I. SUMMARY

Ishihara Sangyo Kaisha (ISK) Biosciences Corporation contracted Golden Pacific Laboratories, LLC (GPL) in Fresno, California, to develop an analytical method and conduct a method validation study for SL-160 and metabolites DTPU, DTPP, and TPSA in water. The objective of this study was to develop and validate an analytical method to meet the US EPA Ecological Effects Test Guidelines, OCSPP 850.6100 Test Guidelines for Environmental Chemistry Methods (ECM). This study has yielded the validated method entitled "Analytical Method for the Determination of SL-160 and its Metabolites DTPU, DTPP, and TPSA in Water by LC-MS/MS" which is contained in Appendix B of this report. 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 SL-160, DTPU, DTPP, and TPSA in surface water. The study was conducted under EPA's Good Laboratory Practice Standards (GLPs) 40 CFR Part 160.

Method Validation

One control sample was used for the surface water matrix in this study. The surface water sample was obtained from the Fresno Irrigation District Herndon Canal No. 39 at a point near the Gates Avenue Bridge. There was no response in the control matrix sample in the chromatograms corresponding to the retention of SL-160 and TPSA. There was an insignificant (< 10% Limit of Detection (LOD) by peak area response) amount of response in the chromatograms of the reagent blank and two of the three control matrix samples corresponding to the retention time of DTPU (primary MS/MS ion). Additionally, there was also an insignificant (< 10% LOD by peak area response) amount of response in the chromatograms of one of the control matrix sample corresponding to the retention time of DTPP (confirmatory MS/MS ion).

Control (untreated) samples of the surface water were analyzed using the provided analytical method. Samples were combined with acetonitrile in a 9:1 ratio and filtered. Quantitation was performed using a liquid chromatography mass spectrometry/mass spectrometry system (LC-MS/MS) monitoring two transition ion pairs for each of the four analytes. Due to limited sensitivity for the TPSA confirmatory ion pair, the confirmatory pair can only be used for peak identity confirmation under 10x the Limit of Quantitation (LOQ).

The method was validated at 0.05, 0.5, and 5 µg/L for the detection of SL-160, DTPU, and DTPP in surface water. The method was also validated at 0.2, 2, and 20 µg/L for TPSA in surface water. The accuracy and precision data for each analyte are listed in the table below. (The TPSA confirmatory data for the LOQ level is omitted due to limited sensitivity at that level).

II. MATERIALS

A. Equipment

The equipment that was used is listed below:

- Balance, Analytical, Mettler Toledo XS204
- Volumetric flasks, glass: 10-mL
- Bottles, amber glass with Teflon lined cap: 30, 60, 120, and 240-mL
- Glass vials, clear with Teflon lined caps: various volumes
- Volumetric glass pipette: various sizes
- Graduated Cylinders: various volumes
- Micropipette, Drummond Wiretrol disposable micropipettes: various volumes
- Disposable Pasteur pipettes, glass
- Repeating Pipette, Eppendorf Stream
- HPLC vials, clear glass: 1.8-mL
- AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC Autosampler

B. Reagents and Standards

The following chemicals were used:

Chemical	Grade	Manufacturer	Distributer	Part Number
Acetonitrile	Optima	Fisher	Fisher	A996-4
Formic Acid (88%)	ACS	Fisher	Fisher	A118P-500
Water	HPLC	Avantor	VWR	MK679510
Water	HPLC	Fisher	Fisher	W5-4

Preparation of Reagent Solutions:

Acetonitrile: HPLC grade water (10:90, v/v): Prepared by adding 100 mL of acetonitrile to 900 mL of HPLC grade water. Mix well.

Mobile Phase A:
0.2% Formic Acid in Acetonitrile: Prepared by adding 2 mL of formic acid to approximately 900 mL of acetonitrile. Solution is brought up to 1000 mL with acetonitrile. Mix well.

Mobile Phase B:
0.2% Formic Acid in HPLC grade water: Prepared by adding 2 mL of formic acid to approximately 900 mL of HPLC grade water. Solution is brought up to 1000 mL with HPLC grade water. Mix well.

1. Reference Substances

The SL-160 and DTPU analytical reference standards were received in good condition on May 14, 2002 and September 24, 2008, respectively. The DTPP and TPSA analytical reference standards were received in good condition on February 13, 2014. All four reference substances were received from Midwest Research Institute, Kansas City, MO. The certificate of analysis for each standard is archived at GPL. The following table contains detailed information for the analytical standards used in this study.

