)	Aliquot Volume (mL)	Dilute with water to a final volume of (mL)	Final Concentration (ng/mL)
30*	1.0	30	1.0
1.0	3.75	25	0.15†
1.0	1.5	25	0.06†
1.0	0.75	25	0.03†
1.0	0.375	25	0.015†
1.0	0.15	25	0.006†

* Solution is in methanol, prepared in section 2.6.2
† Proposed solutions to be used for calibration curve

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

2.6.4 Matrix matched Standards

In case matrix-matched standards (= 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.

Examples for Preparation of matrix-matched standards for calibration

Initial Concentration (ng/mL)	Aliquot Volume (mL)	Dilute with control water matrix to a final volume of (mL)	Final Concentration (ng/mL)
30*	0.10	3.0	1.0
1.0	0.30	2.0	0.15†
0.15	0.40	1.0	0.06†
0.15	0.20	1.0	0.03†
0.15	0.10	1.0	0.015†
0.15	0.040	1.0	0.006†

* Solution is in methanol, prepared in section 2.6.2
† Proposed solutions to be used for calibration curve

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

2.6.5 Stability of Standard Solutions

Stability of M850H040 solutions in methanol (stock and fortification solutions) is at least 32 days when stored under refrigerated conditions. Stability of calibration standard solutions in water is 32 days when stored under refrigerated conditions [Reference 1].

3 Analytical procedure

The methodology described below can be scaled up and/ or down as long as proportions are kept constant.

3.1 Sample Preparation

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

3.2 Weighing and Preparation of Fortified / Treated Samples

For fortified samples, 5.0 ± 0.1 mL of control water sample are measured into a suitable glass container to allow proper mixing, e.g. a disposable culture tube. Appropriate amounts of fortification solutions are added to the matrix according to the table below.

The following fortification scheme may be used:

Sample Type	Sample Volume	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	5.0 mL	-	-	-
Fortification (LOQ)	5.0 mL	0.003 µg/mL	0.050 mL	30 ng/L *
Fortification (10xLOQ)	5.0 mL	0.03 µg/mL	0.050 mL	300 ng/L

* limit of quantitation

Note: Different concentration schemes can be used, if different fortification levels are required.

Total volume of solutions prepared can be changed if overall ratios are maintained. Volume of spiking solution added to generate the fortified sample should not exceed 10 % of sample weight or volume.

3.3 Extraction of Water Samples

No extraction is necessary for water matrices.

3.4 Sample Clean-up

No clean-up is necessary for water matrices.

3.5 Preparation for Measurement

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 with water may be necessary to remain in the linear range of the calibration curve.

3.6 Influence of matrix effects on analysis

During method validation, it was demonstrated that the matrix load in the samples from the water matrices had no significant influence on the analysis (i.e., matrix effects < 20%). Therefore, samples can be analyzed using calibration standard solutions prepared in purified water.

If significant matrix effects are observed, matrix-matched standards can be used.

3.1 Stability of Final Volumes

M850H040 has been shown to be stable in final volume for at least the time period tested, 7 days in drinking water and 8 days in surface water [Reference 1].

4 Quantification and calculation

4.1 Set-up of the analytical run

A sequence for measurement generally consists of:
- Calibration standards
- Control samples
- Procedural recovery samples
- Unknown samples
- Instrument recovery sample

Reagent blanks or blanks can also be injected if considered 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 at least be injected twice. At least 5 calibration levels are needed. At least one Instrument recovery sample has to be measured with each sequence if solvent calibration standards are used. In case of Matrix-matched calibration standards, it can be omitted.

4.2 Instrumental analysis

4.2.1 Instrumentation and Conditions for M850H040

	Parameter		
Chromatographic System	Waters Acquity UPLC system**		
Analytical-column	Acquity BEH C18, 2.1 x 50 mm, 1.7 µm particle size		
Column Temperature	50 ºC		
Injection Volume	10 µL (can be raised or lowered depending on sensitivity)		
Mobile Phase A Mobile Phase B	Water with 0.1% formic acid (**LC1**) Acetonitrile with 0.1% formic acid (**LC2**)		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00 1.00 1.25 2.25 2.50 3.00	85 50 5 5 85 85	15 50 95 95 15 15
Detection System	AB Sciex 5500 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	500 °C		
Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
M850H040 (Reg No 6095223)	429 → 335* 429 → 296	Negative	~ 1.7 min

* Proposed as quantification transition. Any of these transitions could be used for quantitation.

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

Note: 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.

A divert valve can be used to reduce the matrix load on the detection system. Instrument conditions, e.g. injection volume, column, gradient steps may be modified; however, changes have to be documented in the raw data. Changes are acceptable, if the recoveries of the fortification experiments are in the acceptable range of the required guidelines.

If the use of different analytical columns (different stationary phase) or mass transitions is required, then methodology has to be validated by analyzing at least five replicates of fortified samples prepared at e.g. LOQ and 10xLOQ. Assessment of matrix impact by preparation of at least one concentration level of a matrix matched standard is also required.

