INTRODUCTION

This report describes the independent laboratory validation (ILV) of an analytical method for the determination of MON 102100 and its metabolite benzamidine in soil matrices. The representative soils selected for the ILV included a high clay and a high sand soil. The method was developed and validated at Monsanto Company as Method Number AG-ME-1636, "High Throughput Assay for MON 102100 and Benzamidine in Soil" (Appendix E).

The ILV study was conducted according to the protocol in Appendix A and was designed to fulfill the requirements of the US EPA Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation (1). In addition, this study was conducted in compliance with U.S. EPA FIFRA (40 CFR Part 160) Good Laboratory Practice (GLP) standards (2).

The independent laboratory, the Study Director and the analysts chosen to conduct the ILV were unfamiliar with the method, both in its development and its subsequent use in analyzing samples. The independent laboratory used all of its own equipment and supplies with the exception of analytical reference standards supplied by the Sponsor, so that there was no common link between Monsanto Company and the ILV analysts. Throughout the conduct of the study, any communications between Monsanto Company and the Study Director and/or the analysts were logged for inclusion in the report (Appendix B). No one from Monsanto Company was allowed to visit the independent laboratory during the ILV trial to observe, offer help, or assist the chemists or analysts. These steps successfully maintained the integrity of the ILV study.

EXPERIMENTAL PHASE

Storage and Characterization of Control Samples

Characterized soil samples were purchased by EPL BAS to be used as controls. Upon arrival at EPL BAS, the samples were placed in a freezer at a temperature of approximately -20 °C until removed for analysis. Approximately 30-40 mL aliquots of each sample were homogenized for the ILV experiment by milling using a SPEX freezer mill with liquid nitrogen. Full sample details are included in the raw data package.

Prior to purchase by EPL, the control samples were characterized for percent sand, percent silt, percent clay, USDA Textural Class, FAO Textural Class, bulk density (disturbed), cation exchange capacity, moisture, organic matter and pH in1:1 soil: water ratio. Characterization was conducted at AGVISE Laboratories, Northwood, ND USA, under 40 CFR Part 160. Certificates of analysis for the control samples can be found in Appendix C.

Test/Component	High Sand Soil[1]	High Clay Soil[2]
	805-S001	805-X002
Percent Sand	89	29
Percent Silt	4	28
Percent Clay	7	43
USDA Textural Class (hydrometer method)	Sand	Clay
FAO Textural Class (hydrometer method)	Coarse	Fine
Bulk Density (disturbed, gm/cc)	1.24	1.07
Cation Exchange Capacity (meq/100 g)	10.3	31.0
Moisture (%)	9.3	28.4
Organic Matter (%)	2.0	2.1
pH in 1:1 soil: water ratio	6.5	8.0

[1]AGVISE Sample ID 14-1256
[2]AGVISE Sample ID 14-1257

Preparation of Stock Solutions, Calibration Standard Solutions and Fortification Solutions

The reference substance certificates of analysis can be found in Appendix C. Reagents used are of equivalent specifications as those described in the analytical method.

The following reference substances/analytical standards were utilized during the independent laboratory method validation:

Reference Substance/ Analytical Standard:	MON 102100 (tioxazafen) 3-phenyl-5-thiophen-2-yl-1,2,4oxadiazole
Supplier:	Monsanto Company (Sponsor)
Batch/Lot No:	GLP-1103-21325-A
Purity:	99.9%
Expiration date:	31 December 2014
Storage:	Frozen

Reference Substance/ Analytical Standard:	Benzamidine benzenecarboximidamide
Supplier:	Monsanto Company (Sponsor)
Batch/Lot No:	GLP-1205-22096-A and GLP-1405-23425-A
Purity:	98% and 95%
Expiration date:	31 May 2014 and 31 May 2015
Storage:	Ambient

In addition to the reference substances/analytical standards above, the following internal standards were utilized during the independent laboratory method validation.

Reference Substance/ Analytical Standard:	(Phenyl-$^{13}C_6$)MON 102100 3-($^{13}C_6$)phenyl-5-thiophen-2-yl-1,2,4oxadiazole
Supplier:	Monsanto Company (Sponsor)
Batch/Lot No:	GLP-1202-21813-A
Purity:	Not assigned
Expiration date:	29 February 2016
Storage:	Ambient

Reference Substance/ Analytical Standard:	($^{13}C_6$)Benzamidine ($^{13}C_6$)benzenecarboximidamide
Supplier:	Monsanto Company (Sponsor)
Batch/Lot No:	GLP-1207-22176-A
Purity:	Not assigned
Expiration date:	31 July 2016
Storage:	Ambient

Standard stock solutions, calibration standard solutions and fortification solutions were prepared as described in the analytical method. 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 substances and internal standards will be retained until expiry and then disposed of following relevant disposal SOPs with the approval of the Study Monitor.

Fortification of Recovery Samples

The control specimens were fortified as described below:

Matrix	**Reference Substances**	**Reagent Blank**	**Untreated Control Samples**	**Replicates at Lower Fortification Level (LOQ* and 4X LOQ*)**	**Replicates at Higher Fortification Level (10X LOQ* and 40X LOQ)**
High Sand Soil	MON 102100 and Benzamidine	1	2	5 at 0.0050 µg/g (MON 102100) and 0.00125 µg/g (benzamidine)	5 at 0.050 µg/g (MON 102100) and 0.0125 µg/g (benzamidine)
High Clay Soil	MON 102100 and Benzamidine	1	2	5 at 0.0050 µg/g (MON 102100) and 0.00125 µg/g (benzamidine)	5 at 0.050 µg/g (MON 102100) and 0.0125 µg/g (benzamidine)
High Sand Soil	MON 102100 and Benzamidine	1	2	5 at 0.0050 µg/g (MON 102100 and benzamidine)	5 at 0.050 µg/g (MON 102100 and (benzamidine)

High Clay Soil	MON 102100 and Benzamidine	1	2	5 at 0.0050 µg/g (MON 102100 and benzamidine)	5 at 0.050 µg/g (MON 102100 and benzamidine)

*LOQ = Limit of Quantitation

Approximately 0.080-g subsamples of the control matrices were weighed into 1.4-mL polypropylene tubes. Five samples were fortified at 0.0050 µg/g (ppm) for MON 102100 (LOQ) and 0.00125 µg/g (ppm) for benzamidine (LOQ). Five samples were fortified at the upper fortification level of 0.050 µg/g (ppm) for MON 102100 (10x LOQ) and 0.0125 µg/g (ppm) for benzamidine (10x LOQ). Additional fortifications were added for benzamidine analysis: five samples were fortified at 0.0050 µg/g (4x LOQ) and five samples were fortified at 0.050 µg/g (40x LOQ). MON 102100 fortifications of 0.0050 µg/g and 0.050 µg/g were added to the extra fortification samples respectively to keep sample preparation consistent. The fortification solutions were added directly onto the matrix.

Method Principle

Soil samples were fortified, extracted and analyzed according to the analytical method included in the study protocol (Appendix A) and presented in Appendix E.