Analytical Standard	CAS #	Lot #	Purity (%)	Expiration Date
SL-160	104040-78-0	Y-920205	100.0	05/03/2018
DTPU	Not Available	0205	98.5	06/28/2017
DTPP	Not Available	0205	98.9	06/29/2017
TPSA	104040-76-8	0205	100.0	06/22/2017

Upon receipt, the neat standards were stored in a freezer set to maintain ≤ -10 °C (prior to June 18, 2003 the SL-160 reference standard was stored in a freezer set to maintain ≤ 0 °C).

2. Preparation of Standard Solutions

The reference substances were used in the preparation of the fortification and calibration solutions.

a. Stock Solutions

For SL-160, 13.6 mg of the reference standard was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. The stock solution contained 1.36 mg/mL SL-160 (Solution A). For DTPU, 22.1 mg of the reference standard was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 2.18 mg/mL DTPU (Solution B). For DTPP, 11.6 mg of the reference standard was weighed directly into a 10-mL volumetric flask and diluted to the mark with acetonitrile. After correcting for purity, the stock solution contained 1.15 mg/mL DTPP (Solution C). For TPSA, 11.1 mg of the reference standard was weighed directly into a 10 mL volumetric flask and diluted to the mark with acetonitrile. The stock solution contained

1.11 mg/mL TPSA (Solution D).

b. Intermediate Solutions

Each of the stock solutions was diluted in acetonitrile individually to prepare intermediate solutions (see table below).

Analyte	Initial Solution ID	Volume of Solution (mL)	Final Volume (mL)	Final Solution ID	Standard Concentration (µg/mL)
SL-160	A	0.5	68	E	10
DTPU	B	2	218	F	20
DTPP	C	1	57.5	G	20
TPSA	D	0.9	2.5	H	400

The intermediate solutions were assigned an expiration of three months and were stored in a freezer set to maintain ≤ -10 °C (frozen) when not in use.

c. Fortification Solutions

An aliquot of Solution E (10 mL), F (5 mL), G (5 mL), and H (1 mL) were diluted in acetonitrile to a final volume of 200 mL to prepare the 100x LOQ fortification solution containing 500/500/500/2000 ng/mL of SL-160, DTPU, DTPP, and TPSA (Solution I). Solution I was further diluted 10-fold with acetonitrile to prepare the 10x LOQ fortification solution (Solution J). Solution J was diluted 10-fold with acetonitrile to prepare the LOQ fortification solution (Solution K). The fortification solution preparation is summarized below.

Initial Solution ID	Analyte(s)	Initial Concentration (µg/mL)	Volume Added (mL)	Final Volume (mL)	Final Solution ID	Final Concentration (ng/mL)
E	SL-160	10	10	200	I	500/500/500/2000[1]
F	DTPU	20	5			
G	DTPP	20	5			
H	TPSA	400	1			
Initial Solution ID	**Analyte(s)**	**Initial Concentration (ng/mL)**	**Volume Added (mL)**	**Final Volume (mL)**	**Final Solution ID**	**Final Concentration (ng/mL)**
I	All	500/500/500/2000[1]	10	100	J	50/50/50/200[1]
J	All	50/50/50/200[1]	10	100	K	5/5/5/20[1]

[1] Solution concentrations are listed in the following order: SL-160/DTPU/DTPP/TPSA

The fortification solutions were given an expiration of three months and were stored frozen when not in use.

d. Calibration Standards

A 10-mL aliquot of solution J was diluted to a final volume of 100 mL in acetonitrile: HPLC grade water (10:90, v/v) to prepare an intermediate solution (Solution L). Solution L was used to prepare the calibration standards.

All calibration standards were diluted into acetonitrile: HPLC grade water (10:90, v/v). The calibration standards and intermediate solution were assigned an expiration of three months and stored in a refrigerator set to maintain 4 ± 5 °C (refrigerated). The calibration standard preparation is summarized below.

