

2.0 INTRODUCTION

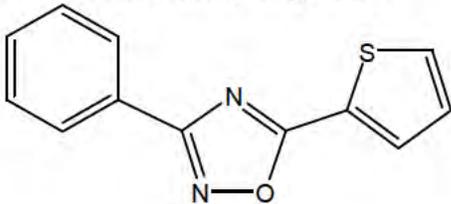
The objective of this study was to independently validate Monsanto EPL-BAS Method 115G761A, entitled “Analytical Method for MON 102100 and Environmental Degradates in Water”. A copy of the method is included in [Appendix 6](#).

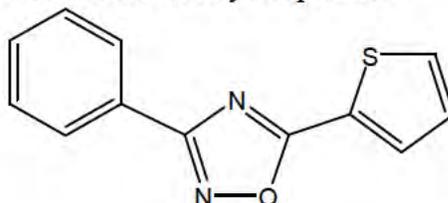
This study was designed to fulfill the requirements of the US EPA Test Guidelines OCSP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [1] and in general accordance with OECD document ENV/JM/MONO(2007)17: Guidance document on pesticide residue methods [2]. In addition, this study was conducted in compliance with US EPA FIFRA (40 CFR Part 160) GLP standards [3].

3.0 MATERIALS AND METHODS

3.1 Test and Reference Substances

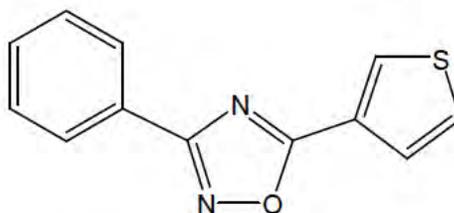
3.1.1 Test Substances

Common name:	Tioxazafen
Monsanto code name:	MON 102100
IUPAC name:	3-Phenyl-5-thiophen-2-yl-1,2,4-oxadiazole
CAS number:	330459-31-9
Ref. substance lot:	GLP-1211-22309-A
GLP purity:	99.7%
Expiration date:	30 November 2014
Storage conditions:	Ambient laboratory temperature
Molecular structure:	

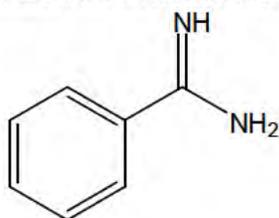


Standard name:	3-Thienyl 102100
Monsanto code name:	MON 102130
IUPAC name:	3-Phenyl-5-thiophen-3-yl-1,2,4-oxadiazole
CAS number:	255866-91-2
Ref. substance lot:	GLP-1402-23161-A
GLP purity:	98.0%
Expiration date:	31 January 2015
Storage conditions:	Ambient laboratory temperature

Molecular structure:

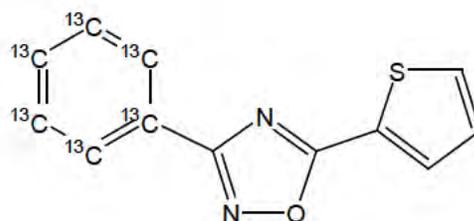


Standard name: Benzamidine
IUPAC name: Benzenecarboximidamide
CAS number: 618-39-3
Ref. substance lot: GLP-1405-23425-A
GLP purity: 95%
Expiration date: 31 May 2015
Storage conditions: Ambient desiccate under N₂
Molecular structure:

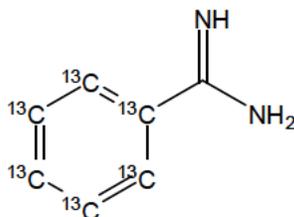


3.1.2 Internal Standards

Standard name: (Phenyl-¹³C₆)MON 102100
IUPAC name: 3-(¹³C₆)Phenyl-5-thiophen-2-yl-1,2,4-oxadiazole
CAS number: Not assigned
Ref. substance lot: GLP-1202-21813-A
Isotopic purity: 100%
Expiration date: 29 February 2016
Molecular formula: ¹³C₆C₆H₈N₂OS
Molecular structure:



Standard name: (¹³C₆)Benzamidine
IUPAC name: (¹³C₆)Benzenecarboximidamide
CAS number: Not assigned
Ref. substance lot: GLP-1207-22176-A
Isotopic purity: 100%
Expiration date: 31 July 2016
Molecular formula: ¹³C₆CH₈N₂
Molecular structure:



Test and reference substances were stored according to the conditions on their individual certificates of analysis. The certificates of analysis are presented in [Appendix 7](#).