Sample Preparation for Analysis

1. 0.080 ± 0.005 g subsamples of soil matrix were weighed into 1.4-mL polypropylene tubes (for 96-well format).
2. 40 µL of 65% acetonitrile (ACN) in water was added to the reagent blank and control samples. 40 µL of the appropriate MON 102100 Working Calibration Solution was added to the samples designated as calibration standards. 40 µL of the appropriate MON 102100 QC Fortification Solution was added to the fortification samples.
3. 40 µL of 65% ACN in water was added to the reagent blank and control samples. 40 µL of the appropriate Benzamidine Working Calibration Solution was added to the samples

designated as calibration standards. 40 µL of the appropriate Benzamidine QC Fortification Solution was added to the fortification samples. One grinding ball was added to each tube.

Description	Volume (µL)	Reagent Solution	Benzamidine Fortification (µg/g, ppm)	MON 102100 Fortification (µg/g, ppm)
Reagent Blank and Controls	80	65% ACN	---	---
Samples fortified at LOQ	40 each	---	0.00125	0.0050
Samples fortified at 10x LOQ	40 each	---	0.0125	0.050
Samples fortified at 4x LOQ for Benzamidine	40 each	---	0.0050	0.0050
Samples fortified at 40x LOQ for Benzamidine	40 each	---	0.050	0.050

4. 720 µL of the Mixed Working Internal Standard Solution was added to each tube.
5. Samples were covered with a shaker cap mat.
6. Samples were sonicated for 3 min.
7. Samples were placed on a Geno/Grinder® at 1200 cpm (cycles per min) for 30 min.
8. Samples were then centrifuged at approximately 6000 × *g* for 5 min at ≤10 °C.
9. Aliquots of each sample were removed and transferred to daughter plates following the table below.

Analyte	Daughter Plate	Transfer Volume (µL)
MON 102100	96-well microplate with clear glass conical inserts	400
Benzamidine	Agilent 96-well plate, 1 mL	50

For MON 102100 Analysis:

10A. 400 µL of toluene was added to the 400 µL aliquot.

11A. Samples were placed on a Geno/Grinder® at 1200 cpm for 2 min.

12A. Samples were centrifuged at approximately 6000 × *g* for 2 min.

13A. At least 100 µL of each sample was then transferred to an amber GC vial and capped.

14A Samples were analyzed by GC-MS/MS.

For Benzamidine Analysis:

10B. 700 µL of 95% ACN, 10 mM ammonium formate was added to the 50 µL aliquot.

11B. Samples were analyzed by LC-MS/MS.

Analytical Instrumentation and Equipment

The instrumental conditions used during the ILV trial were optimized for the available instrumentation. Full instrumental conditions used are given below:

GC-MS/MS Operating Conditions for MON 102100

Instrumentation:	GC Bruker 436
	Bruker CP8400 Autosampler
	Bruker Scion Mass Spectrometer
	MS Workstation data system
Ion Source:	Electron Impact
Column:	Restek Rxi-17 sil MS
	30 m × 0.25 mm, 0.25 µm
Injection Volume:	0.50 µL
GC Carrier Gas:	Helium
Syringe:	5 µL
Air Volume:	1.0 µL

Injection Speed:	5 µL/sec
Fill Volume:	4 µL
Fill Strokes:	5
Fill Speed:	0.30 µL/sec
Viscosity Delay:	3 sec
Inlet Temperature:	250 °C
Injection Pulse Pressure:	25 psi until 0.5 min
Column Flow:	1.0 mL/min

Time (min)	Ramp Rate (°C/min)	Initial Oven Temperature (°C)	Final Oven Temperature (°C)
0-1.0	0	90	90
1.0-6.25	40	90	300
6.25-7.75	10	300	315
7.75-12.75	1	315	315

Typical Mass Spectrometry Operating Conditions for MON 102100

Mode:	EI
Scan Type:	MRM
Resolution:	Q1-unit, Q3-unit
Source Temperature:	230 °C
Solvent Delay:	5 min
Collision Cell He:	2.25 mL/min
Collision Cell N_2:	1.5 mL/min

Analyte	Precursor Ion Q1 (amu)	Product Ion Q3 (amu)	CE (V)	Dwell (ms)
Quantitative Ions				
MON 102100	228.0	119.0	13	125
(Phenyl-$^{13}C_6$) MON 102100 (IS)	234.0	125.0	13	125
Confirmatory Ions				
MON 102100	228.0	111.0	13	125
(Phenyl-$^{13}C_6$)MON 102100 (IS)	234.0	111.0	13	125

LC-MS/MS Operating Conditions for Benzamidine

Instrumentation:	Agilent 1290 LC System
	API 6500 Q-Trap MS/MS Detector
	MDS/Sciex Analyst/MultiQuant Data system
Column Temperature:	40 °C
Injection Volume:	5 µL
Column:	Supelco Ascentis Express HILIC Column
	(2.1 mm × 50 mm, 2.7 µm)
Run Time:	11.0 min
Mobile Phase:	A: 50% methanol, 50 mM Ammonium Formate
	B: 90% ACN, 10 mM Ammonium Formate

Time (min)	Flow (µL/min)	A,%	B, %
0.0	400	0	100
1.80	400	0	100
1.81	1000	100	0
4.19	1000	100	0
4.20	1000	0	0
7.00	1000	0	100
7.10	400	0	100
8.00	400	0	100
11.0	400	0	100

Typical Mass Spectrometry Operating Conditions for Benzamidine

Ionization Mode:	Positive Ion
Scan Type:	Scheduled MRM
Resolution:	Q1-unit, Q3-unit
Curtain Gas (CUR):	35 psi
Collision Gas (CAD):	6
IonSpray Voltage (IS):	5000 V
Temperature (TEM):	500 °C
Entrance Potential (EP):	10 V

Analyte	Q1 Ion	Q3 Ion	Declustering Potential, V	Collision Energy, V	Cell Exit Potential, V
Quantitation Ions					
Benzamidine	121.1	104.0	81	25	14
$^{13}C_6$-Benzamidine (IS)	127.1	110.0	81	25	14
Confirmatory Ions					
Benzamidine	121.1	77.0	81	41	16
$^{13}C_6$-Benzamidine (IS)	127.1	83.1	81	41	16

Calculation of Results

Processing of calibration standard data for the determination of MON 102100 and benzamidine was performed using linear regression (1/x weighting). The linear regression was not forced through zero. For analysis of both soil types, calibration standards were prepared over a concentration range of 0.0080-2.0 µg/mL for MON 102100 and 0.00050-0.60 µg/mL for benzamidine corresponding to injected concentrations of 0.400-99.9 ng/mL for MON 102100 and 0.0238-28.5 ng/mL for benzamidine. Calibration standards for MON 102100 covered the equivalent sample concentration range of 0.0040-1.0 mg/kg (ppm), and calibration standards for benzamidine covered the equivalent sample concentration range of 0.00025-0.30 mg/kg (ppm). All calibration standards and sample extracts contained 0.045 µg/mL of the MON 102100 internal standard and 0.0018 µg/mL of the benzamidine internal standard. The equation for the calibration curve was calculated by plotting the calculated quantitation ratio generated by

dividing the peak area of MON 102100 or benzamidine in the sample by the peak area of the internal standard in the same sample versus analyte concentration ratio (ratio of analyte and internal standard concentrations). Determination of the net concentration of MON 102100 or benzamidine in each recovery sample was conducted by subtracting any background contribution found at the retention time of the analyte in the untreated control sample from that of the gross analyte concentration found in each recovery sample. No corrections were made for net concentration of MON 102100 or benzamidine reported in the recovery samples.