Initial Concentration Solution L (ng/mL)[1]	Volume Added (mL)	Final Volume (mL)	Final Solution ID	Standard Concentration (ng/mL)[1]
5/5/5/20	10	50	M	1/1/1/4
5/5/5/20	4	50	N	0.4/0.4/0.4/1.6
5/5/5/20	2	50	O	0.2/0.2/0.2/0.8
5/5/5/20	1	50	P	0.1/0.1/0.1/0.4
5/5/5/20	0.8	100	Q	0.04/0.04/0.04/ 0.16
5/5/5/20	0.4	100	R	0.02/0.02/0.02/ 0.08

[1] Solution concentrations are listed in the following order: SL-160/DTPU/DTPP/TPSA

C. Safety and Health

Material Safety Data Sheets (MSDS) should be consulted anytime an analyst is to start work with an unfamiliar chemical. Proper personal protective equipment must be used during the execution of this method. Avoid breathing chemical vapor and avoid chemical contact with eyes and skin. MSDS for the chemicals used in this analysis are located in Appendix D. There are no procedural steps that require special precautions to avoid safety or health hazards.

III. METHODS

A. Principal of Analytical Method

The method validation analysis of surface water was performed according to the reference method number GPL-MTH-082 entitled "Analytical Method for the Determination of SL-160 and its Metabolites DTPU, DTPP, and TPSA in Water by LC-MS/MS" (contained in Appendix B of this report). The limit of quantitation (LOQ) and limit of detection (LOD) were defined as listed in the table below.

Analyte	LOQ (µg/L)	LOD (µg/L)
SL-160	0.05	0.01
DTPU	0.05	0.01
DTPP	0.05	0.01
TPSA	0.2	0.088

The method validation surface water was performed on March 26, 2014. All samples were extracted in one analytical set. The set consisted of one reagent blank sample (HPLC grade water), three control samples, seven LOQ laboratory fortification samples, seven 10x LOQ laboratory fortification samples, and seven 100x 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., 546MV01-1).

Aliquots (10 mL) of control matrix water were fortified. An aliquot of each sample was combined in a ratio of 9:1 with acetonitrile. After the acetonitrile was added, the sample was shaken by hand and filtered. Samples were vialed and analyzed by LC-MS/MS.

B. Analytical Procedure

1. Surface Water Control Matrix

The surface water control matrix was obtained from the Fresno Irrigation District Canal "Herndon No. 39" at a point near the Gates Avenue Bridge on 02/25/2014. Sub-portions of this sample were taken and labeled as "Herndon39-140225" and were then transported by GPL personnel to BSK Laboratories in Fresno, California for non-GLP characterization. The non-GLP characterization results of the sample are presented below:

Parameter	Result
pH	7.3
Dissolved Oxygen	9.7 mg/L
Conductivity	42 µmhos/cm
Alkalinity	17 mg/L as $CaCO_3$
Total Hardness	12 mg/L as $CaCO_3$
Total Residues	31 mg/L
Total Organic Carbon	1.6 mg/L
Dissolved Organic Carbon	1.8 mg/L

Sub-portions from the sample "Herndon39-140225-R" (refrigerated after collection) were used for the method validation.

2. Preparation of Samples

Sub-samples (10 mL) of the control water matrix were measured into 16-mL clear glass vials.

3. Fortifications

Method validation samples were fortified as described in the table below. Fortifications were performed using Wiretrol disposable micropipettes directly to fortify the 10-mL samples as follows:

Fortification Level (SL-160/DTPU/DTPP/TPSA)	Amount and Concentration of Spiking Solution Used SL-160/DTPU/DTPP/TPSA
LOQ (0.05/0.05/0.05/0.2 µg/L)	100 µL 5/5/5/20 ng/mL
10x LOQ (0.5/0.5/0.5/2 µg/L)	100 µL 50/50/50/200 ng/mL
100x LOQ (5/5/5/20 µg/L)	100 µL 500/500/500/2000 ng/mL

4. Extraction

After fortification, an aliquot of each sample was combined in a ratio of 9:1 with acetonitrile (i.e. 4.5 mL of sample + 0.5 mL of acetonitrile). After the acetonitrile was added, the sample was shaken by hand for approximately 5 seconds. Each sample extract was then filtered through a 0.45 µm PTFE syringe filter. Samples were vialed and analyzed by LC-MS/MS. Samples requiring additional dilution were diluted into acetonitrile: HPLC grade water (10:90, v/v)

C. Instrumentation

Instrument: AB Sciex API4000 LC/MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC Autosampler

