I. SUMMARY

ISK Biosciences Corporation contracted Golden Pacific Laboratories, LLC (GPL) in Fresno, California, to conduct an Independent Laboratory Validation. The objective of this study was to validate the analytical method (provided by ISK) entitled "Determination of MMTA in Soil" contained in Appendix 2 of the Huntingdon Life Sciences Final Report "MMTA: Validation of Methodology for the Determination of Residues in Two Soil Types" (Document Number JSM0757). The method was successfully validated using Liquid Chromatography (LC) equipped with a tandem mass spectrometer (MS/MS) detector. The analysis was validated for the determination of MMTA in soil in the first method trial. The analytical method was validated to demonstrate method ruggedness and to meet US EPA Ecological Effects Test Guidelines, OCSPP 850.6100 Test Guidelines requirements for environmental chemistry methods and associated independent laboratory validation. The study was conducted under EPA's Good Laboratory Practice Standards (GLPs) 40 CFR Part 160.

Independent Laboratory Validation

One control sample was used in this study. The soil sample was obtained from a previous non-GLP ISK Biosciences study conducted at GPL. There was no response in the control matrix samples in the chromatograms corresponding to the retention of MMTA.

A control (untreated) soil sample was analyzed using the provided analytical method. Soil samples were extracted twice with methanol/water (80:20, v/v) containing 0.1 M ammonium formate, 0.05 M citric acid, and 0.5% v/v hydrochloric acid. The combined extract was taken through an Oasis HLB solid-phase extraction (SPE) clean-up. The organic solvent was evaporated from the eluent; the eluent was brought up to volume, and was submitted for analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

II. MATERIALS

A. Equipment

The equipment that was used is listed below:

- Analytical balance: Mettler Toledo XS204
- Top Loading balance: Mettler Toledo MS3002S/03
- Volumetric flasks, glass: various sizes
- Bottles, amber glass with Teflon lined cap: various sizes
- Volumetric glass pipettes: various sizes
- Polypropylene bottle: 250 mL
- Polypropylene tubes: BD Falcon 15 mL and VWR 50 mL
- Graduated cylinders: various volumes
- Micropipette, Drummond Wiretrol disposable: various volumes
- Disposable pasteur pipettes, glass
- Repeating pipette: Eppendorf Stream
- HPLC vials and caps: 1.8 mL
- SPE cartridges: Oasis HLB cartridges (60 mg, 3 mL)
- SPE manifold: Burdick & Jackson (24 position)
- Evaporator System: TurboVap, Caliper Life Sciences TurboVap® LV
- LC-MS/MS: AB Sciex API5000 LC-MS/MS with Shimadzu LC-20AD XR HPLC Pumps, Shimadzu CBM-20A controller, and SIL-20AC XR autosampler
- Platform shaker: Eberbach 2 speed shaker
- Centrifuge: Eppendorf Multipurpose Centrifuge 5810
- Graduated mixing cylinders: 250 mL

B. Reagents and Standards

The following chemicals were used:

Chemical	Distributer	Part No:
Acetonitrile	Fisher	A996-4
Acetic Acid	Fisher	A38S-500
Ammonium Acetate	Fisher	A639-500
Ammonium Formate, 99%	VWR	AA14517-30
Citric Acid (Anhydrous)	VWR	EM-CX1723-1
Hydrochloric Acid	VWR	EMD-HX0603-3
Methanol	VWR	MK304110
Water	Fisher	W5-4

Preparation of Reagent Solutions:

0.2% acetic acid in water (v/v): Prepared by adding 4 mL of concentrated acetic acid to approximately 800 mL of HPLC-grade water in a 1000-mL volumetric flask. The solution was brought up to volume (2000 mL) with HPLC-grade

water and mixed well.

Acetonitrile/0.2% acetic acid in water (10:90, v/v): Prepared by combining 100 mL of acetonitrile with 900 mL of 0.2% acetic acid in water, and mixing well.

Acetonitrile/0.2% acetic acid in water (50:50, v/v): Prepared by combining 250 mL of acetonitrile with 250 mL of 0.2% acetic acid in water, and mixing well.

Extraction Solvent: *methanol/water (80:20, v/v) containing 0.1 M ammonium formate, 0.05 M citric acid, and 0.5% v/v hydrochloric acid:* Prepared by combining 3200 mL of methanol and 800 mL of HPLC-grade water. To the methanol/water solution, the following was added: 25.2 g of ammonium formate, 42.0 g of citric acid, and 20 mL of concentrated hydrochloric acid. The resulting solution was mixed well.

