# 1 SUMMARY

An analytical method was developed and validated for the determination of MON 102100 and benzamidine in soil. Benzamidine was established as the major environmental transformation product of MON 102100 under anaerobic conditions in soil (Shepler 2013a). Pre-study and in-study method validation conducted during analysis of the terrestrial field dissipation study samples demonstrates the performance and applicability of the method.

Soil samples are milled using a two-stage process that provides adequate homogeneity for reproducible measurement of MON 102100 and benzamidine in 80-mg subsamples. The fine-milled matrix is weighed frozen into 96-well format tubes followed by the addition of a 65% acetonitrile in water solution containing stable isotopically-labeled internal standards for both MON 102100 and benzamidine. The sample tubes are capped and agitated on a high-speed shaker for extraction. Following centrifugation, aliquots of the extract supernatant are transferred to two separate 96-well plates for further processing and analysis of MON 102100 and/or benzamidine. The extracts designated for MON 102100 analysis are partitioned with toluene, and the toluene solution is analyzed by EI GC-MS/MS. The extracts designated for benzamidine analysis are diluted 15-fold with 95% acetonitrile in 10 mM ammonium formate followed by ESI LC-MS/MS analysis.

## 2 BACKGROUND

MON 102100 (tioxazafen) is a nematicide currently under development by Monsanto Company. It is planned to be registered for use as a seed treatment on corn, soy, and cotton. A U.S. terrestrial field dissipation study was conducted to determine the mobility and persistence potential of MON 102100 and its major degradation products in soil when applied as a pre-coated soybean seed treatment under field conditions (Jacobson et

al. 2014). The analytical method described in this report was developed and validated to support the field dissipation study.

The degradation of MON 102100 in soil was evaluated under aerobic (Shepler 2013b) and anaerobic (Shepler 2013a) conditions. Benzamidine was identified as a major transformation product under anaerobic conditions and was selected, together with MON 102100, for monitoring in the terrestrial field dissipation study. A single extraction method was developed for the targeted analysis of MON 102100 and benzamidine in soil matrix. The extraction solvent contains stable-labeled internal standards for both MON 102100 and benzamidine. Aliquots of the extract supernatant were transferred to two separate 96-well plates for further processing and analysis of MON 102100 and/or benzamidine. Extracts designated for MON 102100 analysis were partitioned with toluene and analyzed by EI GC-MS/MS. The benzamidine extracts were subsequently diluted 15-fold with 95% acetonitrile in 10 mM ammonium formate followed by ESI LC-MS/MS analysis.

# 3 MATERIALS AND METHODS

## 3.1 Test Systems

Soil was evaluated during pre-study and in-study method validation.

## 3.2 Principle of the Method

Soil samples collected from the field undergo a typical preliminary homogenization in a vertical cutter mixer (VCM) with dry ice. A secondary fine milling procedure is performed on a subsample of the homogenized bulk sample to produce a sample uniformity that allows reproducible analytical results on subsamples as small as 80-mg.

The analytical method starts with the dispensing of 80±5-mg subsamples of the fine milled matrix into 96-well format tubes followed by the addition of a 65% acetonitrile in water solution containing stable-labeled internal standards for both MON 102100 and benzamidine. Samples are extracted using a high-speed plate shaker followed by centrifugation to remove solid materials from the liquid column. 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 aliquot is directly partitioned with toluene. A portion of the toluene is transferred to a new plate for analysis by EI GC-MS/MS. The benzamidine aliquot is diluted 15-fold with 95% ACN in 10 mM ammonium formate to adjust the sample composition and pH for hydrophilic interaction chromatography (HILIC). Following mixing, samples are analyzed by ESI LC-MS/MS. The limit of quantitation (LOQ) for MON 102100 was determined to be 0.0050 mg/kg (ppm) and the LOQ for benzamidine was determined to be 0.00125 mg/kg (ppm).

When MON 102100 concentrations exceeded the range of the calibration curve, additional dilution of samples was performed using toluene without internal standard. The accuracy of this approach was demonstrated during the pre-study validation process.