HPLC Column: Phenomenex Luna C18

50 x 3.00 mm, 3 µm (100 Å)
Part # 00B-4251-Y0
Serial # 586432-1

Guard Column: C18 Security Guard Cartridge
4 x 2.00 mm
Part # AJ0-4286

Data System: Analyst Chromatography Data System version 1.5.2, AB Sciex

Mobile Phases:

A) 0.2% Formic Acid in Acetonitrile
B) 0.2% Formic Acid in Water

Flow Rate: 0.5 mL/minute
Run Time: 8.0 minutes
Injection Volume: 10 µL
Gradient Program:

Time (minutes)	%A	%B
0.0	30	70
5.0	90	10
6.0	90	10
6.1	30	70
8.0	30	70

Column Heater: NA

Approximate Retention Times:

SL-160: 3.2 minutes
DTPU: 2.4 minutes
DTPP: 2.7 minutes
TPSA: 1.5 minutes

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

AB Sciex API-4000 Acquisition Parameters (TurbolonSource, ESI interface, MRM mode, positive mode, Unit/Unit Resolution)						
Analyte	**Quantitation**	**Q1 (m/z)**	**Q3 (m/z)**	**Dwell (msec)**	**DP**	**CE**
SL-160	Primary	407.9	181.8	50	60	31
	Confirmatory	407.9	139.1	50	60	58
DTPU	Primary	343.9	300.9	50	46	18
	Confirmatory	343.9	281.1	50	46	36
DTPP	Primary	300.9	281.1	50	50	31
	Confirmatory	300.9	238.1	50	50	42
TPSA	Primary	227.0	145.8	50	56	33
	Confirmatory	227.0	126.0	50	56	42

Parameter	Setting
CUR:	25
GS1:	40
GS2:	40
IS:	5500
TEM:	600
CAD:	6
EP:	10
CXP:	12

The instrument parameters were optimized for analyte sensitivity and resolution prior to the chromatographic run. The exact parameters were documented with the data set.

D. Potential Interferences

1. Matrix Interference

The detection technique is highly selective for this method. No interferences arising from co-eluting compounds from the surface water were observed.

2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. There was no response in the reagent blank sample in the chromatograms corresponding to the retention of SL-160, DTPP, and TPSA. However, there was an insignificant (< 10% LOD by peak area response) amount of response in the chromatograms of the reagent blank and two of the three control matrix samples corresponding to the retention time of DTPU (primary MS/MS ion). This indicates that the LC-MS/MS mobile phases may have potentially been contaminated with a low level of DTPU.

3. Labware Interference

This method uses mostly disposable labware. No interferences from the labware use were observed.

E. Confirmatory Techniques

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

F. Time Required for Analysis

Two hours were required for one person to prepare an analysis set (25 samples) from the time samples were prepared to LC-MS/MS analysis. Automated LC-MS/MS analysis was performed overnight. An additional 1.5 hours was spent on data calculation and tabulation the following day. This method requires approximately 4 hours of direct analyst involvement. However, due to the analysis time (8 minutes per sample), at most, two calendar days (including analysis time, i.e. no analyst direct involvement) may be needed to prepare an analysis set and to calculate and tabulate the data.

G. Modification or Potential Problems

There were no modifications to the method. There were no potential problems encountered.

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 SL-160, DTPU, DTPP, and TPSA as ng/mL using the linear regression function with 1/x weighting. The calibration 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 at a maximum interval of six sample injections, as well as at the beginning and end of the injection sequence. Six different standard concentrations were injected within the analytical set. Calibration standard injections were repeated if necessary to maintain the minimum interval of one calibration standard injection every six sample injections. The concentrations (ng/mL) of SL-160, DTPU, DTPP, and TPSA detected in method validation sample extracts were interpolated from the standard calibration curve. The concentration as µg/L of residue found in samples was then calculated with Microsoft® Excel using the following equation:

$$\mu g/L = \frac{(ng/mL\ from\ curve) \times (Aliquot\ Factor) \times (Final\ Volume\ (mL)) \times 1000\ mL \times 1\ \mu g}{(Sample\ amount\ (mL)) \times 1\ Liter \times 1000\ ng}$$

The aliquot factor is determined as follows:

$$Aliquot\ Factor = \frac{Sample\ Volume\ (mL)}{Aliquot\ Volume\ (mL)}$$

The final volume is determined as follows:

Final Volume (mL) = (Aliquot Volume (mL) + Acetonitrile added (mL)) x Dilution Factor

Any additional dilution (dilution factor) must also be used when calculating the final volume.