3.2 Test System

The test system used for the validation was untreated surface water, ground water, and drinking water provided by Monsanto Company. The samples were stored at 4 °C in a refrigerator until needed for analysis.

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Monsanto Method 115G761A ([Appendix 6](#), Section II: Reagents and Reagent Solutions and Section III: Equipment and Instrument). Identical or equivalent equipment and materials outlined in the method validation were used.

3.3.1 Equipment

- VWR Pyrex[®] volumetric flasks, glass class A (assorted volumes)
- VWR Pyrex[®] mixing cylinders, glass class A (assorted volumes)
- VWR Pyrex[®] disposable Pasteur pipets
- Manual micro pipettor 20 µL (Transferpette[®])
- Manual micro pipettor 200 µL (Transferpette[®])
- Manual micro pipettor 1000 µL (Transferpette[®])
- Manual micro pipettor 10000 µL (VWR International, LLC)
- VWR pipet tips (assorted volumes)
- BD Falcon[®] 15-mL conical centrifuge tubes
- Fisherbrand[™] borosilicate disposable culture tubes, 15-mL
- Wheaton[®] 10-mL amber glass vials
- Agilent Technologies autosampler vials with screw caps
- Waters XBridge[®] Phenyl Column 3.5 µm 2.1 × 100 mm (PN 186003323)
- Agilent 1200 HPLC System (Agilent Technologies)

Analytical balance (Mettler Toledo)
API 4000™ Tandem Mass Spectrometer, MS/MS (Applied Biosystems®)
Heraeus™ Megafuge™ 11R Centrifuge (Thermo Scientific™)
Vortex Mixer
Ultrasonic cleaner (Branson 5510)
Various general laboratory glassware and utensils

3.3.2 Reagents

Water (CPS-Millipore)
Methanol (EMD)
Isopropanol (Pharmaco-Aaper)
Acetonitrile (EMD)
Formic acid (FLUKA)
Heptafluorobutyric acid anhydride (HFBA) (Sigma Aldrich®)

3.4 Experimental Design

3.4.1 Establishment of the Method

Prior to performing the ILV, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined, and the test system was verified as free of interferences at appropriate retention times.

3.4.2 Sample Validation Sets, Fortification, and Sample Preparation Procedure

Sample Validation Sets

Each analytical set consisted of at least 13 samples: one reagent blank, two untreated controls, five untreated controls fortified with MON 102100, 3-thienyl 102100, and benzamidine at the method LOQ (0.10 ppb), and five untreated controls fortified with MON 102100, 3-thienyl 102100, and benzamidine at 10× LOQ (1.0 ppb).

Data are summarized in [Table 1](#) for surface water, [Table 2](#) for ground water, and [Table 3](#) for drinking water. Residue data sheets are included in [Appendix 2](#).

Calibration standard solutions (0.06 to 5.0 ng/mL) and blanks were also included in each sample set.

Fortification

Control (untreated) water samples were fortified with 0.010 mL of the appropriate fortification standard solutions of MON 102100, 3-thienyl 102100, and benzamidine to achieve the desired fortification level (LOQ or 10× LOQ). The fortification standard solutions had a concentration of 0.1 µg/mL for the LOQ fortification and a concentration of 1.0 µg/mL for the 10× LOQ fortification. The fortification standard solutions of 0.1 µg/mL and 1.0 µg/mL were diluted with untreated control (UTC) water to the final concentration, of 0.100 ng/mL (ppb) and 1.00 ng/mL (ppb) respectively. These fortification samples were then used as treated samples to perform the ILV.

Sample Preparation Procedure

The following extraction steps were followed for each sample.

1. Pipette 10 mL of water sample into a 15-mL tube. For reagent blank sample, pipette 10 mL of Millipore water into a 15-mL tube; for untreated control samples and fortification samples, pipette 10 mL of water matrix into a 15-mL tube. Fortify samples with the appropriate amount of the mixed intermediate calibration solutions.