## 3.3 Analytical Procedure

MON 102100 and benzamidine were analyzed using analytical method ME-1636. A copy of the method is shown in Appendix I. A flow diagram of the extraction procedure for both MON 102100 and benzamidine is presented in Figure 1. Residues are calculated

in the method as mg/kg (ppm) MON 102100 and benzamidine *per se* (benzamidine is not calculated in MON 102100 equivalents).

### 3.4 Standards

All analytical reference standards and stable-label internal standards were obtained from the Monsanto Analytical Reference Standards Program. Characterization data for these materials is archived by Monsanto Company, St. Louis, MO.

#### 3.4.1 Analytical Reference Standards

| | |
|---|---|
| Common Name: | Tioxazafen |
| Chemical Name: | 3-Phenyl-5-thiophen-2-yl-1,2,4-oxadiazole |
| CAS Registry Number: | 330459-31-9 |
| Monsanto Code Number: | MON 102100 |
| Molecular Weight: | 228.27 |
| Purity: | ≥98% |

| | |
|---|---|
| Common Name: | Benzamidine |
| Chemical Name: | Benzenecarboximidamide |
| CAS Registry Number: | 618-39-3 |
| Molecular Weight: | 120.15 |
| Purity: | ≥95% |

The chemical structures of MON 102100 and its metabolite, benzamidine, are shown in Figure 2.

#### 3.4.2 Internal Standards

| | |
|---|---|
| Common Name: | (Phenyl-$^{13}C_6$)MON 102100 |
| Chemical Name: | 3-($^{13}C_6$)Phenyl-5-thiophen-2-yl-1,2,4-oxadiazole |
| Molecular Weight: | 234.22 |

| | |
|---|---|
| Common Name: | ($^{13}C_6$)Benzamidine |
| Chemical Name: | ($^{13}C_6$)Benzenecarboximidamide |
| Molecular Weight: | 126.11 |

The structures of the internal standards are provided in Figure 3.

### 3.5 Calculation of Residues

Analyte concentrations are reported in mg/kg (ppm) of matrix. The instrument software calculates the standard curve and applies the necessary Quantitation Factor (i.e. correction for dilution or sample weight). 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 or relative concentration (i.e., ratio of analyte concentration to internal standard concentration). A linear regression model with 1/x weighting is used for quantitation.

The calculation performed by the instrument may be checked manually by applying the linear equation shown below.

$$x\ (\mu g/g\ or\ mg/kg) = \frac{(y - B)(Quant.Factor)}{A}$$

The Quantitation (Quant.) Factor must include adjustment for sample weights (target weight divided by actual sample weight) and any additional sample dilution performed due to responses above the standard curve range.

**Example Calculation**

when:

x = Analyte Amount (mg/kg)
y = Response Ratio = 0.004
Curve constant A: 9.14613
Curve Constant B: 0.0013570
Quant. Factor: 0.95

$$Analyte\ Amount(mg/kg) = \frac{(0.004 - 0.0013570)(0.95)}{9.14613} = 0.0003\ \text{mg/kg}$$

When calculating the standard curve, there is an option within the software to use the relative concentration instead of the concentration. When using this setting the analyte amount (x) is expressed as a ratio of sample concentration to IS concentration. Therefore, the result of the linear equation must be multiplied by the IS concentration in order to obtain the analyte amount in mg/kg.

$$x\ (\mu g/g\ or\ mg/kg) = \frac{(y - B)(Quant.Factor)}{A} \times IS\ Concn(\mu g/g\ or\ mg/kg)$$