Example Calculations (Quantitation Transition)

Example calculations performed in Microsoft Excel 2007 are found below.

Relative Error Accuracy (RE, %) for a MON 102100 Standard with 0.4995 ng/mL Concn.

$$RE\ (\%) = \frac{(\text{Calc. Std. Concn. (ng/mL)} - \text{Nominal Std. Concn. (ng/mL)}) \times 100}{\text{Nominal Std. Concn. (ng/mL)}}$$

Where:

Lab Standard ID: CMW091714-2A, Set S001S

Calc. Std. Concn. (ng/mL) = 0.518

Nominal Std. Concn. (ng/mL) = 0.4995

To Solve:

RE (%) = ((0.518 – 0.4995) ×100) / 0.4995 = 3.704

MON 102100 recovery at 0.0050 µg/g (LOQ Fortification Level)

Laboratory Sample ID: 805-S001-S1SA, Set S001S

$$\mu g/g\ (ppm)\ Found = \frac{\text{Amount Found (ng/mL)} \times \text{Final Vol. (mL)} \times \text{Dilution Factor}}{\text{Sample Weight (g)} \times 1000\ mg/g}$$

Where:

Amount Found (ng/mL) = 0.429

Final Vol. (mL) = 0.800

Dilution Factor = 1

Sample Weight (g) = 0.081

To Solve:

(0.429 × 0.800 × 1) / (0.081 × 1000) = 0.00424 µg/g (ppm)

Fortification Level µg/g (ppm) =

$$\frac{\textit{Volume Spiking Solution (mL)} \times \textit{Concn. of Spiking Solution (µg/mL)}}{\textit{Sample Weight (g)}}$$

Where:

Volume Spiking Solution (mL) = 0.040

Concn. Of Spiking Solution (µg/mL) = 0.00999

Sample Weight (g) = 0.081

To Solve:

(0.040 × 0.00999) / (0.081) = 0.00493 µg/g (ppm)

$$\textit{Bkgd-Corrected Recovery (\%)} = \frac{\textit{(µg/g Found in Spike – µg/g Found in Control)} \times 100}{\textit{Fortification Level (µg/g)}}$$

Where:

µg/g (ppm) Found in Spike = 0.00424

Average µg/g (ppm) Found in Controls = (0.0000 + 0.0000) / 2 = 0.0000

Fortification Level µg/g (ppm) = 0.00493

To Solve:

$((0.00424 – 0.0000) \times 100) / 0.00493 = 85.89^{\dagger}\%$

†Slight difference appears in data summary tables due to rounding in presented data.

Calc. stands for Calculated

Std. stands for Standard

Concn. stands for Concentration

Vol. stands for Volume

Bkgd stands for background

Statistical Treatment of Data

The mean recoveries and associated standard deviations for the fortified samples were calculated using the "AVERAGE" and "STDEV" functions of Microsoft Excel 2007. Percent relative standard deviation, (RSD, %) was calculated by dividing the standard deviation by the mean, and then multiplying by 100. Slight rounding differences may be found.

Confirmation of Residue Identity (Specificity)

Confirmation of the identities of the analytes was performed, to demonstrate the specificity of the method, by monitoring one additional SRM transition simultaneous to the primary detection (quantitation) transition for each analyte. Representative confirmatory ion chromatograms for MON 102100 and benzamidine are found in Appendix D.

Matrix Interference (Selectivity)

Untreated control matrix samples and samples fortified at the lowest fortification level for each analyte/matrix combination were analyzed to demonstrate the selectivity of the method. Fortified samples were corrected for the background signal found in unfortified control matrix samples.

Problems Encountered, Changes or Modifications Made and Critical Steps

One modification was required for the MON 102100 analysis. The final aliquot removed in the MON 102100 preparation was transferred to an amber GC vial. This was because the autosampler on the GC-MS/MS is not equipped to accommodate 96-well plates.

The sponsor requested three changes for the final attempt at method validation for the benzamidine method. The HPLC gradient was modified from the one displayed in Appendix 1 of the protocol (Appendix A) to the one presented below. The equilibration time at the end of the method is 3.0 min at 400 µL per min and 100% B.

Time (min)	Flow (µL)	A,%	B, %
0.0	400	0	100
1.80	400	0	100
1.81	1000	100	0
4.19	1000	100	0
4.20	1000	0	100
7.00	1000	0	100
7.10	400	0	100
8.00	400	0	100
11.0	400	0	100

Additional benzamidine fortification levels were added at 4x LOQ and 40x LOQ for a total of twenty fortifications instead of ten. MON 102100 fortifications of 0.005 µg/g and 0.05 µg/g were added to the extra fortification samples respectively to keep sample preparation consistent. Two additional calibration points were added to the lower end of the curve at 50% and 25% of the lowest calibration standard in the method. The calibration for benzamidine had a total of fourteen calibration points.

Monsanto Company Standard Operating Procedure

Effective Date: June 10, 2014 **AG-ME-1636-02** Page 1 of 30

High Throughput Assay for MON 102100 and Benzamidine in Soil

Overview

Purpose & Scope

This procedure describes the analytical method used by Environmental Sciences Technology Center personnel for the determination of MON 102100 and/or benzamidine in soil. The method is an internal standard method in which analyte-specific stable-labeled internal standards are used to compensate for procedural losses and matrix-based ionization effects in mass spectral analysis.

SOP Revision Summary

Following is a synopsis of the changes in this SOP from its last version:

- Updated sample homogenization description.
- Modified method soil sample processing descriptions to reflect the available and preferred options.
- Corrected column headings and units in tables contained within the validation summary document.
- Additional fortification level at 10X LOQ was added for QC fortification solution preparation.
- Updated significant figures and formatting.
- Corrected typographical errors.
- Clarifications were added on the procedures.

Method Summary

Soil samples are milled to appropriate homogeneity to allow reproducible measurement of 80-mg subsamples. The milled matrix is weighed into 96-well format tubes followed by the addition of a 65% ACN solution containing ISs for both MON 102100 and benzamidine. The sample tubes are capped and then agitated on a high speed shaker for extraction. Aliquots of the extract are transferred to separate 96-well plates for further processing for analysis of MON 102100 and/or benzamidine. The MON 102100 is partitioned with toluene for analysis by EI GC-MS/MS. The benzamidine aliquot is subsequently diluted 15-fold with 95% ACN in 10 mM ammonium formate for analysis by LC-MS/MS with electrospray ionization. The working range of the method is 0.00125 to 0.30 mg/kg (ppm) for benzamidine and 0.0050 to 1.0 mg/kg (ppm) for MON 102100.