Fortification Level	Mixed Intermediate Calibration Solution (µg/mL)	Mixed Intermediate Calibration Solution Added (µL)	Final Concentration (ng/mL, ppb)
LOQ	0.100	10.0	0.100
10× LOQ	1.00	10.0	1.00

Centrifuge the samples for 10 minutes at $2700 \times g$ at 4 °C to clear suspended material from the liquid column.

2. Aliquot the supernatant into a clean glass tube.
3. **Samples:** Transfer 0.750 mL of centrifuged sample to an autosampler vial.
Calibration Standards: Transfer 0.750 mL of the appropriate working calibration standard solution to an autosampler vial.
Double Blanks and Single Blanks: Transfer 0.750 mL of Millipore water to an autosampler vial.
4. Add 0.250 mL of the working internal standard solution (20 ng/mL) into each autosampler vial except the double blank vial. Instead, add 0.250 mL of Mobile Phase B (MPB) to the double blank vial.
5. Mix the solution well.
6. Submit for analysis by LC-MS/MS. Vials may be stored refrigerated for up to three days pending instrumental analysis.

3.4.3 Sample Processing and Analysis

The samples were processed and analyzed as described in Monsanto EPL-BAS Method No. 115G761A ([Appendix 6](#)), with minor modifications as described below.

3.4.4 Preparation of Fortification and Calibration Standard Solutions

Primary individual stock solutions for all the reference standards (test substances and internal standards) were prepared in acetonitrile at a concentration of approximately 1.0 mg/mL by weighing approximately 10.0 mg of each analytical standard into separate 10-mL volumetric flasks.

The mixed intermediate calibration solution for the three test substances was prepared at a concentration of 10 µg/mL by combining an appropriate amount of each primary stock solution and diluting to 10 mL with 65/35 acetonitrile/water. The mixed intermediate calibration solution for the three test substances was prepared at a concentration of 1.0 µg/mL by diluting a 1.0-mL aliquot of the 10-µg/mL mixed intermediate solution to

10 mL with 65/35 acetonitrile/water. A second mixed intermediate calibration solution for the three test substances was prepared at a concentration of 0.10 µg/mL by diluting a 1.0-mL aliquot of the 1.0-µg/mL mixed intermediate solution to 10 mL with 65/35 acetonitrile/water. A third mixed intermediate calibration solution for the three test substances was prepared at a concentration of 0.01 µg/mL by diluting a 1.0-mL aliquot of the 0.10-µg/mL mixed intermediate solution to 10 mL with 65/35 acetonitrile/water.

Mixed intermediate calibration solutions at concentrations of 0.100 µg/mL and 1.00 µg/mL were used to fortify the LOQ and 10× LOQ level samples, respectively.

Working calibration solutions for the three test substances were prepared at concentrations of 0.06, 0.1, and 0.2 ng/mL by diluting 0.06, 0.1, and 0.2 mL, respectively, of the 0.01 µg/mL mixed intermediate calibration solution to 10 mL in water. Working calibration solutions for the three test substances were prepared at concentrations of 0.5, 1.0, and 2.0 ng/mL by diluting 0.05, 0.1, and 0.2 mL, respectively, of the 0.1 µg/mL mixed intermediate calibration solution to 10 mL in water. The working calibration solution for the three test substances was prepared at a concentration of 5.0 ng/mL by diluting 0.05 mL of the 1.0 µg/mL mixed intermediate calibration solution to 10 mL in water.

The mixed intermediate solution for the two internal standards was prepared at a concentration of 1.0 µg/mL by combining an appropriate amount of each primary stock solution and diluting to 10 mL with MPB (50/475/475 isopropanol/methanol/acetonitrile with formic acid and HFBA).

The working solution for the two internal standards was prepared at a concentration of 20 ng/mL by diluting a 0.50-mL aliquot of the 1.0 µg/mL mixed intermediate internal standard solution to 25 mL with MPB (50/475/475 isopropanol/methanol/acetonitrile with formic acid and HFBA).

All stock solutions and intermediate solutions were stored in amber glass bottles in a freezer (approximately -20 °C) when not in use.