Recovery of the analyte from fortified samples was calculated as follows:

$$\%\ Recovery = \frac{(Measured\ Concentration,\ \mu g/L) \quad x\ 100}{(Theoretical\ Concentration,\ \mu g/L\ added)}$$

An example calculation for surface water for a SL-160 laboratory fortification (primary ion) in set 546MV01, sample 546MV01-8 LOQ sample fortified at 0.0500 µg/L, is as follows:

standard curve equation: $y = 1.85 \times 10^{5}(x) + 113$

where x = SL-160 concentration in ng/mL and

y = peak response = 8553.3

SL-160 concentration from the curve =0.0455 ng/mL

$$Aliquot\ Factor = \frac{10\ mL}{4.5\ (mL)} = 2.22$$

Final Volume (mL) = 4.5 mL + 0.5 mL

$$\mu g/L = \frac{(0.0455\ ng/mL) \times (2.22) \times (5\ mL) \times 1000\ mL \times 1\ \mu g}{(10\ mL) \times 1\ Liter \times 1000\ ng} = 0.0505\ \mu g/L$$

$$\%\ recovery = \frac{0.0505\ \mu g/L}{0.0500\ \mu g/L}\ X\ 100 = 101\%$$

No measurable residues were measured in any control samples. Laboratory fortification samples were not corrected for reported control responses. 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.

1.0 INTRODUCTION

SL-160 (Flazasulfuron) is a broad spectrum herbicide. An analytical method is required for the determination of residues of SL-160 and its metabolites DTPU (1-(4,6-dimethoxypyrimidin-2-yl)-1-(3-trifluoromethyl-2-pyridyl)urea), DTPP (4,6-dimethoxy-2-(3-trifluoromethyl-2-pyridylamino) pyrimidine), and TPSA (3-trifluoromethyl-2-pyridinesulphonamide). This method is concerned with the measurement of SL-160, DTPU, DTPP, and TPSA residues in water.

The target limit of quantitation (LOQ) and the limit of detection (LOD) for each analyte are defined in the table below.

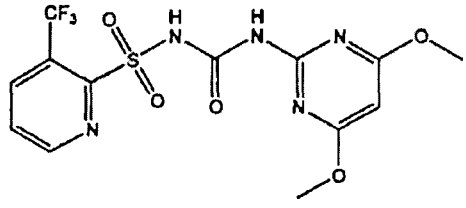
Analyte	LOQ (µg/L)	LOD (µg/L)
SL-160	0.05	0.01
DTPU	0.05	0.01
DTPP	0.05	0.01
TPSA	0.2	0.088

The water analysis is conducted using LC-MS/MS with monitoring of 2 transition ion pairs for each analyte. This method was validated under Golden Pacific Laboratories (GPL) study number 140546 in March of 2014 to satisfy the requirements of the Ecological Effects Test Guidelines OCSPP 850.6100. The results are discussed in the corresponding method validation report. This method is an appendix of the GPL study number 140546 report.

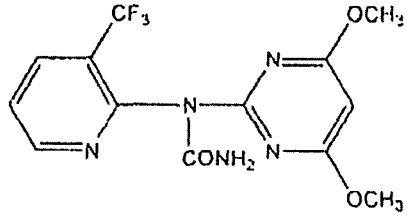
(Note: Due to limited sensitivity for the TPSA confirmatory ion pair, the confirmatory ion pair can be used only for peak identity confirmation under 10x LOQ for TPSA).

2.0 REFERENCE SUBSTANCES

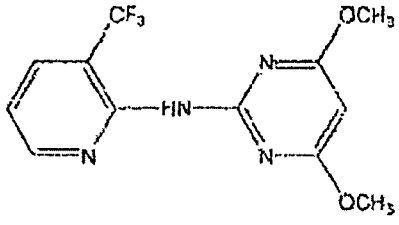
Ishihara Sangyo Kaisha (ISK) Biosciences Corporation provided the reference substances (received from Midwest Research Institute). The reference substances are used to prepare calibration and fortification solutions, and to determine procedural recoveries.