Safety Precautions

Follow current Monsanto safety policies. Important precautions include:

- Some solvents are volatile and flammable. Care must be taken to keep them away from any source of ignition.
- Ensure proper ventilation to avoid excessive exposure to solvent vapors.
- Read and follow all safety warnings on reagent containers.
- Ensure proper safety requirements are followed when operating liquid handlers or autosamplers.

Abbreviations The following abbreviations are used in this procedure:

Abbreviation	Definition
ACN	acetonitrile
amu	atomic mass units
ARS	Analytical Reference Standard
Cal.	Calibration
CE	collision energy
cps	counts per second
CXP	collision exit potential
CV	coefficient of variation
Concn	Concentration
DP	declustering potential
EP	entrance potential
ESI	electrospray ionization
EI	electron impact
HPLC	high performance liquid chromatography
g	gram
Int.	Intermediate
IS	Internal Standard
kg	kilogram
LC-MS/MS	liquid chromatography/tandem mass spectrometry
GC-MS/MS	gas chromatography/tandem mass spectrometry
LOQ	limit of quantitation
mg mL	milligram milliliter
mm	millimeter
MRM	multiple reaction monitoring
ms	millisecond
MS	mass spectrometry
N	number of samples
ppm	parts-per-million
µg	microgram
V	volts
x *g*	relative centrifugal force

Contents

Materials

Equipment The following equipment is used in this procedure. Specific brands are listed to aid the analyst in finding items. In most cases, equivalent equipment from other vendors can be used.

Equipment	Number/Specification
Analytical balance	Capable of weighing 0.0001 g
GC system (MON 102100 parent)	Agilent 7890A System with Gerstel MPS auto sampler
Mass spectrometer (MON 102100 parent)	Agilent 7000 Triple Quadrupole with EI ionization source
GC column (MON 102100 parent)	Agilent DB-17 MS 30 m × 0.25 mm, 0.25 µm (part number122-4732)
GC data acquisition system	PC workstation with Agilent MassHunter and Gerstel Maestro software
HPLC system (benzamidine)	Shimadzu Prominence 20A System: Solvent Degasser, at least 2 Pumps, Autosampler, Column Compartment and Controller
HPLC switching valve (benzamidine)	Rheodyne, 6 port
Mass spectrometer (benzamidine)	AB Sciex API 5000™ with Turbo-V ion source
HPLC column (benzamidine)	Supelco Ascentis Express HILIC Column 50 mm × 2.1 mm, 2.7 µm
LC data acquisition system	PC workstation with AB Sciex Analyst® software
Freezer mill (secondary milling)	SPEX SamplePrep Model 6870 with 100 mL milling vessel
High-speed plate shaker	Genogrinder 2000
Sonicator	Branson B-22-4 Ultrasonic Cleaner
Graduated cylinders (100 mL, 1 L)	suitable for procedure
Volumetric flasks (1 L)	suitable for procedure
Mechanical pipettes	suitable for procedure
Repeating/dispensing pipette	Eppendorf Repeater Xstream
Liquid handler	Tomtec, Inc. Quadra 4 or comparable pipetting system
Water purification system (or HPLC quality water)	Millipore Compact Milli-Q Plus
96-Well microplate with clear glass conical inserts (with sealing mat)	National Scientific, K96-1.1MB
96 Deep-well plate, glass-coated polypropylene (1 mL)	National Scientific, P96U-1.0G
96 Deep-well plate, clear	Axygen Scientific, VWR 10011-940
Polypropylene extraction tubes (1.4 mL)	Thermo Scientific Cat. No. 4140MTX
96-Well plate cap mat (shaker)	Sun-SRI Cat No. 400-079
Matrix empty latch rack for 1.4 ml tubes	Thermo Scientific Cat. No. 4898
96-Well plate, 1 mL (autosampler)	Agilent No. 5042-1387
96-Well pre-slit cap mat	Thermo Scientific Cat. No. 276011

Grinding ball	About 3 mm diameter, stainless steel
96-Well mat WEBSEAL, blue	XPERTEK 972150
96-Well micro mat flat bottom	XPERTEK 971805

Chemicals & Reagents

The following reagents are used in this method. **Note:** Specific brands are listed to aid the analyst in finding items. In most cases, equivalent reagents from other vendors can be used. It is important to use high quality reagents to avoid chromatographic interferences. It is recommended to verify the isotopic purities of the internal standard materials prior to use.

Chemical/Reagent	Number/Specification
Toluene, ACS grade	EMD, TX0735-6
ACN, HPLC grade	Burdick & Jackson Cat. No. 015-4
Methanol, ultrapure grade	EMD Cat. No. MX04881
Ammonium formate, ≥95% purity	Fisher Cat. No. A666-500
Water, HPLC or higher purity grade	Milli-Q water or equivalent
MON 102100	Monsanto ARS Program
Benzamidine	Monsanto ARS Program
(Phenyl-$^{13}C_6$)MON 102100	Monsanto ARS Program
($^{13}C_6$)Benzamidine	Monsanto ARS Program

Reagent/Solution Preparation

Prepare the following reagent solutions for use in sample analysis. The absolute volume of the solutions may be varied at the discretion of the analyst, as long as the correct proportions of the components are maintained. A six month expiration date will be assigned to these solutions unless a shorter expiration is specified on the label. Solutions may be stored at room temperature in glass containers.

Solution	Preparation
65% ACN	Add 1300 mL ACN to 700 mL water
Stock Ammonium Formate	200 mM ammonium formate: Dissolve 6.30 g of ammonium formate in 500 mL of water
Benzamidine HPLC Mobile Phase A	50% methanol, 50 mM ammonium formate: Add 250 mL of stock ammonium formate (200 mM) to 250 mL water and 500 mL methanol
Benzamidine HPLC Mobile Phase B	90% ACN, 10 mM ammonium formate: Add 100 mL of stock ammonium formate (200 mM) to 100 mL water and 1800 mL ACN
Benzamidine HPLC Injection Needle Wash	95% ACN, 10 mM ammonium formate: Add 25 mL of stock ammonium formate (200 mM) to 475 mL ACN
Benzamidine Sample Dilution Solution	95% ACN, 10 mM ammonium formate: Add 25 mL of stock ammonium formate (200 mM) to 475 mL ACN
GC Wash Solvent 1	50% ethyl acetate in acetone: Add 250 mL of ethyl acetate and 250 mL acetone
GC Wash Solvent 2	100% toluene

Standard Calibration and QC Solution Preparation

Overview

All standard calibration and fortification solutions must be properly labeled and stored in amber glass vials with airtight lids at approximately -20 °C. Preparation procedures which result in equivalent solutions may be substituted. Various additional solutions may be prepared.

Stability

The solution stability of MON 102100 and benzamidine was demonstrated during the validation of the crop methods AG-ME-1579-01 (benzamidine) and AG-ME-1604-01 (MON 102100) and is summarized in the following table.