All working solutions were stored refrigerated in amber glass bottles.

3.5 LC-MS/MS Instrumentation

Instrumentation

Agilent 1200 HPLC System

AB SCIEX API 4000™ LC-MS/MS

Software: AB SCIEX Analyst Software, Analyst® 1.6.2

Waters XBridge® Phenyl Column 3.5 µm, 2.1 × 100 mm

3.6 Data Acquisition and Reporting

Peak integration was performed by Analyst® Software version 1.6.2. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for the analytes of interest. The overall purpose of the external

calibration curve was to display acceptable linearity ($r^2 \geq 0.9801$) of the detector response over the assigned calibration range. The recoveries of the analyte from the fortified samples were calculated by multi-point calibration.

Recovery results were computed for each sample. The equation used for quantification is presented in [Appendix 3](#). A statistical treatment of the data includes the calculation of means, SDs, RSDs as percentages (%), and the 95% confidence intervals. All statistics were calculated using Microsoft[®] Office Excel[®] 2010.

The MON 102100, 3-thienyl 102100, benzamidine, (phenyl-¹³C₆)MON 102100, and (¹³C₆)benzamidine transitions from m/z 229 to 111, m/z 229 to 111, m/z 121 to 104, m/z 235 to 111, and m/z 127 to 110, respectively, were used to quantitate the analytes.

Prior to performing the ILV, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined, and the test system was verified as free of interferences at the appropriate retention times.

To accommodate differences in LC-MS/MS instrumentation, slight modifications to the mobile phase gradient were made. Three minutes of equilibration time was added to the end of the original gradient. A 10- μ L injection was used for benzamidine analysis and a 75- μ L injection was used for MON 102100 and 3-thienyl 102100 analyses. The modified gradient program used is presented in [Table 5](#).

**TABLE 5 HPLC SYSTEM OPERATING PARAMETERS FOR MONSANTO
 EPL-BAS METHOD NO. 115G761A (TRIAL 1)**

HPLC System: Agilent Model 1200
 Software: Applied Biosystems®, Analyst® Software 1.6.2
 Analytical Column: Waters XBridge® Phenyl Column 3.5 µm 2.1 × 100 mm
 Column Temperature: 20 °C
 Injection Volume: 10.0 µL for benzamidine and 75.0 µL for MON 102100 and 3-thienyl 102100
 Run Time: 33.0 minutes
 Mobile Phase: (A): 0.1% formic acid, 0.1% HFBA in water
 (B): 0.1% formic acid, 0.1% HFBA in 50/475/475 isopropanol/methanol/acetonitrile (v/v/v)
 Needle Wash: Flush port: 15.0 seconds using 50/50 methanol/water (v/v; prior to injection)
 Gradient:

Time (min)	A (%)	B (%)	Flow (µL/min)
0.0	98	2	250
5.0	98	2	250
5.5	70	30	250
23.0	30	70	250
26.0	10	90	250
26.5	5	95	250
30.0	98	2	250
33.0	98	2	250

TABLE 6 MS/MS OPERATING PARAMETERS

Tandem Mass Spectrometry System, Applied Biosystems[®], API 4000[™]
 Software: Applied Biosystems[®], Analyst[®] Software 1.6.2

The following parameters were used for operation of the mass spectrometer:

Parameter		Setting				
Ion Source:		Turbo IonSpray				
Scan Type:		MRM				
Polarity:		Positive				
Curtain Gas (CUR):		40				
Temperature (TEM):		500				
Ion Spray Voltage (IS):		5500				
Collision Gas (CAD):		12				
Ion Source Gas 1 (GS1):		40				
Ion Source Gas 2 (GS2):		30				
Interface Heater (IHE):		ON				
Entrance Potential (EP):		10				
Resolution Q1:		Unit				
Resolution Q3:		Unit				
Analyte	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)	CXP (V)	
MON 102100 (Quantitation):	229	82.9	50	20	10	
3-Thienyl 102100 (Quantitation):	229	82.9	50	20	10	
(Phenyl- ¹³ C ₆)MON 102100 (IS):	235	111	50	30	15	
Benzamidine (Quantitation):	121	104	109	24	9	
(¹³ C ₆)Benzamidine (IS):	127	110	69	31	9	

amu = atomic mass units; DP = declustering potential; CE = collision energy; CXP = collision exit potential; V = volts

APPENDIX 3 CALCULATIONS

The amount found (ng/mL), for each analyte, is calculated automatically after each sequence run with the Applied Biosystems[®] Analyst[®] Software version 1.6.2 using linear regression (weighted 1/x). Calibration standard curves are generated as the ratio of the analyte response (e.g., peak area) to the internal standard response, for each standard level, plotted against analyte concentration (or the ratio of the analyte concentration to the internal standard concentration).