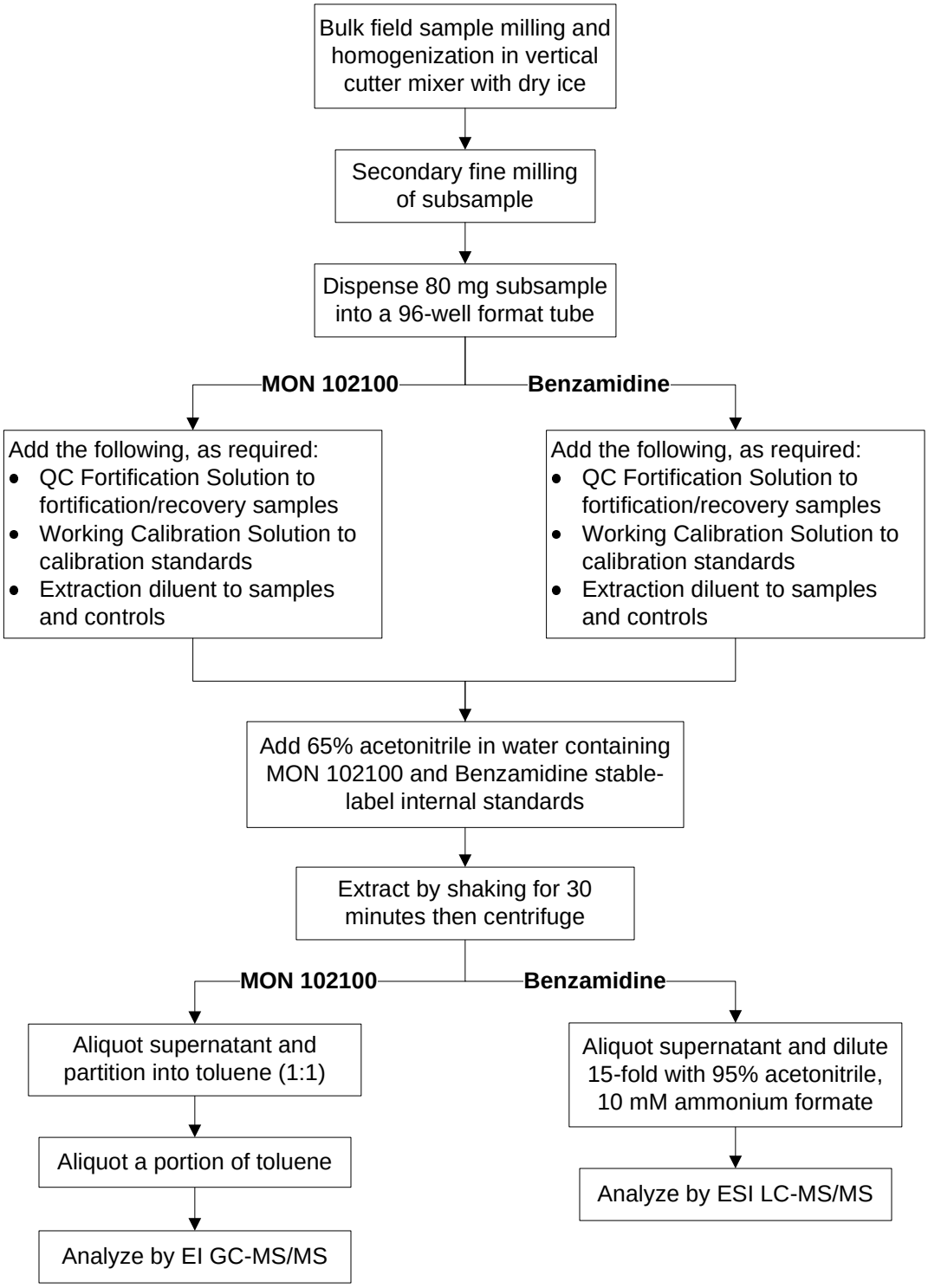
when:

x = Analyte Amount (Relative Concentration)
y = Response Ratio = 0.004
Curve constant A: 0.73169
Curve Constant B: 0.0013570
Quant. Factor: 0.95
IS Concn: 0.08 mg/kg

$$Analyte\ Amount(mg/kg) = \frac{(0.004 - 0.0013570)(0.95)}{0.73169} x\ 0.08\ mg/kg$$
$$= 0.0003\ \text{mg/kg}$$

