

INTRODUCTION

This report describes the validation of a residue analytical method for the determination of MON 102100 (tioazafen, 3-phenyl-5-thiophen-2-yl-1,2,4-oxadiazole) and its potential environmental degradates, MON 102130 (3-thienyl 102100, 3-phenyl-5-thiophen-3-yl-1,2,4-oxadiazole) and benzamidine, in drinking, surface and ground water. Validation of the method was conducted over the concentration range of 0.10 - 1.0 ng/mL (ppb). Common names, chemical names and molecular formulae for the analytes used in this study are presented in [Tables 1-3](#).

This study was conducted to fulfill data requirements outlined in the US EPA Ecological Effects Test Guidelines, OCSPP 850.6100 ([1](#)) and in compliance with US EPA FIFRA Good Laboratory Practices, 40 CFR 160. The validation also complies with the requirements of OECD method ENV/JM/MONO/2007/17 and Canada PMRA Regulatory Directive Dir98-02 ([2-3](#)) according to the study protocol amendment #1 ([Appendix A](#)).

EXPERIMENTAL PHASE

Sample Origin, Preparation, Storage and Characterization

The control samples of drinking, ground and surface water were sourced directly by EPL BAS. On arrival, the samples were placed in a refrigerator set to maintain a specimen temperature of 2 °C where they were stored at all times until removed for analysis. No sample preparation or homogenization was necessary prior to sample analysis. Full sample details are included in the raw data package.

Prior to use, the control samples were characterized for pH, calcium, magnesium, hardness, conductivity, total suspended solids, turbidity, alkalinity, total organic carbon and dissolved organic carbon. Certificates of analysis for the control samples can be found in [Appendix B](#).

Test/Component	Drinking Water ¹	Ground Water ²	Surface Water ³
	761-X001	761-X002	761-X003
pH	8.3	8.1	8.5
Calcium (ppm)	22	63	61
Magnesium (ppm)	8.9	27	55
Hardness (mg equiv. CaCO ₃ /L)	92	272	382
Conductivity (mmhos/cm)	1.17	0.59	3.12
Total Suspended Solids (ppm)	10	8	8
Turbidity (NTU)	0.39	0.31	6.77
Alkalinity (mg CaCO ₃ /L)	387	201	521
Total Organic Carbon (ppm)	2.3	1.6	23.4
Dissolved Organic Carbon (ppm)	1.9	0.6	22.3

¹An aliquot of 759-X001 (AGVISE Sample ID 14-25)

²An aliquot of 759-X002 (AGVISE Sample ID 14-26)

³An aliquot of 759-X004 (AGVISE Sample ID 14-27)

Calculation of Standard Calibration Curve

The reference substance/analytical standards and internal standards used during the conduct of method validation are detailed in [Tables 1-3](#). Certificates of analysis for the reference

substances and internal standards can be found in [Appendix B](#). Standard stock solutions, calibration standard solutions and fortification solutions were prepared as described in the analytical method ([Appendix C](#)). Full details of these materials are included in the raw data package for the study along with details of the preparation of all analytical and fortification standards prepared from the primary reference substances. The reference and internal standards will be retained until expiry and then disposed of following relevant Testing Facility disposal SOP's with the approval of the Study Monitor.

Calculation of a standard curve began with the injection of a series of calibration standards prepared as described in [Appendix C](#) and acquisition of the peak areas for the following transitions:

MON 102100	<i>m/z</i> 228.9/111.1 <i>m/z</i> 228.9/82.9	(Quantitative) (Confirmatory)
MON 102130	<i>m/z</i> 228.9/111.1 <i>m/z</i> 228.9/82.9	(Quantitative) (Confirmatory)
Benzamidine	<i>m/z</i> 121.0/104.1 <i>m/z</i> 121.0/77.0	(Quantitative) (Confirmatory)
(Phenyl- ¹³ C ₆)MON 102100	<i>m/z</i> 235.0/111.0	(Quantitative)
(¹³ C ₆)Benzamidine	<i>m/z</i> 127.0/110.0	(Quantitative)

Confirmation of Residue Identity

The method was specific for the determination of MON 102100, MON 102130 and benzamidine by virtue of the chromatographic separation and selective detection system used. Confirmation was performed to demonstrate the selectivity of the primary method by monitoring one additional precursor-to-product ion transition simultaneous to the primary detection transition for each analyte. Untreated control matrix samples and samples fortified at the lowest

fortification level for each analyte/matrix combination were provided to demonstrate the selectivity of the method.

Statistical Treatment of Data

Statistical treatment of the data was performed using AB Sciex Analyst[®] Chromatography Software, MultiQuant Data Analysis Software and Microsoft Excel. Statistical treatments included but were not limited to the calculation of linear regression equations and coefficients of determination (r^2) for describing the linearity of calibration curves; and means, standard deviations, and relative standard deviations of the results for the fortified samples.

Example calculations performed in Excel are found below.

MON 102100 recovery at 0.10 ng/mL (LLMV Fortification Level)

Laboratory Sample ID: 761-X001-S1, Set V001

Amount Found (ng/mL, Instrumental Response) = Calculated Concn.