Solution Components	Solution Type	Concentration or Range *	Solvent	Approx. Storage (° C)	Demonstrated Stability (Days)
MON 102100	Stock Solution	500. µg/mL	ACN	-20	97
MON 102100	Working Calibration Standard Solutions	0.25 to 1000 ng/mL	Toluene	-20	31
Benzamidine	Stock Solution	526 µg/mL 1000. µg/mL (MON 102100 equivalents)	ACN/Water	-20	204
Benzamidine	Working Calibration Standard Solutions	1.05 to 316 ng/mL 2.0 to 600. ng/mL (MON 102100 equivalents)	ACN/Water	-20	204

*Stability of benzamidine was established in method AG-ME-1579-01. In AG-ME-1579-01 benzamidine was expressed in MON 102100 equivalents. This method reports benzamidine *per se*; therefore, both values are shown for clarity.

MON 102100 Calibration Stock Solution (0.50 mg/mL)

Weigh 20-25 mg (record to at least 0.1 mg) of MON 102100 standard into a 60-mL amber glass bottle. Add the appropriate volume (to the nearest 0.1 mL) of ACN to prepare a 0.50 mg/mL solution of MON 102100 (purity adjusted). An adjustable positive-displacement mechanical pipette capable of delivering up to 50 mL is recommended. The solution should be sonicated briefly to ensure complete dissolution.

MON 102100 QC Stock Solution (0.50 mg/mL)

Prepare a separate 0.50 mg/mL MON 102100 (purity adjusted) QC Stock Solution using the procedure above for the MON 102100 Calibration Stock Solution. Preparation of this requires separate weighing.

Benzamidine Calibration Stock Solution (0.50 mg/mL)

Weigh 20-25 mg (record to at least 0.1 mg) of benzamidine standard into a 60-mL vial. Add the appropriate volume (to the nearest 0.1 mL) of 65% ACN in water to prepare a 0.50 mg/mL solution of benzamidine (purity adjusted). An adjustable positive-displacement mechanical pipette capable of delivering up to 50 mL is recommended. The solution should be sonicated briefly to ensure complete dissolution.

Benzamidine QC Stock Solution (0.50 mg/mL)

Prepare a separate 0.50 mg/mL solution of benzamidine (purity adjusted) QC Stock Solution using the procedure above for the Benzamidine Calibration Stock Solution. Preparation of this requires separate weighing.

Intermediate Calibration Solutions

Prepare the following intermediate calibration standard solutions in 20-mL amber glass vials by dilution of the appropriate stock solution with 65% ACN in water. These solutions will be used for the preparation of working solutions.

Intermediate Calibration Solution (µg/mL)	Aliquot Solution ID	Aliquot Volume (mL)	Diluent Volume (mL)
10.	MON 102100 Calibration Stock Solution (0.50 mg/mL)	0.200	9.8
1.0	10. µg/mL MON 102100	1.00	9.0
0.10	1.0 µg/mL MON 102100	1.00	9.0
5.0	Benzamidine Calibration Stock Solution (0.50mg/mL)	0.100	9.9
0.50	5.0 µg/mL Benzamidine	1.00	9.0
0.050	0.50 µg/mL Benzamidine	1.00	9.0

Working Calibration Standard Solutions

Solutions may be prepared in the following manner. Other concentrations may be used as long as the preparation is documented. A suggested scheme for working calibration solution preparation is shown below. For each working solution add the listed aliquot of the intermediate calibration solution to an amber glass vial and dilute with the specified volume of 65% ACN diluent. Additional standard levels may be prepared as necessary.

MON 102100 Working Calibration Solutions

MON 102100 Working Cal. Solution (µg/mL)	Dilute this MON 102100 Int. Solution (µg/mL)	MON 102100 Aliquot Volume (mL)	65% ACN Volume (mL)	Matrix Equivalent MON 102100 Concn (mg/kg, ppm)*	Level in Mass Hunter
0.0080	0.10	0.800	9.2	0.0040	1
0.010	0.10	1.00	9.0	0.0050	2
0.012	0.10	1.2	8.8	0.0060	3
0.020	0.10	2.0	8.0	0.010	4
0.040	1.0	0.400	9.6	0.020	5
0.080	1.0	0.800	9.2	0.040	6
0.12	1.0	1.2	8.8	0.060	7
0.20	10.	0.200	9.8	0.10	8
0.40	10.	0.400	9.6	0.20	9
0.80	10.	0.800	9.2	0.40	10
1.2	10.	1.2	8.8	0.60	11
2.0	10.	2.0	8.0	1.0	12

**0.040 mL of working calibration standard solution used. Although no matrix is used in standards, study samples use 0.080 g of matrix. The values listed in this column represent the concentration of MON 102100 in solution given a target 0.080 g matrix sample.*

Benzamidine Working Calibration Solutions

Benzamidine Working Cal. Solution (µg/mL)	Dilute this Benzamidine Int. Solution (µg/mL)	Benzamidine Aliquot Volume (mL)	65% ACN Volume (mL)	Matrix Equivalent Benzamidine Concn (mg/kg, ppm)*
0.0020	0.10	0.200	9.8	0.0010
0.0025	0.10	0.250	9.75	0.00125
0.0030	0.10	0.300	9.7	0.0015
0.0050	0.10	0.500	9.5	0.0025
0.010	0.10	1.00	9.0	0.0050
0.020	1.0	0.200	9.8	0.010
0.030	1.0	0.300	9.7	0.015
0.050	1.0	0.500	9.5	0.025
0.10	10.	0.100	9.9	0.050
0.20	10.	0.200	9.8	0.10
0.30	10.	0.300	9.7	0.15
0.60	10.	0.600	9.4	0.30

**0.040 mL of working calibration standard solution used. Although no matri x is used in standards, study samples use 0.080 g of matrix. The values listed in this column represent the concentration of benzamidine in solution given a target 0.080 g matrix sample.*

QC Fortification Solutions

Solutions may be prepared in the following manner. Other concentrations may be used as long as the preparation is documented. A suggested scheme for QC fortification solution preparation is shown below. For each fortification solution, add the listed aliquot of the designated solution to an amber glass vial and dilute with the specified volume of 65% ACN. Additional fortification solution levels may be prepared as necessary.

MON 102100 QC Fortification Solutions

MON 102100 QC Solution Concn (µg/mL)	Aliquot Solution ID	Aliquot Volume (mL)	65% ACN Volume (mL)	MON 102100 Fortification (mg/kg, ppm)*
10.	0.50 mg/mL MON 102100 QC Stock	0.200	9.8	N/A
1.0	10. µg/mL MON 102100 QC Stock	1.00	9.0	N/A
0.010	1.0 µg/mL MON 102100 QC Stock	0.100	9.9	0.0050 (LOQ QC)
0.10	10. µg/mL MON 102100 QC Stock	0.100	9.9	0.050 (10x LOQ QC)
0.20	10. µg/mL MON 102100 QC Stock	0.200	9.8	0.10 (Mid QC)
1.8	10. µg/mL MON 102100 QC Stock	1.80	8.2	0.90 (High QC)
8.0	10. µg/mL MON 102100 QC Stock	8.0	2.0	4.0 (Dilution QC)