Additional calculations may be completed as follows:

Fortification Level of Laboratory Fortified Samples (ng/mL) =

$$\frac{\text{Volume Spiking Solution (mL)} \times \text{Spiking Solution Concentration (ng/mL)}}{\text{(Sample Volume [mL] + Fortification Solution Volume [mL])}}$$

$$\text{Recovery (\%)} = \frac{(\text{ng/mL Found}) \times 100}{\text{Fortification Level (ng/mL)}}$$

APPENDIX 6 MONSANTO EPL-BAS METHOD NO. 115G761A

Analytical Method for MON 102100 and Environmental Degradates in Water

EPL-BAS Method No. 115G761A

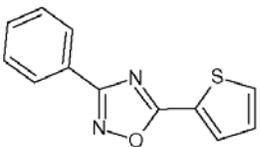
Method Summary

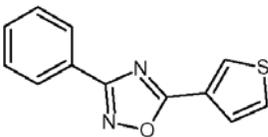
Water samples are first centrifuged to remove suspended solid particles. Sample supernatants are mixed (3:1, v/v) with a solution of stable-labeled (¹³C₆) internal standards in an organic solvent comprised of formic acid and heptafluorobutyric acid anhydride in a mixture of acetonitrile, methanol and isopropanol. Samples are agitated and analyzed by LC-MS/MS for the analytes MON 102100 (tiozafen), MON 102130 (3-thienyl 102100) and benzamidine. The lower limit of method validation (LLMV) of the method is 0.10 ng/mL (ppb) and the working range of the method is 0.10 ng/mL (ppb) to 20 ng/mL (ppb) for each analyte. The method is validated for these analytes in drinking water, ground water and surface water.

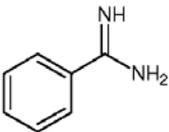
I. Reference Substances and Reference Substance Solutions

A. Reference Substances and Internal Standards

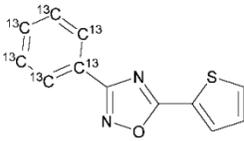
The following reference standards are used:

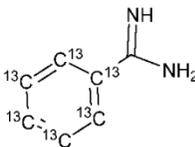
Common name	Tioxazafen
Monsanto code name:	MON 102100
Chemical name (IUPAC):	3-phenyl-5-thiophen-2-yl-1,2,4-oxadiazole
CAS-Registry-No.:	330459-31-9
Chemical structure:	
Molecular formula:	C ₁₂ H ₈ N ₂ OS
Molecular weight:	228.27

Common name	3-Thienyl 102100
Monsanto code name:	MON 102130
Chemical name (IUPAC):	3-phenyl-5-thiophen-3-yl-1,2,4-oxadiazole
CAS-Registry-No.:	255866-91-2
Chemical structure:	
Molecular formula:	C ₁₂ H ₈ N ₂ OS
Molecular weight:	228.27

Common name	Benzamidine
Chemical name (IUPAC):	Benzenecarboximidamide
CAS-Registry-No.:	618-39-3
Chemical structure:	
Molecular formula:	C ₇ H ₈ N ₂
Molecular weight:	120.15

In addition, the following internal standards are used:

Common name	(Phenyl-¹³C₆)MON 102100
Chemical Name (IUPAC):	3-(¹³ C ₆)Phenyl-5-thiophen-2-yl-1,2,4-oxadiazole
CAS-Registry-No.:	Not assigned
Chemical structure:	
Molecular formula:	¹³ C ₆ C ₆ H ₈ N ₂ OS
Molecular weight:	234.22

Common name	(¹³C₆)Benzamidine
Chemical Name (IUPAC):	(¹³ C ₆)Benzenecarboximidamide
CAS-Registry-No.:	Not assigned
Chemical structure:	
Molecular formula:	¹³ C ₆ CH ₈ N ₂
Molecular weight:	126.11

B. Reference Substance and Internal Standard Solutions

Sample volumes may be adjusted as long as proportionality is maintained.