Mobile Phase B: 0.01 M Ammonium Acetate (aq): Prepared by dissolving 0.77 g of ammonium acetate into approximately 200 mL of HPLC-grade water in a 1000-mL volumetric flask. The solution was brought up to volume (1000 mL) with HPLC-grade water and mixed well.

Needle-Wash, acetonitrile/water (50:50, v/v): Prepared by combining 500 mL of acetonitrile with 500 mL of HPLC-grade water and mixing well.

1. Reference Substances

The MMTA analytical reference standard were received in good condition on November 14, 2014, from MRI Global, Kansas City, MO. The certificate of analysis for the standard is in the archives at GPL. The following table contains detailed information for the analytical standard used in this study.

Analytical Standard	Chemical Name	CAS #	Lot #	Purity (%)	Expiration Date
MMTA	3-(2-ethoxyethoxy)-2 methyl-4-(methylsulfonyl) benzoic acid	NA	20140226	100	07/21/2017

Upon receipt, the neat reference standards were stored in a freezer set to maintain ≤ -10 °C (frozen). The Sponsor was responsible for the archival of the reference substance retention sample.

2. Preparation of Standard Solutions

The MMTA reference substance was used in the preparation of the fortification and calibration solutions. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

a. Stock Solution

On January 13, 2015, 10.9 mg of MMTA reference standard was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. The stock solution contained 1.09 mg/mL MMTA (Solution A). The stock solution was stored in a freezer set to maintain ≤ - 10°C (frozen) when not in use.

b. Fortification Solutions

A 0.1-mL aliquot of Solution A was diluted to 54.5 mL with acetonitrile, resulting in a solution that contained 2.00 µg/mL MMTA (Solution B). Further, a 5 mL aliquot of Solution B was diluted to 50 mL with acetonitrile, resulting in a solution that contained 200 ng/mL MMTA (Solution C). Aliquots of Solution C were used to fortify soil at the LOQ level. Aliquots of Solution B were used to fortify soil at the 10x LOQ level. The fortification solutions were stored frozen when not in use.

c. Intermediate and Calibration Solutions

A 5-mL aliquot of Solution B was diluted to 100 mL with acetonitrile/0.2% acetic acid in water (10:90, v/v), resulting in a solution containing 100 ng/mL MMTA (Solution D). The calibration standards were prepared by making the following dilutions in acetonitrile/0.2% acetic acid in water (10:90, v/v):

Initial Soln. ID	Initial Soln. Concentration (ng/mL)	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (ng/mL) MMTA	Final Soln. ID
D	100	1	10	10.0	E
D	100	0.5	10	5.00	F
D	100	0.5	20	2.50	G
E	10.0	1	10	1.00	H
E	10.0	0.5	10	0.500	I
E	10.0	0.5	20	0.250	J
H	1.00	1	10	0.100	K
H	1.00	0.5	10	0.0500	L
H	1.00	0.5	20	0.0250	M

The intermediate solution and calibration standards were given an expiration of one calendar day and were stored frozen when not in use.

C. Safety and Health

Material Safety Data Sheets (MSDS) and/or Safety Data Sheets (SDS) were available. Proper personal protective equipment was worn during the execution of this method. Staff avoided breathing chemical vapor and avoided chemical contact with eyes and skin. Caution was used when handling concentrated acetic acid. There were no other procedural steps that required special precautions to avoid safety or health hazards.

III. METHODS

A. Principle of Analytical Method

The analysis of soil for MMTA was performed according to the reference method titled "Determination of MMTA in Soil" (contained in Huntingdon Life Sciences Document Number: JSM0757). The limit of quantitation (LOQ) for MMTA was 1 ppb (ng/g). The limit of detection (LOD) was 0.25 ppb.

The method validation was performed on January 15, 2015. All of the samples were extracted in one analytical set. The set consisted of one reagent blank sample, two control samples, five LOQ laboratory fortification samples and five 10x LOQ laboratory fortification samples. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number; the sample set designation and a sample number (e.g., 581ILV01-5).