** 0.040 mL of QC fortification solution added to 0.080 g of control sample*

Benzamidine QC Fortification Solutions

Benzamidine QC Solution Concn (µg/mL)	Aliquot Solution ID	Aliquot Volume (mL)	65% ACN Volume (mL)	Benzamidine Fortification (mg/kg, ppm)*
10.	0.50 mg/mL Benzamidine QC Stock	0.200	9.8	N/A
1.0	10 µg/mL Benzamidine QC Stock	1.00	9.0	N/A
0.10	1.0 µg/mL Benzamidine QC Stock	1.00	9.0	N/A
0.0025	0.10 µg/mL Benzamidine QC Stock	0.500	19.5	0.00125 (LOQ QC)
0.025	1.0 µg/mL Benzamidine QC Stock	0.250	9.75	0.0125 (10x LOQ QC)
0.050	1.0 µg/mL Benzamidine QC Stock	0.500	9.5	0.025 (Mid QC)
0.60	10. µg/mL Benzamidine QC Stock	0.600	9.4	0.30 (High QC)

** 0.040 mL of QC fortification solution added to 0.080 g of control sample*

(Phenyl-$^{13}C_6$)MON 102100 IS Stock Solution (1.0 mg/mL)

Weigh 20-25 mg (record to at least 0.1 mg) of (phenyl-$^{13}C_6$)MON 102100 standard in a 60-mL amber glass vial. Add the appropriate volume (to the nearest 0.1 mL) of ACN to prepare a 0.50 mg/mL solution of (phenyl-$^{13}C_6$)MON 102100 (e.g., 40.2 mL of diluent for 20.1 mg of (phenyl-$^{13}C_6$)MON 102100). An adjustable positive-displacement mechanical pipette capable of delivering up to 50 mL is recommended. The solution should be sonicated briefly to ensure complete dissolution.

($^{13}C_6$)Benzamidine IS Stock Solution (1.0 mg/mL)

Weigh 20-25 mg (record all to at least 0.1 mg) of ($^{13}C_6$)benzamidine standard in a 60-mL amber glass vial. Add the appropriate volume (to the nearest 0.1 mL) of 65% ACN to prepare a 1.0 mg/mL solution of ($^{13}C_6$)benzamidine. An adjustable positive-displacement mechanical pipette capable of delivering up to 50 mL is recommended. The solution should be sonicated briefly to ensure complete dissolution.

Intermediate IS Solutions

Prepare the following intermediate IS solutions by dilution of the appropriate IS stock solution with 65% ACN

Intermediate IS Solution (µg/mL)	Aliquot Solution ID	Aliquot Volume (mL)	65% ACN Volume (mL)
10.	(Phenyl-$^{13}C_6$)MON 102100 IS Stock Solution (0.50 mg/mL)	0.200	9.8
10.	($^{13}C_6$)Benzamidine IS Stock Solution (1.0 mg/mL)	0.100	9.9
1.0	($^{13}C_6$)Benzamidine IS Stock Solution (10. µg/mL)	1.00	9.0

Mixed IS Working Solution (0.050 µg/mL (Phenyl-$^{13}C_6$)MON 102100 0.0020 µg/mL ($^{13}C_6$)Benzamidine)

Prepare the IS working solution the day to be used for analysis by diluting intermediate IS solutions with 65% ACN. An example dilution scheme is shown below.

$^{13}C_6$-Benzamidine Int. IS Solution (µg/mL)	(Phenyl-$^{13}C_6$)MON 102100 Int. IS Solution (µg/mL)	($^{13}C_6$)Benzamidine Aliquot (mL)	(Phenyl-$^{13}C_6$)MON 102100 Aliquot (mL)	Diluent Volume (mL)	Mixed IS Working Solution ($^{13}C_6$)Benz Concn (µg/mL)	Mixed IS Working Solution (Phenyl-$^{13}C_6$)MON 102100 Concn (µg/mL)
1.0	10.	0.200	0.500	99.3	0.0020	0.050

Sample Dilution Solution

Dilution of samples for which the MON 102100 analyte response is beyond the range of calibration standards is achieved by adding toluene without IS for MON 102100 analysis.

Sample Preparation Procedure

Sample Storage

Homogenized samples will be maintained frozen at approximately -20 °C for extended storage periods.

Sample Homogenization

Raw sample material must be thoroughly milled and homogenized using a two-step milling process to reproducibly measure 80-mg subsamples. The first step involves preliminary bulk homogenization of the frozen sample with dry ice using an appropriate milling device such as a vertical cutter mixer. After bulk homogenization, a 30-40-mL subsample is milled further using cryogenic cooling with liquid nitrogen to a powder-like state. This can be performed with a SPEX Freezer Mill or other comparable device. Typical milling conditions on a SPEX Freezer Mill are below.

	Off-Line Precooling Method	On-Line Precooling Method
Cycles	4	4
Off-line Precool (external to instrument)	8 minutes	N/A
On-line Precool (on instrument)	2 minutes	15 minutes
Run Time	2 minutes	2 minutes
Cool Time	1 minute	1 minute
Rate	9 cps	9 cps

Soil Sample Processing

The following describes the preparation of soil samples for MON 102100 and benzamidine analysis.
A typical analytical set will include study samples, QCs and standards.

Step	Action
1	Weigh 80 ± 5 mg of milled matrix into a 1.4-mL polypropylene tube (for 96-well format) and record the weight. Soil matrices must be kept frozen on dry ice and transferred frozen during this process. A common control matrix absent of any significant interference of MON 102100 and benzamidine (and their ISs) will be used for QCs and control blanks. **Note: The exact sample mass will be used to adjust the dilution factor for correction of sample concentration (e.g., target sample weight 0.0800 g /actual sample weight of 0.0828 g, enter dilution factor of 0.966 in MassHunter and Analyst).*
2A	Add 40.0 µL of the following solution to the designated sample type: • 65% ACN to test samples and controls • MON 102100 Working Calibration Standard Solutions to calibration standards • MON 102100 QC Fortification Solutions to QC samples (e.g. LOQ QC, 10x LOQ QC, Mid QC, High QC and/or Dilution QC)
2B	Add 40.0 µL of the following solution to the designated sample type: • 65% ACN to test samples and controls • Benzamidine Working Calibration Standard Solutions to calibration standards • Benzamidine QC Fortification Solutions to QC samples (e.g. LOQ QC, 10x LOQ QC, Mid QC, High QC)
2C	Add 1 grinding ball to each tube.
3	Add 720. µL of the Mixed Working IS Solution (0.0020 µg/mL ($^{13}C_6$)benzamidine and 0.050 µg/mL (phenyl-$^{13}C_6$)MON 102100) to each tube (including tubes designated for standards) using an automated liquid handler or other pipetting device.
4	Cover the 96-well plate with a shaker cap mat. Ensure the cap mat is sealed well before proceeding.
5	Place the plate in a sonicator and sonicate for approximately 3 minutes.
6	Shake samples on the Genogrinder to extract analyte from matrix (e.g. at 1200 cycles per minute for 30 minutes). Examine the plate and tubes for leaks. If leaks are detected discard and re-prepare.

QC Sample	Fortification Level (mg/kg, ppm)
LOQ QC	0.0050
10x LOQ QC	0.050
Mid QC	0.10
High QC	0.90
Dilution QC	Variable, depending on dilution level needed. Up to a 10x dilution.