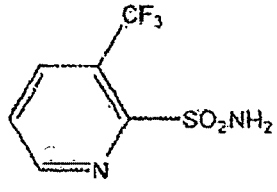
Common Name: SL-160
IUPAC Chemical Name: 1-(4,6-dimethoxypyrimidin-2-yl)-3-(3-trifluoromethyl-2 pyridylsulphonyl)urea
CAS No.: 104040-78-0
Source: ISK
Lot No.: Y-920205
Purity: 100.0
Expiration Date: 05/03/2018



Common Name: DTPU
IUPAC Chemical Name: 1-(4,6-dimethoxypyrimidin-2-yl)-1-(3-trifluoromethyl-2-pyridyl)urea
CAS No.: NA
Source: ISK
Lot No.: 0205
Purity: 98.5
Expiration Date: 06/28/2017



Common Name: DTPP
IUPAC Chemical Name: 4,6-dimethoxy-2-(3-trifluoromethyl-2-pyridylamino) pyrimidine
CAS No.: NA
Source: ISK
Lot No.: 0205
Purity: 98.9
Expiration Date: 06/29/2017



Common Name: TPSA
IUPAC Chemical Name: 3-trifluoromethyl-2-pyridinesulphonamide
CAS No.: NA
Source: ISK
Lot No.: 0205
Purity: 100.0
Expiration Date: 06/22/2017

A copy of the certificates of analysis of the reference substances will be kept in the archives at GPL. The reference substances will be stored in a freezer set to maintain ≤ -10 °C (frozen).

3.0 PRINCIPLE OF THE METHOD

An aliquot of the representative water sample is combined in a ratio of 9:1 with acetonitrile. After the acetonitrile is added, the sample is shaken by hand and filtered. It is then analyzed for SL-160, DTPU, DTPP, and

TPSA residues using LC-MS/MS.

Six concentrations of the reference substance in acetonitrile: HPLC grade water (10:90, v/v) are used for calibration standards. A calibration plot is generated for each analyte and used for quantitation purposes.

4.0 EQUIPMENT AND MATERIALS

Unless otherwise indicated, the equipment and materials listed below may be substituted with functionally equivalent equipment.

- Balance, Analytical, Mettler Toledo XS204
- Volumetric flasks, glass: 10 mL
- Bottles, amber glass with Teflon lined cap: 30, 60, and 120, 240 mL
- Glass vials, clear with Teflon lined cap: various volumes
- Volumetric glass pipette: various volumes
- Graduated Cylinders: various volumes
- Micropipette, Drummond Wiretrol disposable micropipettes: various volumes
- Disposable Pasteur pipettes, glass
- Repeating Pipette, Eppendorf Stream
- HPLC vials, clear glass: 1.8 mL
- AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC Autosampler

5.0 CHEMICALS/REAGENTS

Alternate suppliers of reagents having comparable specifications may be used.

- Acetonitrile, Fisher #A996-4
- Water, HPLC Grade, Fisher #W5-4
- Formic Acid, 88%, Fisher #A118P-500

5.1 Solution Preparation

Acetonitrile: HPLC grade water (10:90, v/v): Prepared by adding 100 mL of acetonitrile to 900 mL of HPLC grade water and mixing well.

Mobile Phase A:
0.2% Formic Acid in Acetonitrile: Prepared by adding 2 mL of formic acid to approximately 900 mL of acetonitrile. Solution is brought up to 1000 mL with acetonitrile. Mix well.

Mobile Phase B:
0.2% Formic Acid in HPLC grade water: Prepared by adding 2 mL of formic acid to approximately 900 mL of HPLC grade water.

Solution is brought up to 1000 mL with HPLC grade water. Mix well.

6.0 STANDARD SOLUTIONS

The reference substances are used in the preparation of the fortification and calibration solutions. The reference substances are stored frozen.

6.1 SL-160, DTPU, DTPP, and TPSA Fortification Solutions

Each reference substance is first weighed separately into 10-mL volumetric flasks and made up to volume with acetonitrile to give a stock solution with a concentration of approximately 1.0 mg/mL after being corrected for purity. The stock solutions are individually diluted further in acetonitrile to produce intermediate solutions which are then diluted in combination to produce the 100x LOQ fortification solution "I" (see table below). Additionally serial dilutions are made in acetonitrile to produce the 10x LOQ (Solution J) and LOQ level (Solution K) fortification solutions. Alternative dilution schema may be used to reach the same concentration levels.