MON 102100	Weigh 10 mg (recorded to at least 0.1 mg) of MON 102100
Calibration	reference material into a 10-mL class A volumetric flask and
Stock Solution	dilute to volume with acetonitrile (ACN). The solution may
(1.0 mg/mL)	be sonicated briefly to ensure complete dissolution. Store
	frozen (approximately -20 °C) in an amber bottle.
Benzamidine	Weigh 10 mg (recorded to at least 0.1 mg) of benzamidine
Calibration	reference material into a 10-mL class A volumetric flask and
Stock Solution	dilute to volume with ACN. The solution may be sonicated
(1.0 mg/mL)	briefly to ensure complete dissolution. Store frozen
	(approximately -20 °C) in an amber bottle.

3-Thienyl 102100 Calibration Stock Solution (1.0 mg/mL) Weigh 10 mg (recorded to at least 0.1 mg) of 3-thienyl 102100 reference material into a 10-mL class A volumetric flask and dilute to volume with ACN. The solution may be sonicated briefly to ensure complete dissolution. Store frozen (approximately -20 °C) in an amber bottle.

Intermediate Calibration Solutions Prepare the following Intermediate Calibration Solutions by dilution of the appropriate Calibration Stock Solution with 65/35 ACN/water. These solutions are all prepared in 10-mL class A volumetric flasks and stored frozen (approximately -20 °C) in amber bottles.

Intermediate Calibration Solution	Source Solution ID	Aliquot Volume (mL)
10 µg/mL Intermediate Calibration Solution	MON 102100 Calibration Stock Solution (1.0 mg/mL)	0.100
	3-Thienyl 102100 Calibration Stock Solution (1.0 mg/mL)	0.100
	Benzamidine Calibration Stock Solution (1.0 mg/mL)	0.100
1.0 µg/mL Intermediate Calibration Solution	10 µg/mL Intermediate Calibration Solution	1.00
0.10 µg/mL Intermediate Calibration Solution	1.0 µg/mL Intermediate Calibration Solution	1.00
0.01 µg/mL Intermediate Calibration Solution	0.10 µg/mL Intermediate Calibration Solution	1.00

Working Calibration Standard Solutions Prepare the following Working Calibration Standard Solutions by dilution of the appropriate Intermediate Calibration Solution in water. All solutions are prepared in 10-mL class A volumetric flasks and stored refrigerated in amber bottles.

Working Calibration Standard Solution (ng/mL)	Dilute this Intermediate Calibration Solution (µg/mL)	Aliquot Volume (mL)	Final Calibration Level (ng/mL)†
0.080	0.01	0.080	0.0600
0.10	0.01	0.100	0.0750
0.20	0.01	0.200	0.150
0.50	0.10	0.050	0.375
1.0	0.10	0.100	0.750
2.0	0.10	0.200	1.50
5.0	1.0	0.050	3.75
10	1.0	0.100	7.50
20	1.0	0.200	15.0

†Final Calibration Level (ng/mL) assumes 0.750 mL of the Working Calibration Standard Solution (ng/mL) is brought to a final volume of 1.00 mL.

(Phenyl-¹³C₆) MON 102100 Internal Standard Stock Solution (1.0 mg/mL) Weigh 10 mg (recorded to at least 0.1 mg) of (phenyl-¹³C₆) MON 102100 reference material into a 10-mL class A volumetric flask and dilute to volume with ACN. The solution may be sonicated briefly to ensure complete dissolution. Store frozen (approximately -20 °C) in an amber bottle.

(¹³C₆)Benzamidine Internal Standard Stock Solution (1.0 mg/mL) Weigh 10 mg (recorded to at least 0.1 mg) of (¹³C₆) benzamidine reference material into a 10-mL class A volumetric flask and dilute to volume with ACN. The solution may be sonicated briefly to ensure complete dissolution. Store frozen (approximately -20 °C) in an amber bottle.