Sub-samples (20 g) of control soil sample were fortified. Soil samples were extracted twice with methanol/water (80:20, v/v) containing 0.1 M ammonium formate, 0.05 M citric acid, and 0.5% v/v hydrochloric acid. The combined extract was taken through an Oasis HLB solid-phase extraction (SPE) clean-up. The organic solvent was evaporated from the eluent; the eluent was brought up to volume, and was submitted for analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

B. Analytical Procedure

1. Control Matrix

The soil control matrix sample was obtained from North Rose, NY from a previous non-GLP ISK Biosciences study conducted at GPL. The non-GLP soil characterization of the soil was provided in an email from the Sponsor and listed below:

Parameter	0-3 Inch	3-6 Inch	6-12 Inch	12-18 Inch	18-24 Inch
Texture	Loam	Silt Loam	Silt Loam	Silt Loam	Silt Loam
% Sand	38	36	22	22	22
% Silt	47	51	55	57	59
% Clay	15	13	23	21	19
pH	7.4	7.5	5.3	5.9	6.2
Organic Matter, %	5.7	5.5	0.7	0.3	0.2
CEC (meq/100g)	19.6	22.2	12.1	14.3	13.5
Bulk Density (gm/cc)	1.01	0.99	1.17	1.22	1.23
% Moisture at 1/3 Bar	30.3	29.3	24.2	23.4	23.2
% Moisture at 15 Bar	11.7	11.8	9.3	9.5	8.2

The sample was kept frozen when not in use.

2. Preparation of Samples

Sub-samples (20.0 ± 0.05 g) of the control soil matrix were measured into 250-mL polypropylene bottles.

3. Fortifications

Independent laboratory validation samples were fortified at the LOQ (1 ppb) or 10x the LOQ (10 ppb). Fortifications were performed using Wiretrol disposable micropipettes to directly fortify the 20-g samples as follows:

Fortification Level	Amount and Concentration of Spiking Solution Used
LOQ (1 ppb)	100 µL of a 200 ng/mL MMTA solution
10x LOQ (10 ppb)	100 µL of a 2.00 µg/mL MMTA solution

4. Extraction

After fortification, 100 mL of extraction solvent (methanol/water (80:20, v/v) containing 0.1 M ammonium formate, 0.05 M citric acid, and 0.5% v/v hydrochloric acid) was added to each sample. Each sample bottle was capped and shaken on a platform shaker for 30 minutes at approximately 200 rpm. After shaking, the samples were centrifuged at a setting of 3500 rpm for approximately 3 minutes. The supernatant was decanted into a 250-mL graduated mixing cylinder.

An additional 80 mL of extraction solvent was added to each sample. Each sample bottle was capped and shaken on a platform shaker for 30 minutes at approximately 200 rpm. After shaking, the samples were centrifuged at a setting of 3500 rpm for approximately 3 minutes. The supernatant was decanted into the 250-mL graduated mixing cylinder, combining the extracts. Each sample was brought up to a volume of 200 mL with extraction solvent.

5. Clean-Up

SPE (Oasis HLB) cartridges were conditioned with 3 mL of methanol followed by equilibration using 3 mL of 0.2% acetic acid in water. The eluate was discarded. An aliquot of the extract (10 mL) was transferred into a 50-mL polypropylene tube. An aliquot (20 mL) of 0.2% acetic acid in water was added to the polypropylene tube. The diluted extract was mixed well and loaded onto the SPE cartridge. The eluate was discarded. Once the sample was loaded onto the cartridge, the cartridge was eluted into a 15 mL graduated polypropylene tube using an 8-mL aliquot of acetonitrile/0.2% acetic acid in water (50:50, v/v).

The eluent volume was reduced to less than 4 mL under a steady stream of nitrogen in a TurboVap set to 40 °C. An aliquot (1-mL) of acetonitrile was added to the sample extract. The extract was diluted to a final volume of 10 mL using 0.2% acetic acid in water. The extracts were vialed for analysis by LC-MS/MS.

C. Instrumentation

Instrument:	AB Sciex API5000 LC/MS/MS with Shimadzu LC-20AD XR HPLC Pumps, Shimadzu CBM-20A Controller, Shimadzu SIL-20AC XR Autosampler
HPLC Column:	Waters Acquity UPLC BEH C18 50 x 2.1 mm, 1.7 µm Part # 186002350 Serial # 01763007415522
Guard Column:	NA
Data System:	Analyst Chromatography Data System version 1.5.2, AB Sciex
Mobile Phases:	A) 100% Methanol B) 0.01 M Ammonium Acetate (aq.)