Initial Solution ID	Analyte(s)	Concentration of Initial Solution (µg/mL)	Volume of Solution (mL)	Final Volume (mL)	Final Solution ID	Standard Concentration (µg/mL)
A	SL-160	1000	0.5	50	E	10
B	DTPU	1000	0.5	50	F	10
C	DTPP	1000	0.5	50	G	10
D	TPSA	1000	1	2.5	H	400
Initial Solution ID	**Analyte(s)**	**Concentration of Initial Solution (µg/mL)**	**Volume of Solution (mL)**	**Final Volume (mL)**	**Final Solution ID**	**Standard Concentration (ng/mL)**
E	SL-160	10	10	200	I	500/500/500/2000[1]
F	DTPU	10	10			
G	DTPP	10	10			
H	TPSA	400	1			
Initial Solution ID	**Analyte(s)**	**Concentration of Initial Solution (ng/mL)**	**Volume of Solution (mL)**	**Final Volume (mL)**	**Final Solution ID**	**Standard Concentration (ng/mL)**
I	All	500/500/500/2000[1]	10	100	J	50/50/50/200[1]
J	All	50/50/50/200[1]	10	100	K	5/5/5/20[1]

[1] Solution concentrations are listed in the following order: SL-160/DTPU/DTPP/TPSA

The fortification solutions will be stored frozen. They are prepared every 3 months or as needed.

6.2 Calibration Solutions

Solution J is diluted in acetonitrile: HPLC grade water (10:90, v/v) to produce an intermediate curve solution of 5/5/5/20 µg/mL SL-160/DTPU/DTPP/TPSA (Solution L). Solution L will be diluted to make calibration standards at appropriate concentrations. Calibration standards are made in acetonitrile: HPLC grade water (10:90, v/v). Typical concentrations are listed in the table below. Alternative dilution schema may be used to reach the same concentration levels.

Initial Solution ID	Analyte(s)	Concentration of Initial Solution (ng/mL)[1]	Volume of Solution (mL)	Final Volume (mL)	Final Solution ID	Standard Concentration (ng/mL)[1]
L	All	5/5/5/20	10	50	M	1/1/1/4
L	All	5/5/5/20	4	50	N	0.4/0.4/0.4/1.6
L	All	5/5/5/20	2	50	O	0.2/0.2/0.2/0.8
L	All	5/5/5/20	1	50	P	0.1/0.1/0.1/0.4
L	All	5/5/5/20	0.8	100	Q	0.04/0.04/0.04/ 0.16
L	All	5/5/5/20	0.4	100	R	0.02/0.02/0.02/ 0.08

[1] Solution concentrations are listed in the following order: SL-160/DTPU/DTPP/TPSA

The calibration solutions will be stored in refrigerated. Calibration solutions are prepared every three months or as required.

7.0 ANALYTICAL PROCEDURE

A 10-mL sample aliquot of sample of water is measured into a glass test tube or other clear glass vial. Samples requiring fortification are fortified at the appropriate concentration. Possible targeted levels are as follows:

	Fortification Levels (µg/L)			
	SL-160	DTPU	DTPP	TPSA
LOQ	0.05	0.05	0.05	0.2
10x LOQ	0.5	0.5	0.5	2
100x LOQ	5	5	5	20

Laboratory fortifications are prepared using a syringe or Wiretrol pipette.

Following fortification of the fortified samples, an aliquot of each sample is combined with acetonitrile in a 9:1 ratio (e.g. 4.5 mL sample + 0.5 mL acetonitrile). Samples are gently shaken by hand for approximately 5 seconds. Next, the sample is filtered through a disposable plastic syringe with a PTFE 0.45-µm filter into a new glass test tube or glass vial. From

the filtered extract, an aliquot is transferred to a chromatography vial and analyzed by LC-MS/MS. Samples having higher residue levels are diluted to an appropriate final volume using acetonitrile: HPLC grade water (10:90, v/v) so that the response falls within the calibration range of the standards. Sample extracts should be stored refrigerated.

8.0 QUANTITATION

8.1 Instrumentation

An AB Sciex API4000 LC-MS/MS system is used. The HPLC consists of Shimadzu LC-20AD HPLC Pumps, a Shimadzu SCL-10A VP Controller, and Shimadzu SIL-20AC Autosampler. Data is acquired using Analyst Software.