Intermediate Internal Standard Solution Prepare the following Intermediate Internal Standard Solution by dilution of the appropriate Internal Standard Stock Solutions with Mobile Phase B (MPB). This solution is prepared in a 10-mL class A volumetric flask and is stored frozen (approximately -20 °C) in an amber bottle.

Intermediate Internal Standard Solution ($\mu\text{g/mL}$)	Source Solution ID	Source Aliquot Volume (mL)
1.0	(Phenyl- $^{13}\text{C}_6$)MON 102100 Internal Standard Stock Solution (1.0 mg/mL)	0.010
	($^{13}\text{C}_6$)Benzamidine Internal Standard Stock Solution (1.0 mg/mL)	0.010

Internal Standard Working Solution (20 ng/mL) Prepare the following Internal Standard Working Solution on the day of analysis by dilution of the Intermediate Internal Standard Solution with MPB. This solution is prepared in a 25-mL class A volumetric flask.

Internal Standard Working Solution (ng/mL)	Source Solution ID	Source Aliquot Volume (mL)
20	Intermediate Internal Standard Solution (1.0 $\mu\text{g/mL}$)	0.500

II. Reagents and Reagent Solutions

A. Reagents

Acetonitrile (ACN), HPLC Grade
 Isopropanol (IPA), Analytical Grade
 Methanol (MeOH), HPLC Grade
 Heptafluorobutyric acid anhydride (HFBA), Analytical Grade
 Formic acid (FA), Analytical Grade
 Water, HPLC Grade or equivalent

B. Reagent Solutions

65/35 (v/v) ACN/ Water: For every liter of solution prepared, combine 650 mL of ACN with 350 mL of water. Invert to mix. Store ambient.

Mobile Phase A (MPA): Add 1 mL of formic acid and 1 mL of HFBA into 1000 mL of water. Invert to mix. Store ambient.

Mobile Phase B (MBP): Add 1 mL of formic acid and 1 mL of HFBA into 1000 mL of an organic solvent mixture with the composition of 50/475/475 (v/v/v) isopropanol/methanol/acetonitrile.

III. Equipment and Instrument

Balance, Analytical, capable of weighing to the nearest 0.1 mg
Centrifuge, with rotor to accommodate 15-mL culture tube
Culture Tubes, conical or round-bottom, 15-mL with screw-top lids
Vortex Mixer
HPLC System, Agilent 1200
Mass Spectrometer, AB SCIEX 6500
Autosampler Vials (1.8 mL) with pre-slit screw-top lids
Pipettes, Air-displacement, 10-100 μ L capacity with disposable tips
Pipettes, Air-displacement, 100-1000 μ L capacity with disposable tips
Graduated Cylinders, various volumes up to 2000 mL
Class A Volumetric Pipettes and Flasks, various volumes

IV. Sample Preparation Procedure

Water Sample Processing The following describes the preparation of water samples for analysis by LC-MS/MS. A typical analytical set will include (at a minimum) samples for analysis, QC fortifications and calibration standards. Sample contact with plastic materials should be minimized.

Step	Action									
1	Pipette approximately 10-15 mL of sample into a disposable 15-mL culture tube. For QC fortification samples, pipette 10-15 mL of water into a disposable 15-mL culture tube. Centrifuge the samples for 10 minutes at 4000 rpm to clear suspended materials from the liquid column.									
2	Aliquot the supernatant into a clean glass vial.									
3	Samples: Transfer 0.750 mL of centrifuged sample into a 1.8-mL autosampler vial. QC Fortifications: Transfer 0.675 mL of centrifuged sample into a 1.8-mL autosampler vial. Calibration Standards: Transfer 0.750 mL of the appropriate Working Calibration Standard Solution into a 1.8-mL autosampler vial.									
4	Add 75 µL of the following solutions to the appropriate QC Fortification samples. <table border="1" data-bbox="570 1020 1291 1161"> <thead> <tr> <th>QC Sample</th> <th>Working Calibration Standard Solution (ng/mL)</th> <th>Final QC Fortification Concentration (ng/mL)</th> </tr> </thead> <tbody> <tr> <td>LLMV</td> <td>1</td> <td>0.10</td> </tr> <tr> <td>10X LLMV</td> <td>10</td> <td>1.0</td> </tr> </tbody> </table>	QC Sample	Working Calibration Standard Solution (ng/mL)	Final QC Fortification Concentration (ng/mL)	LLMV	1	0.10	10X LLMV	10	1.0
QC Sample	Working Calibration Standard Solution (ng/mL)	Final QC Fortification Concentration (ng/mL)								
LLMV	1	0.10								
10X LLMV	10	1.0								
5	Add 0.250 mL of the Working Internal Standard Solution (20 ng/mL) into each autosampler vial.									
6	Mix the solution well (a vortex mixer may be used).									
7	Submit for analysis by LC-MS/MS. Vials may be stored refrigerated up to 3 days pending instrumental analysis.									