Flow Rate: 0.5 mL/minute
Run Time: 4.0 minutes
Injection Volume: 10 µL
Gradient Program:

Time (minutes)	%A	%B
0.0	10	90
0.2	10	90
2.0	95	5
2.5	95	5
3.0	10	90
4.0	10	90

Column Heater: 45 °C

Approximate Retention Time:

MMTA: 1.2 minutes

Mass Spectrometer Parameters (operated in LC-MS/MS mode):

AB Sciex API 5000 Acquisition Parameters (TurboIonSource, ESI interface, MRM mode, negative mode, Unit/Unit Resolution)				
Analyte	**Q1 (m/z)**	**Q3 (m/z)**	**Dwell (msec)**	**CE**
MMTA (Prim.)	287.0	184.0	100	-33
MMTA (Conf.)	287.0	243.0	100	-20

Parameter	Setting
DP:	-60
CUR:	35
GS1:	40
GS2:	40
IS:	-3000
TEM:	500
CAD:	8
EP:	-10
CXP	-10

D. Potential Interferences

1. Matrix Interference

No matrix interferences were observed for MMTA.

2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. No interferences were observed.

3. Labware Interference

This method uses disposable labware and washable glassware. No interferences from the labware or glassware use were observed.

E. Confirmatory Techniques

The independent laboratory validation set was run by LC-MS/MS with monitoring of two ion transition pairs for MMTA. As this method is highly selective, no additional confirmatory technique was used.

F. Time Required for Analysis

Approximately 4 to 6 hours were required for one person to prepare an analysis set from the time samples were prepared to LC-MS/MS analysis. Automated LC-MS/MS analysis was performed overnight. An additional 0.5 hours was spent on data calculation and tabulation the following day. An analytical set of 13 samples can be prepared, analyzed and tabulated during one calendar day.

G. Modification or Potential Problems

Two minor modifications were made to the method: 1) An AB Sciex API5000 LC/MS/MS was used instead of an AB Sciex API4000, and 2) 250-mL graduated glass mixing cylinders were used in place of the 250-mL polypropylene bottles to collect the decanted extract. The AB Sciex API5000 instrument was used due to the pressure limitations of the pumps connected to the AB Sciex API4000. No potential problems with the method were observed.

H. Methods of Calculation

Analyst Chromatography Data System version 1.5.2, a product of AB Sciex, was used to acquire, integrate and calculate the concentrations of MMTA as ng/mL using the linear regression function with 1/x weighting. The calibration curve was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak area

response was designated as the dependent variable and plotted on the y-axis. From this regression curve, a slope, a correlation coefficient and other parameters of the standard curve were calculated. Calibration standards were injected every three to five sample injections as well as at the beginning and end of the injection sequence. Nine different standard concentrations were injected within the analytical set. The concentrations (ng/mL) of MMTA detected in method validation sample extracts were interpolated from the standard calibration curve. The concentration as ppb of residue found in samples was then calculated with Microsoft® Excel using the following equation:

$$ppb = \frac{(ng/mL\ from\ curve) \times (Aliquot\ Factor) \times (Final\ Volume\ (mL))}{Sample\ Amount\ (g)}$$

Where the aliquot factor is:

Aliquot Factor = Extraction Volume (mL) ÷ Aliquot Volume (mL)

Recovery of each of the analytes from fortified samples was calculated as follows:

$$\%\ Recovery = \frac{Measured\ Concentration\ (ppm)}{Theoretical\ Concentration\ (ppm)} \times 100$$

An example calculation for soil, for a MMTA (primary ion) laboratory fortification sample in set 581ILV01, sample 581ILV01-7 LOQ sample fortified at 1.00 ppb, is as follows:

standard curve equation: $y = 9.33\,(x) \times 10^4 + (-41.3)$

where x = MMTA concentration in ng/mL and

y = peak response = 8555.4

MMTA concentration from the curve =0.0921 ng/mL

Aliquot Factor = 200 mL ÷ 10 mL = 20

$$ppb = \frac{(0.0921\ ng/mL) \times (20) \times (10\ mL)}{(19.99\ g)} = 0.921\ ppb$$

$$\%\ Recovery = \frac{0.921\ ppb}{1.00\ ppb} \times 100 = 92.1\%$$

No detectable residues were measured in any control samples. Laboratory fortification samples were not corrected for control responses (no responses were observed). Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.