# 9 FIGURES

**Figure 1: Flow Diagram of Extraction Procedure for MON 102100 and Benzamidine in Soil.**



# 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.

**SOP Revision Summary**

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

- Updated Validation Summary appendix.
- Updated ARS solution stability information.
- Updated acceptance criteria.
- Corrected column headings and units in tables contained within the validation summary document.
- Updated significant figures and formatting.
- Corrected typographical errors.
- Clarifications were added to 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 stable-label internal standards 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 aliquot 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 |
| approx. | approximately |
| ARS | Analytical Reference Standard |
| CE | collision energy |
| cps | cycles per second |

| | |
|---|---|
| CXP | collision exit potential |
| Concn | Concentration |
| DP | declustering potential |
| EP | entrance potential |
| ESI | electrospray ionization |
| EI | electron impact |
| HPLC | high performance liquid chromatography |
| g | gram |
| IS | Internal Standard |
| kg | kilogram |
| LC-MS/MS | liquid chromatography/tandem mass spectrometry |
| GC-MS/MS | gas chromatography/tandem mass spectrometry |
| LOD | limit of detection |
| LOQ | limit of quantitation |
| m | meter |
| mg | milligram |
| min | minute |
| mL | milliliter |
| mm | millimeter |
| MRM | multiple reaction monitoring |
| ms | millisecond |
| MS | mass spectrometry |
| N | number of samples |
| ppm | parts-per-million |
| psi | pounds-per-square inch |
| RSD | relative standard deviation |
| sec | second |
| 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 may 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 number 122-4732) |
| 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 6870 Freezer/Mill® (or comparable) with 100 mL milling vessel |
| High-speed plate shaker | SPEX Geno/Grinder® 2010 or comparable |
| 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. Quadra4™ 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 (for polypropylene extraction tubes) | 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. Specific brands are listed to aid the analyst in finding items. In most cases, equivalent reagents from other vendors may 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 | J.T. Baker. 4218-03 |
| 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 |

(Refer to Appendix **A** for analyte and internal standard compound structures.)

## 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 reagent container label. Solutions may be stored at room temperature in glass containers.

| Solution | Preparation |
|---|---|
| 65% ACN | Mix 1300 mL of ACN and 700 mL of 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 of water and 500 mL of methanol |
| Benzamidine HPLC Mobile Phase B | 90% ACN, 10 mM ammonium formate: Add 100 mL of stock ammonium formate (200 mM) to 100 mL of water and 1800 mL of ACN |
| Benzamidine HPLC Injection Needle Wash | 95% ACN, 10 mM ammonium formate: Add 25 mL of stock ammonium formate (200 mM) to 475 mL of ACN |
| Benzamidine Sample Dilution Solution | 95% ACN, 10 mM ammonium formate: Add 25 mL of stock ammonium formate (200 mM) to 475 mL of ACN |

| | |
|---|---|
| GC Wash Solvent 1 | 50% ethyl acetate in acetone: Add 250 mL of ethyl acetate and 250 mL of acetone |
| MON 102100 Partitioning Solvent and GC Wash Solvent 2 | 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 that 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 (benzamidine) and AG-ME-1604 (MON 102100), as well as separate ARS solution stability studies. The combined results are summarized in the following table.

| Solution Components | Solution Type | Concentration or Range[1] | Solvent | Approx. Storage (°C) | Demonstrated Stability (Days) |
|---|---|---|---|---|---|
| MON 102100 | Stock Solution | 500 µg/mL | ACN | -20 | 97 |
| MON 102100 | Intermediate and Working Calibration Standard Solutions | 0.001 to <500 µg/mL | ACN/Water | -20 | 182 |
| Benzamidine | Standard Solutions | 0.0011 to 526 µg/mL<br>0.002 to 1000 µg/mL (MON 102100 equivalents) | ACN/Water | -20 | 204 |

[1] Stability of benzamidine was established in method AG-ME-1579. In AG-ME-1579, 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 (recorded 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 (recorded 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 or intermediate calibration 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 Intermediate Calibration Solution | 1.00 | 9.0 |
| 0.10 | 1.0 µg/mL MON 102100 Intermediate Calibration Solution | 1.00 | 9.0 |
| 10 | Benzamidine Calibration Stock Solution (0.50 mg/mL) | 0.200 | 9.8 |
| 1.0 | 10 µg/mL Benzamidine Intermediate Calibration Solution | 1.00 | 9.0 |
| 0.10 | 1.0 µg/mL Benzamidine Intermediate Calibration Solution | 0.500 | 9.5 |