Peak area, MON 102100 = 9012

Peak area, MON 102100 Internal Standard = 910356

Internal Standard (ITSD) Concentration = 5.0 ng/mL

Linear Regression equation: $y = 0.67188 x + (0.00135)$

Where: $y = (\text{MON 102100 peak area} / \text{ITSD peak area}) = 0.0098994$

$x = (\text{MON 102100 Concn.} / \text{ITSD Concn.}) = (\text{Calculated Concn.} / 5)$

To solve:

$(0.0098994) - (0.00135) = (0.67188) * (\text{Concn.} / 5)$

Calculated Concn. = $[(0.0098994 - 0.00135)] * 5 / 0.67188 = \underline{0.0636 \text{ ng/mL}}$

$$\text{Amount Found (ng/mL)} = \frac{\text{Amount Found (ng/mL)} * \text{Final Volume (mL)}}{\text{Sample Volume (mL)}}$$

Where:

$$\text{Amount Found} = 0.0636 \text{ ng/mL}$$

$$\text{Final Volume} = 1 \text{ mL}$$

$$\text{Sample Volume} = 0.750 \text{ mL}$$

To solve:

$$\text{Amount Found (ng/mL)} = [(0.0636 \text{ ng/mL}) * (1 \text{ mL})] / [0.750 \text{ mL}]$$

$$\text{Amount Found (ng/mL)} = \underline{0.08480 \text{ ng/mL}}$$

$$\text{Fortification Level (ng/mL)} = \frac{\text{Vol. of Fortification Solution (mL)} * \text{Concn. of Fortification Solution (ng/mL)}}{\text{Sample Volume (mL)}}$$

Where:

$$\text{Vol. of Fortification Solution} = 0.075 \text{ mL}$$

$$\text{Concn. of Fortification Solution} = 1.000 \text{ ng/mL}$$

$$\text{Sample Volume} = 0.750 \text{ mL}$$

To solve:

$$\text{Fortification Level (ng/mL)} = (0.075 \text{ mL} * 1.000 \text{ ng/mL}) / 0.750 \text{ mL}$$

$$\text{Fortification Level (ng/mL)} = \underline{0.1000 \text{ ng/mL}}$$

Recovery (%) =

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

Where:

Amount Found (ng/mL) = 0.08480 ng/mL

Fortification Level (ng/mL) = 0.1000 ng/mL

To solve:

$$\text{Recovery (\%)} = (0.08480/0.1000) * 100$$

$$\text{Recovery (\%)} = \underline{84.80 \%}$$

Concn. stands for Concentration

Vol. stands for Volume

Table 1. Identities and Structures of MON 102100 and Related Internal Standard

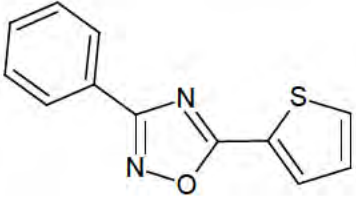
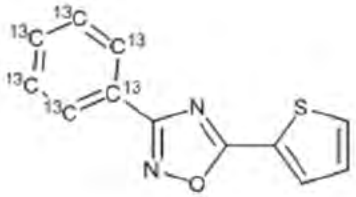
Identifying Information	Structure and IUPAC Name
<p>Common Name of Compound: Tioxazafen</p> <p>Molecular Formula: C₁₂H₈N₂OS</p> <p>Molecular Weight: 228.27</p> <p>Appearance: White solid</p> <p>CAS Number: 330459-31-9</p>	 <p>3-phenyl-5-thiophen-2-yl-1,2,4-oxadiazole</p>
<p>Common Name of Compound: (Phenyl-¹³C₆)MON 102100</p> <p>Molecular Formula: ¹³C₆C₆H₈N₂OS</p> <p>Molecular Weight: 234.22</p> <p>Appearance: White solid</p> <p>CAS Number: Not Assigned</p>	 <p>3-(¹³C₆)phenyl-5-thiophen-2-yl-1,2,4-oxadiazole</p>

Table 2: Identities and Structures of MON 102130 and Related Internal Standard

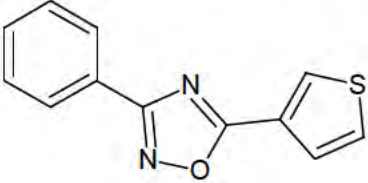
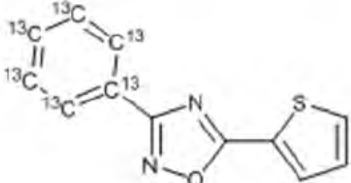
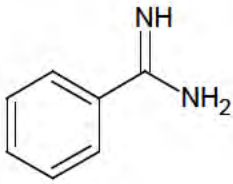
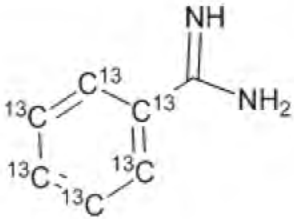
Identifying Information	Structure and IUPAC Name
<p>Common Name of Compound: 3-Thienyl 102100</p> <p>Molecular Formula: C₁₂H₈N₂OS</p> <p>Molecular Weight: 228.27</p> <p>Appearance: White solid</p> <p>CAS Number: 255866-91-2</p>	 <p>3-phenyl-5-thiophen-3-yl-1,2,4-oxadiazole</p>
<p>Common Name of Compound: (Phenyl-¹³C₆)MON 102100</p> <p>Molecular Formula: ¹³C₆C₆H₈N₂OS</p> <p>Molecular Weight: 234.22</p> <p>Appearance: White solid</p> <p>CAS Number: Not Assigned</p>	 <p>3-(¹³C₆)phenyl-5-thiophen-2-yl-1,2,4-oxadiazole</p>