QC Sample	Fortification Level (mg/kg, ppm)
LOQ QC	0.00125
10x LOQ QC	0.0125
Mid QC	0.025
High QC	0.30

7	Place plate in a ≤10 °C centrifuge and spin to clear suspended materials from the liquid column and form a solid pellet (e.g., 5 minutes at 6000 x g).
8	Remove cap mat and transfer appropriate volume of supernatant to clean 96-well microplate(s) as needed for further analysis (see table below for suggested volumes). This transfer can be performed using a liquid handler, multichannel pipette or other pipetting device.

Analyte	Daughter Plate	Transfer Volume (mL)
MON 102100	96-well microplate with clear glass conical inserts	0.400
Benzamidine	Agilent 96-well plate, 1 mL	0.050

For MON 102100 Analysis

Step	Action
9A	*Add 400. µL of toluene.
10A	Shake samples on the Genogrinder at 1200 cycles per minute for 2 minutes. Examine the plate and tubes for leaks. If leaks are detected discard and re-prepare.
11A	Place plate in a ≤10 °C centrifuge and spin so that all liquid is at the bottom of the tubes (e.g., 2 minutes at 6000 x g).
12A	Remove cap mat and transfer at least 100. µL of the toluene layer (top) of the extract to a clean 96-well micro plate with clear glass conical inserts or a glass-lined polypropylene plate and cover with an autosampler cap mat. This transfer can be performed using a liquid handler, multichannel pipette or other pipetting device.
13A	Analyze by EI GC-MS/MS within storage time determined during method validation.

* Prior to Step 9A samples may be gently evaporated until approximately 150-200 µL remains to remove the majority of the ACN from the sample. The acceptability of method performance with and without this evaporation step was demonstrated during validation (see validation summary in **Appendix C**).

For Benzamidine Analysis

Step	Action
9B	Add 700. µL of Benzamidine Sample Dilution Solution (95% ACN, 10 mM ammonium formate) to the 50. µL aliquot that was transferred in Step 8. Cover the plate with an autosampler cap mat. This transfer can be performed using a liquid handler, multichannel pipette or other pipetting device.
10B	Analyze by ESI LC-MS/MS within storage time determined during method validation..

Extract Dilution

High-level samples producing an analyte response greater than that of the highest standard of the calibration curve must be diluted to within the analyte response range of the standards and reanalyzed. Due to partitioning of MON 102100 as well as the IS during extraction, the samples are diluted with a solvent that does not contain IS to maintain the response ratio during sample dilution. It is not necessary to enter an additional dilution factor in calculations. The amount of dilution will be estimated so that the response of analyte and IS after dilution will be within the analyte response range of the standards. Note: because high-level samples are diluted with extraction solvent rather than a solution containing IS, the response ratio may not be within the range of response ratios of the standards.

Note: Dilution of samples for analysis of benzamidine using diluent containing internal standard did not pass validation acceptance criteria. Therefore, only a dilution scheme for MON 102100 analysis is shown below.

Dilutions for MON 102100 analysis will be made by the following procedure:

Step	Action
1	Transfer an appropriate aliquot of the processed sample (from Step 12A above) to a new tube or well.
2	Add an appropriate volume of MON 102100 Sample Dilution Solution (toluene) and mix thoroughly.
3	Cap, mix and analyze by EI GC-MS/MS.

Instrumental Analysis

Sample Analysis Guidance and Acceptance Criteria

Acceptance criteria for study samples utilizing this analytical method are:

Calibration Curve:

- Back-calculated calibration standard concentrations used to determine results must be within ±20% of their respective nominal concentrations.
- Calibration points may be removed for a documented analytical reason or a back-calculated inaccuracy outside ±20%. Values falling outside these limits can be removed and not included in the calculation, provided they do not change the established regression model (e.g. linear 1/x weighting). If a calibration standard(s) is removed the reason must be documented in the raw data (i.e. inaccuracy >20%).
- A minimum of six concentration levels (excluding blanks) and at least 75% of the total number of calibration standards must be represented in the final curve.

Quality Control Samples:

- The acceptance criterion for mean accuracy should be within 70-120% of the corrected, back-calculated nominal value.
- The acceptance criterion for precision is ≤20% RSD at each fortification level.
- A maximum of 1 outlier (i.e. falls outside of acceptance criteria and fails Grubbs outlier test) may be discarded at each QC fortification level. Proper justification and documentation of discarded outliers must be performed.
- Any response in QC control samples falling within the retention window of a given analyte must be ≤30% of the response at the LOQ level. In cases where this response is exceeded, the presence of the target analyte (i.e. inadvertent contamination) versus an unknown interference will be assessed using an appropriate confirmatory technique.
- Any response from the matrix in QC control samples within the retention window of the IS must be ≤5% of the response for the IS peak in the control matrix sample with IS.
- Capability of dilution is demonstrated by including dilution QC samples in the study. The dilution QC samples must meet acceptance criteria for quality control sample accuracy and precision.

Injection Carryover:

The potential for carryover will be evaluated in each analytical or batch run by placing a double blank after the highest calibration curve point.

- The response for analyte in the carryover sample must be ≤20% of the response at the LOQ level
- The response for IS (if used) in the carryover sample must be ≤5% of the response of the average IS response in the two replicate QC control samples

Instrument Setup

Instrument operation is controlled by acquisition methods containing all autosampler, LC or GC, switching valve (if utilized) and mass spectrometer operating parameters. Precursor and product ions for the analytes are shown below along with choices for possible use in confirmatory analyses. Alternate ions may be used for quantitation or confirmation if they provide better data (sensitivity and/or specificity). The use of a minimum of one quantitation transition and one confirmatory transition is required for each batch run. The following equipment and conditions are instrument/system dependent and may be modified to obtain optimal instrument performance and maximize sensitivity. Actual method parameters must be documented in the raw data.

System Conditions for Analysis

GC-MS/MS System Conditions for Analysis of MON 102100

GC: Agilent 7890A
Autosampler: Gerstel MPS
Mass spectrometer: Agilent 7000 Triple Quadrupole
Ion source: electron impact
Column: DB-17 MS 30m × 0.25mm, 0.25 µm
Injection volume: 0.50 µL
Autosampler temperature: 10 °C
GC carrier gas: helium

Autosampler Conditions

Injection
Syringe: 5 µL
Injection volume: 0.5 µL
Air volume (below): 1.0 µL
Injection speed: 50.0 µL/sec
Fill volume: 4 µL
Fill strokes: 2

Fill speed: 0.30 µL/sec
Viscosity delay: 3 sec
Eject speed: 25.0 µL/sec

Cleaning
Fill speed: 5.0 µL/sec
Viscosity delay: 3 sec.
Eject speed: 50.0 µL/sec

Wash solvent 1: 50% ethyl acetate in acetone
Wash solvent 2: toluene
Wash 1: preclean: 0, postclean: 4
Wash 2: preclean: 1, postclean: 4

GC Conditions

Inlet
Inlet temperature: 250 °C
Pulsed splitless
Injection pulse pressure: 25 psi until 0.5 min
Purge flow to split vent: 20 mL/min at 0.5 min