**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 Calibration Solution (µg/mL) | Dilute this MON 102100 Intermediate Solution (µg/mL) | MON 102100 Aliquot Volume (mL) | 65% ACN Volume (mL) | Matrix Equivalent MON 102100 Concn (mg/kg, ppm)[1] | 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 |

[1] A *0.040 mL aliquot of working calibration standard solution is 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 Calibration Solution (µg/mL) | Dilute this Benzamidine Intermediate Solution (µg/mL) | Benzamidine Aliquot Volume (mL) | 65% ACN Volume (mL) | Matrix Equivalent Benzamidine Concn (mg/kg, ppm)[1] |
|---|---|---|---|---|
| 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 |

[1] *A 0.040 mL aliquot of working calibration standard solution is 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 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)[1] |
|---|---|---|---|---|
| 10 | 0.50 mg/mL MON 102100 QC Stock Solution | 0.200 | 9.8 | N/A |
| 1.0 | 10 µg/mL MON 102100 QC Solution | 1.00 | 9.0 | N/A |
| 0.010 | 1.0 µg/mL MON 102100 QC Solution | 0.100 | 9.9 | 0.0050 (LOQ QC) |
| 0.10 | 10 µg/mL MON 102100 QC Solution | 0.100 | 9.9 | 0.050 (10x LOQ QC) |
| 0.20 | 10 µg/mL MON 102100 QC Solution | 0.200 | 9.8 | 0.10 (Mid QC) |
| 1.8 | 10 µg/mL MON 102100 QC Solution | 1.80 | 8.2 | 0.90 (High QC) |
| 8.0 | 10 µg/mL MON 102100 QC Solution | 8.0 | 2.0 | 4.0 (Dilution QC) |

[1] *Concentration represents 0.040 mL of QC Fortification Solution added to a targeted 0.080 g of control sample weight.*

**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)[1] |
|---|---|---|---|---|
| 10 | 0.50 mg/mL Benzamidine QC Stock | 0.200 | 9.8 | N/A |
| 1.0 | 10 µg/mL Benzamidine QC Solution | 1.00 | 9.0 | N/A |
| 0.10 | 1.0 µg/mL Benzamidine QC Solution | 1.00 | 9.0 | N/A |
| 0.0025 | 0.10 µg/mL Benzamidine QC Solution | 0.500 | 19.5 | 0.00125 (LOQ QC) |
| 0.025 | 1.0 µg/mL Benzamidine QC Solution | 0.250 | 9.75 | 0.0125 (10x LOQ QC) |
| 0.050 | 1.0 µg/mL Benzamidine QC Solution | 0.500 | 9.5 | 0.025 (Mid QC) |
| 0.60 | 10 µg/mL Benzamidine QC Solution | 0.600 | 9.4 | 0.30 (High QC) |

[1] *Concentration represents0.040 mL of QC Fortification Solution added to a targeted 0.080 g of control sample weight.*

**(Phenyl-$^{13}C_6$) MON 102100 IS Stock Solution (0.50 mg/mL)**

Weigh 20-25 mg (recorded 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 0.5 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 (recorded 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 or intermediate 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 Intermediate IS Solution (10 µg/mL) | 1.00 | 9.0 |

**Mixed IS Working Solution**

Prepare the Mixed IS Working Solution (0.050 µg/mL (phenyl-$^{13}C_6$)MON 102100 and 0.0020 µg/mL ($^{13}C_6$)benzamidine) the day to be used for sample preparation by diluting intermediate IS solutions with 65% ACN. An example dilution scheme is shown below.

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

**Sample Dilution Solution**

Dilution of samples for which the MON 102100 response is beyond the range of calibration standards is achieved by adding toluene without IS for MON 102100 analysis. Dilution using this approach has been tested and validated up to a 10-fold dilution. Additional dilution beyond 10 fold should be confirmed using dilution QCs.