Table 3: Identities and Structures of Benzamidine and Related Internal Standard

Identifying Information	Structure and IUPAC Name
<p>Common Name of Compound: Benzamidine</p> <p>Molecular Formula: $C_7H_8N_2$</p> <p>Molecular Weight: 120.15</p> <p>Appearance: White crystalline solid</p> <p>CAS Number: 618-39-3</p>	 <p>benzenecarboximidamide</p>
<p>Common Name of Compound: $(^{13}C_6)$Benzamidine</p> <p>Molecular Formula: $^{13}C_6CH_8N_2$</p> <p>Molecular Weight: 126.11</p> <p>Appearance: White solid</p> <p>CAS Number: Not Assigned</p>	 <p>$(^{13}C_6)$benzenecarboximidamide</p>

Analytical Method for MON 102100 and Environmental Degradates in Water

EPL-BAS Method No. 115G761A

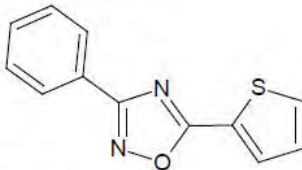
Method Summary

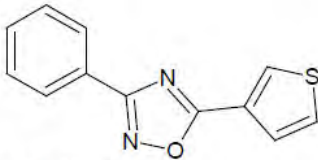
Water samples are first centrifuged to remove suspended solid particles. Sample supernatants are mixed (3:1, v/v) with a solution of stable-labeled ($^{13}\text{C}_6$) internal standards in an organic solvent comprised of formic acid and heptafluorobutyric acid anhydride in a mixture of acetonitrile, 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.

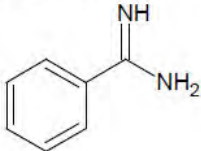
I. Reference Substances and Reference Substance Solutions

A. Reference Substances and Internal Standards

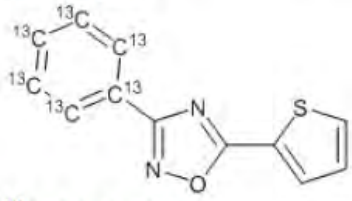
The following reference standards are used:

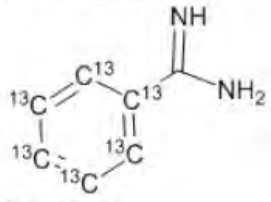
Common name	Tioxazafen
Monsanto code name:	MON 102100
Chemical name (IUPAC):	3-phenyl-5-thiophen-2-yl-1,2,4-oxadiazole
CAS-Registry-No.:	330459-31-9
Chemical structure:	
Molecular formula:	C ₁₂ H ₈ N ₂ OS
Molecular weight:	228.27

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- ¹³ C ₆)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	(¹³ C ₆)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.

**QC
 Fortification
 Solutions**

Prepare the following QC Fortification Solutions by dilution of the appropriate solution with water. These solutions are all prepared in 10-mL class A volumetric flasks and stored refrigerated in an amber bottles. Additional QC Fortification Solutions may be prepared as needed.

QC Fortification Solution (ng/mL)	Source Solution ID	Aliquot Volume (mL)	QC Level
100	10 µg/mL Intermediate Calibration Solution	0.100	N/A
10	0.10 µg/mL Intermediate Calibration Solution	1.00	10X LLMV (1 ppb)
1	0.01 µg/mL Intermediate Calibration Solution	1.00	LLMV (0.1 ppb)
2	0.01 µg/mL Intermediate Calibration Solution	2.00	2X LLMV (0.2 ppb)

**(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 (µg/mL)	Source Solution ID	Source Aliquot Volume (mL)
1.0	(Phenyl- ¹³ C ₆)MON 102100 Internal Standard Stock Solution (1.0 mg/mL)	0.010
	(¹³ 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 µg/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	<p>Samples: Transfer 0.750 mL of centrifuged sample into a 1.8-mL autosampler vial.</p> <p>QC Fortifications: Transfer 0.675 mL of centrifuged sample into a 1.8-mL autosampler vial.</p> <p>Calibration Standards: Transfer 0.750 mL of the appropriate Working Calibration Standard Solution into a 1.8-mL autosampler vial.</p>		
4	Add 75 µL of the following solutions to the appropriate QC Fortification samples.		
	QC Sample	QC Fortification 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