Other parameters such as ion source temperature, gas flows, and voltages, are highly specific to the equipment used and therefore not listed. Those parameters need to be adapted to the actual instrument used. To obtain stable measurements, it is recommended to condition the instrument properly.

4.2.2 Calibration procedures

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

The calibration curve is obtained by direct injection of solvent or matrix standards into LC-MS/MS in the range of e.g. 0.006 ng/mL to 0.15 ng/mL. In a given injection run, the same injection volume is used for all samples and standards.

Linear calibration functions are preferred for evaluation. If other functions are used (e.g. quadratic), this should be fully justified.

4.2.3 Calculation of Residues and Recoveries

Calculation of results is based on area measurements.

For procedural recoveries, the sample volume will be considered to be 5.0 mL in the final calculation of residues [ng/L]. This approach requires that the sample volume has to be within a measurement precision of +/- 0.1 mL for fortification samples (matrix). The recovery is the percentage of the fortified amount of the analyte (µg or ng), which is recovered after the entire sample work-up steps.

Outside of this range, the actual sample weight must be used of the calculation, for this approach the amount of fortified analyte(s) as to be calculated individually, depending on the sample weight.

Calculation is described by the equation given below:
The residues of all analytes in ng/L are calculated as shown in equations **I** and **II**:

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

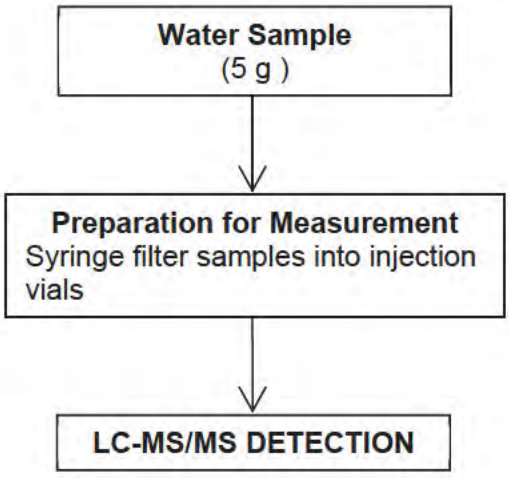
II. **Residue [ng/L]** $= \frac{V_{End} \times C_A \times 1000}{G \times A_F}$

V_{end}	=	Final volume of the extract after all dilution steps [mL]
C_A	=	Concentration of analyte as read from the calibration curve [ng/mL]
G	=	Volume of the sample extracted [mL]
A_F	=	Aliquotation factor
1000	=	Factor remaining after all unit conversions

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

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

5 Flowchart


Water Sample
(5 g)
Preparation for Measurement
Syringe filter samples into injection vials
LC-MS/MS DETECTION

6 Method management and time requirements

Description of the amount of time required for sample preparation, analysis and data reduction.
The analysis of one series of samples (= 13 unknown samples, 2 fortified samples for recovery experiments, 1 blank sample) requires 0.5 working days (4 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

7 Conclusion and method capabilities

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The limit of quantification is defined as the lowest fortification level successfully tested.
The limit of quantification is 30 ng/L.
The limit of detection is 20% of the limit of quantification.
The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

Selectivity
The tested untreated surface and drinking water samples showed no significant interferences (< 20 %) at the retention time of the analyte of interest.

Confirmatory Techniques
The LC-MS/MS determination for M850H040 is a highly selective detection technique. Quantitation is possible at two different mass transitions. Therefore, no additional confirmatory technique is required.

Potential Problems
M850H040 has been observed to be unstable in acidic aqueous solutions. Therefore, acid should be avoided for stored solutions.

9 Appendix

9.1 Example of Calculation

Example: M850H040, 429 → 335; surface water sample fortified at 30 ng/L:

Concentration in the final volume [ng/mL]

Concentration [ng/mL]:

$$= \frac{Response\ -Intercept}{Slope} = C_A$$

Residue in the sample [mg/kg]

Residue [ng/L]:

$$= \frac{V_{End}\ \times\ C_A\ \times 1000}{G\ \times\ A_F}$$

Recovery %:

$$= \frac{(\boldsymbol{Residue\ in\ fortified\ sample\ -Residue\ in\ control}) \times \mathbf{100}}{\boldsymbol{Amoun}\text{t of analyte fortified}}$$

The following values were used in this calculation:

Response of fortified sample	31645
Response of control sample	0
Slope:	1120000
Intercept:	-63.1
Sample Volume (G):	5.0 mL
Final Volume (V_{end}):	5.0 mL
Aliquotation factor A_F:	1 (= 100%)
Conversion factor mL → L:	1000

Concentration (ng/mL):

$$= \frac{31645\ -(-63.1)}{1120000} = 0.0282\ ng/mL$$

Residue (ng/L):

$$= \frac{5.0\ mL\ \times\ 0.0282\ ng/mL\ \times 1000}{5.0\ mL\ \times\ 1} = 28.2\ ng/L$$

Recovery %:

$$= \frac{(28.2\ \text{ng/L}\ -0.00\ \text{ng/L})\ \times 100}{30\ \text{ng/L}} = 94.0\%$$