Oven

Time (min)	Ramp Rate (°C/min)	Initial Oven Temperature (°C)	Final Oven Temperature (°C)
0-1.0	0	90	90
1.0-6.25	40	90	300
6.25-7.75	10	300	315
7.75-12.75	0	315	315

Total run time: 12.75 min

MS transfer line: 300 °C Column flow: 1.0 mL/min				
Mass Spectrometer Conditions				
Mode: EI Scan type: MRM Resolution Q1: unit Resolution Q3: unit Source temperature: 230 °C Solvent delay: 5 min Collision cell He: 2.25 mL/min Collision cell N_2: 1.5 mL/min				
Analyte	Precursor Ion Q1 (amu)	Product Ion Q3 (amu)	CE (V)	Dwell (ms)
MON 102100	228	119	13	125
(Phenyl-$^{13}C_6$) MON 102100 (IS)	234	125	13	125
Confirmatory Ions				
MON 102100	228	111	13	125
(Phenyl-$^{13}C_6$) MON 102100 (IS)	234	111	13	125

LC-MS/MS System Conditions for Analysis of Benzamidine

HPLC: Shimadzu Prominence 20A
Mass spectrometer: AB Sciex API 5000/5500
Ion source: Turbo-V
Column: Supelco Ascentis Express HILIC Column, 50 mm × 2.1 mm, 2.7 µm
Injection volume: 5 µL
Autosampler temperature: 4 °C
Column oven temperature: 40 °C
Mobile Phase A: 50% methanol, 50 mM ammonium formate
Mobile Phase B: 90% ACN, 10 mM ammonium formate
HPLC Gradient Conditions:

Time (min.)	%B	Total Flow (mL/min)	Divert
0-0.5	100	0.400	To waste
0.5-1.69	100	0.400	To MS
1.7-1.8	100	0.400	To waste
1.8-4.1	0	1.00	To waste
4.2-7.0	100	1.00	To waste
7.1-8.0	100	0.400	To waste

Run time: 8 min (MS data collection 1.7 min with 42 sec delay time)

Mass Spectrometer Conditions

Mode: positive ion
Scan type: MRM
Resolution Q1: unit
Resolution Q3: unit
Probe type: ESI

Duration: 1.7 min
Curtain gas (CUR): 35
Collision gas (CAD): 6
Gas 1: 45 N_2
Gas 2: 45 N_2

IonSpray voltage (IS): 5000 V
Entrance Potential (EP): 10
Interface heater: on
Temperature (TEM): 500 °C
Scan time (ms): 150

Analyte	Precursor Ion Q1 (amu)	Product Ion Q3 (amu)	DP (V)	CE (V)	CXP (V)
Benzamidine	121.1	104.0	81	25	14
($^{13}C_6$)Benzamidine (IS)	127.1	110.0	81	25	14
Confirmatory Ions					
Benzamidine	121.1	77.0	81	41	16
($^{13}C_6$)Benzamidine (IS)	127.1	83.1	81	41	16

Data Processing

Process the LC-MS/MS data using the Analyst™ quantitation wizard. Process the GC-MS/MS data using the MassHunter quantitation wizard. A method may be created which processes the data for the MRM transition pairs established in the acquisition method. The method detects and integrates the analyte peaks based on retention time and MRM transition. Chromatograms may be smoothed prior to integration, as long as the smoothing algorithm is consistent throughout the entire sample set. Manual peak integration should be used when the automated procedure is not effective due to baseline noise. Dilution factors, if applicable, must be added during data processing if not input prior to the start of the instrument run.

Calculations

Overview

Analyte concentrations are calculated using the Analyst® (for LC-MS/MS) or Mass Hunter (for GC-MS/MS) software. The software calculates the standard curve and applies the dilution factor to account for dilution or concentration during processing. 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. A linear regression model is used for quantitation with or without weighting (e.g. linear 1/x weighted). All the samples from a study must be analyzed with the same type of calibration curve for a given analyte.

Analyte Concentration

Analyte concentrations are reported in mg/kg (ppm) of matrix. The MassHunter and Analyst systems automatically calculate the raw concentration of the injected sample relative to the standard curve (*calculated concentration*). This value is also automatically multiplied by any value entered in the *dilution factor* column.

Assumptions:

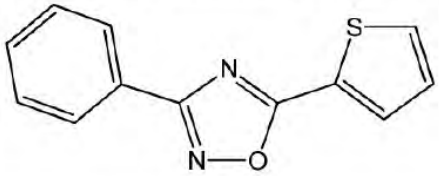
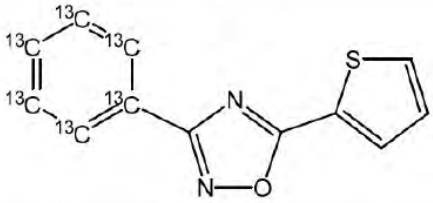
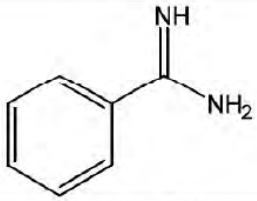
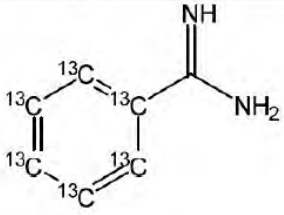
1) The nominal dilution of the sample during extraction (1:10 for MON 102100 and 1:150 for benzamidine) is incorporated into the calibration standard concentrations that are entered into the MassHunter or Analyst software. The calibration standard concentrations are entered as matrix equivalent concentrations ('Matrix Equivalent Concn (mg/kg, ppm)' in the Working Calibration Solution tables above). Calibration standard solutions are diluted equivalently to samples in the sample processing procedure of the method; therefore, these entered concentrations are 10 times and 150 times (for MON 102100 and benzamidine, respectively) their actual injected concentrations, so the dilution factor is eliminated.
2) The error in the 10x or 150x correction above due to actual sample weight is entered as the ratio of the target and actual sample weight (e.g., 0.080 g target / 0.0845 g actual = 0.9467) into the MassHunter or Analyst dilution factor column.
3) Entry of a separate dilution factor is <u>not</u> required for samples with MON 102100 analyte responses higher than the highest calibration standard that are diluted into the range of the curve (samples are diluted with a solvent that does not contain IS, so the original analyte/IS response ratio is maintained).

Documentation

The analytical raw data packages will include (at a minimum): the sample processing worksheet, instrumental sample queue/run record, calibration curves, MRM chromatograms, results tables, instrument acquisition parameters.

Appendices

Appendix A: Chemical Structures

	MON 102100 3-Phenyl-5-thiophen-2-yl-1,2,4-oxadiazole $C_{12}H_8N_2OS$ Average molecular weight: 228.27
	(Phenyl-$^{13}C_6$) MON 102100 (Phenyl-$^{13}C_6$)3-phenyl-5-thiophen-2-yl-1,2,4-oxadiazole $^{13}C_6C_6H_8N_2OS$ Average molecular weight: 234.22
	Benzamidine Benzenecarboximidamide $C_7H_8N_2$ Average molecular weight: 120.15
	($^{13}C_6$)Benzamidine ($^{13}C_6$)Benzenecarboximidamide $^{13}C_6CH_8N_2$ Average molecular weight: 126.11