Capability of dilution for benzamidine in soil matrix was not established during method validation.

# 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 Cycle 1 | 2 minutes | 2 minutes |
| Cool Time | 1 minute | 1 minute |
| Run Time Cycle 2 | 2 minutes | 2 minutes |
| Rate | ≥6 cps | ≥6 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. This procedure is written for processing samples for analysis of both MON 102100 and benzamidine. However, the solutions and procedures can be modified for single analytes, as required. These modifications should be documented in the raw data.

| **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®).* |

<table>
<tr><td>2A</td><td>Add 40.0 µL of the following solution to the designated sample type:<ul><li>65% ACN to test samples and controls</li><li>MON 102100 Working Calibration Standard Solutions to calibration standards</li><li>MON 102100 QC Fortification Solutions to QC samples (e.g. LOQ QC, 10x LOQ QC, Mid QC, High QC and/or Dilution QC)</li></ul>
<table>
<tr><th>QC Sample</th><th>Fortification Level (mg/kg, ppm)</th></tr>
<tr><td>LOQ QC</td><td>0.0050</td></tr>
<tr><td>10x LOQ QC</td><td>0.050</td></tr>
<tr><td>Mid QC</td><td>0.10</td></tr>
<tr><td>High QC</td><td>0.90</td></tr>
<tr><td>Dilution QC</td><td>Variable, depending on dilution level needed. Up to a 10x dilution.</td></tr>
</table></td></tr>
<tr><td>2B</td><td>Add 40.0 µL of the following solution to the designated sample type:<ul><li>65% ACN to test samples and controls</li><li>Benzamidine Working Calibration Standard Solutions to calibration standards</li><li>Benzamidine QC Fortification Solutions to QC samples (e.g. LOQ QC, 10x LOQ QC, Mid QC, High QC)</li></ul>
<table>
<tr><th>QC Sample</th><th>Fortification Level (mg/kg, ppm)</th></tr>
<tr><td>LOQ QC</td><td>0.00125</td></tr>
<tr><td>10x LOQ QC</td><td>0.0125</td></tr>
<tr><td>Mid QC</td><td>0.025</td></tr>
<tr><td>High QC</td><td>0.30</td></tr>
</table></td></tr>
<tr><td>2C</td><td>Add 1 grinding ball to each tube.</td></tr>
<tr><td>3</td><td>Add 0.720 mL of the Mixed IS Working 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.</td></tr>
<tr><td>4</td><td>Cover the 96-well plate with a shaker cap mat. Ensure the cap mat is sealed well before proceeding.</td></tr>
<tr><td>5</td><td>Place the plate in a sonicator and sonicate for approximately 3 minutes.</td></tr>
<tr><td>6</td><td>Shake samples on the Geno/Grinder® to extract analytes 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.</td></tr>
<tr><td>7</td><td>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).</td></tr>
<tr><td>8</td><td>Remove cap mat and transfer appropriate volume of supernatant to clean 96-well microplate(s) as needed for further analysis (see table below). This transfer can be performed using an automated liquid handler, multichannel pipette or other pipetting device.
<table>
<tr><th>Analyte</th><th>Daughter Plate</th><th>Transfer Volume (mL)</th></tr>
<tr><td>MON 102100</td><td>96-well microplate with clear glass conical inserts</td><td>0.400[a]</td></tr>
<tr><td>Benzamidine</td><td>Agilent 96-well plate, 1 mL</td><td>0.050</td></tr>
</table>
[a] If 0.400 mL can not be recovered, a minimum volume of 0.200 mL can be taken, as long as an equal volume of toluene is used in step 9A.</td></tr>
</table>

**For MON 102100 Analysis**

| Step | Action |
|---|---|
| 9A | *Add 0.400 mL of toluene. |
| 10A | Shake samples on the Geno/Grinder® 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 0.100 mL 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 an automated liquid handler, multichannel pipette or other pipetting device. |
| 13A | Analyze by EI GC-MS/MS within the storage time determined during method validation (see Section 8 of Appendix C). |

* Prior to Step 9A samples may be gently evaporated until approximately 0.150 – 0.200 mL 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 0.700 mL of Benzamidine Sample Dilution Solution (95% ACN, 10 mM ammonium formate) to the 0.050 mL 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 the storage time determined during method validation (see Section 8 of Appendix C). |

**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 (e.g., 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 nominal value at each QC level.
- The acceptance criterion for precision is ≤20% RSD at each fortification level.