V. Instrumental Analysis

Instrument Setup Instrument operation is controlled by acquisition methods containing all HPLC, source interface and mass spectrometer operating parameters. The typical precursor and product ions for the analytes are shown below (along with typical confirmatory ions). Alternate ions may be selected if necessary to improve data sensitivity and/or

specificity. The following equipment and operating conditions may be modified to obtain optimal instrument performance. Actual method parameters used must be recorded in the raw data.

HPLC Conditions					
HPLC: Agilent 1200					
Column: Waters Xbridge Phenyl (2.1 x 100mm, 3.5 µm)					
Injection Volume: 20 µL					
Column Oven Temperature: 20 °C					
Autosampler Temperature: 20 °C					
Mobile Phase A: 0.1% FA, 0.1% HFBA in Water					
Mobile Phase B: 0.1% FA, 0.1% HFBA in IPA/MeOH/ACN (50:475:475)					
Flow Rate: 0.250 mL/min					
Gradient Profile Information					
Time (min)	% MPA	% MPB	Divert		
0.0	98	2	To waste		
5.0	98	2	To waste		
5.5	70	30	To MS		
23.0	30	70	To MS		
23.5	10	90	To MS		
26.0	5	95	To MS		
26.5	98	2	To waste		
30.0	98	2	To waste		
Mass Spectrometer Conditions					
Mass Spectrometer: AB SCIEX 6500 Q-Trap with (what ion source-turbo V??)					
Ion Source: ESI					
Mode: Positive Ion					
Scan Type: Scheduled MRM					
Resolution (Q1 and Q3): Unit					
Curtain Gas (CUR): 20			Gas 1: 60		
Collision Gas (CAD): Medium			Gas 2: 60		
IonSpray Voltage (IS): 5500 V			Entrance Potential (EP): 10 V		
Temperature (TEM): 600 °C					
Analyte	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)	CXP (V)
Benzamidine (Quantitation)	121.0	104.1	30	29	13
Benzamidine (Confirmatory)	121.0	77.0	30	42	13
¹³ C ₆ Benzamidine (IS)	127.0	110.0	40	31	14
MON 102100 (Quantitation)*	228.9	111.1	70	41	14

MON 102100 (Confirmatory)*	228.9	82.9	70	53	14
(Phenyl- ¹³ C ₆)MON 102100 (IS)**	235.0	111.0	55	40	12

* MON 102100 refers to both MON 102100 and 3-thienyl 102100.
** (Phenyl-¹³C₆)MON 102100 is utilized as internal standard for both MON 102100 and 3-thienyl 102100 analytes.

VI. Calculations

The amount found (ng/mL), for each analyte, is calculated in MultiQuant software from the linear regression equation (weighted 1/x) of the calibration standard curve. Calibration standard curves are generated as the ratio of the analyte response (e.g., peak area) to the internal standard response, for each standard level, plotted against concentration.

Additional calculations may be completed as follows:

Fortification Level of Laboratory Fortified Samples (ng/mL) =

$$\frac{\text{Volume Spiking Soln. (mL)} \times \text{Spiking Soln. Conc. (ng/mL)}}{[\text{Sample Volume (mL)} + \text{Fortification Solution Volume (mL)}]}$$

Recovery (%) =

$$\frac{(\text{ng/mL Found}) \times 100}{\text{Fortification Level (ng/mL)}}$$