- A maximum of one 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 the 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.
- 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.

**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<br>Autosampler: Gerstel MPS<br>Mass spectrometer: Agilent 7000 Triple Quadrupole<br>Ion source: electron impact<br>Column: DB-17 MS 30m × 0.25mm, 0.25 µm<br>Injection volume: 0.50 µL<br>Autosampler temperature: 10 °C<br>GC carrier gas: helium |
| Autosampler Conditions |
| Injection<br>Syringe: 5 µL<br>Injection volume: 0.5 µL<br>Air volume (below): 1.0 µL<br>Injection speed: 50.0 µL/sec<br>Fill volume: 4 µL<br>Fill strokes: 2<br><br>Fill speed: 0.30 µL/sec<br>Viscosity delay: 3 sec<br>Eject speed: 25.0 µL/sec<br><br>Cleaning<br>Fill speed: 5.0 µL/sec<br>Viscosity delay: 3 sec<br>Eject speed: 50.0 µL/sec<br><br>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
Injection mode: 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<br>Curtain gas (CUR): 35<br>Collision gas (CAD): 6<br>Gas 1: 45 $N_2$<br>Gas 2: 45 $N_2$ | IonSpray voltage (IS): 5000 V<br>Entrance potential (EP): 10<br>Interface heater: on<br>Temperature (TEM): 500 °C<br>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 or relative concentration (i.e., ratio of analyte concentration to internal standard concentration). A linear regression model is used for quantitation with or without weighting (e.g., 1/x weighted). All samples from a study must be analyzed with the same type of calibration curve (i.e., plot axes and weighting) for a given analyte.

**Analyte Concentration**

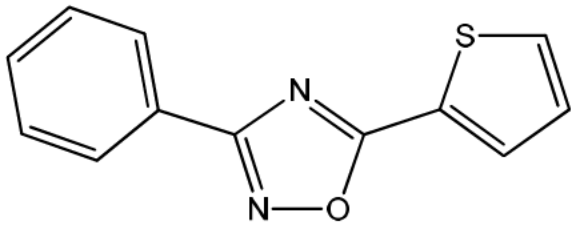
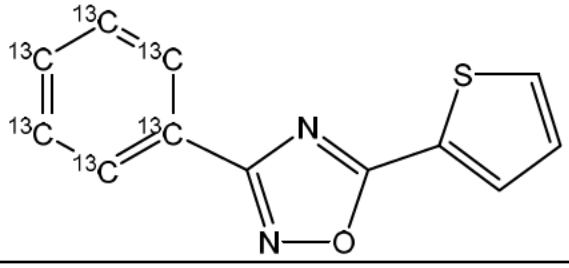
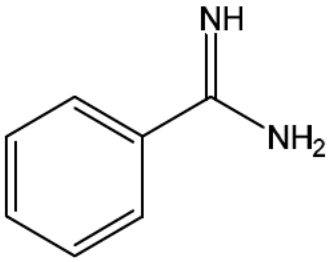
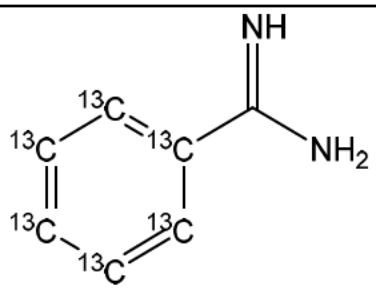
Analyte concentrations are reported in mg/kg (ppm) of matrix. The MassHunter and Analyst® systems automatically calculate the raw concentration of the injected sample relative to the standard curve (*calculated concentration*). This value is also automatically multiplied by any value entered in the *dilution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

# Appendices

## Appendix A: Chemical Structures

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