8.2 Typical LC-MS/MS Conditions

HPLC Column: Phenomenex Luna C18(2)
50 x 3.00 mm, 3 µm (100 Å)
Part # 00B-4251-Y0
Serial # 586432-1

Guard Column: C18 Security Guard Cartridge
4 x 2.00 mm
Part # AJ0-4286

Data System: Analyst Chromatography Data System version 1.5.2, AB Sciex

Mobile Phases:
A) 0.2 % Formic Acid in Acetonitrile
B) 0.2% Formic Acid in Water

Flow Rate: 0.5 mL/minute
Run Time: 8.0 minutes
Injection Volume: 10 µL
Gradient Program:

Time (minutes)	%A	%B
0.0	30	70
5.0	90	10
6.0	90	10
6.1	30	70
8.0	30	70

Column Heater: Not Applicable

Approximate Retention Times:

SL-160: 3.2 minutes

DTPU: 2.4 minutes

DTPP: 2.7 minutes

TPSA: 1.5 minutes

Note: Retention times may vary slightly on different equipment.

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

AB Sciex API-4000 Acquisition Parameters (TurbolonSource, ESI interface, MRM mode, positive mode, Unit/Unit Resolution)						
Analyte	**Quantitation**	**Q1 (m/z)**	**Q3 (m/z)**	**Dwell (msec)**	**DP**	**CE**
SL-160	Primary	407.9	181.8	50	60	31
	Confirmatory	407.9	139.1	50	60	58
DTPU	Primary	343.9	300.9	50	46	18
	Confirmatory	343.9	281.1	50	46	36
DTPP	Primary	300.9	281.1	50	50	31
	Confirmatory	300.9	238.1	50	50	42
TPSA	Primary	227.0	145.8	50	56	33
	Confirmatory	227.0	126.0	50	56	42

Parameter	Setting
CUR:	25
GS1:	40
GS2:	40
IS:	5500
TEM:	600
CAD:	6
EP:	10
CXP:	12

Mass spectrometer parameters should be optimized for the instrument prior to sample analysis.

8.4 LC-MS/MS Detector Response Calibration

The LC-MS/MS responses (peak areas) are determined for a series of external calibration standards. Through the Analyst Software, the concentrations of the standards injected and their corresponding peak responses are compiled. The calibration standards from the analytical set are plotted and the standard linear regression, slope and y-intercept values are calculated using a 1/x weighted curve.

The concentration of the sample that is injected (ng/mL) is taken as the X-axis and the detector response (peak area) is taken as the Y-axis to give Equation 2.

$$y = mx + b \qquad \text{[Eq. 1]}$$

Where: y = peak area response for analyte injected (sample/standard)
m = slope of the regression line
x = amount (ng/mL) of analyte found in the sample/standard
b = intercept of the regression line

$$\text{peak area} = m\ (\text{ng/mL in the sample/standard}) + b \qquad \text{[Eq. 2]}$$

8.5 Sample Analysis

For samples, the amount found (ng/mL) of each of the analytes may be calculated from the observed peak area, using Equation 3.

$$x\ (\text{ng/mL})\ \text{in sample} = \frac{\text{peak area} - b}{m} \qquad \text{[Eq. 3]}$$

Both samples and standards must be analyzed under the same LC-MS/MS conditions and within the same analytical sequence.

9.0 CALCULATION OF RESIDUES

From the standard calibration curve, analyte concentrations (µg/L) in unknown samples are determined using the following equation:

$$\mu g/L = \frac{(\text{ng/mL from curve}) \times (\text{Aliquot Factor}) \times (\text{Final Volume (mL)}) \times 1000\ \text{mL} \times 1\ \mu g}{(\text{Sample amount (mL)}) \times 1\ \text{Liter} \times 1000\ ng} \qquad \text{[Eq. 4]}$$

The aliquot factor is determined as follows:

$$\text{Aliquot Factor} = \frac{\text{Sample Volume (mL)}}{\text{Aliquot Volume (mL)}} \qquad \text{[Eq. 5]}$$

The final volume is determined as follows:

Final Volume (mL) = (Aliquot Volume (mL) + Acetonitrile added (mL)) x Dilution Factor

[Eq. 6]

Any additional dilution (dilution factor) must also be used when calculating the final volume.

$$\% \text{ Recovery} = \frac{\text{measured residues } (\mu g/L)}{\text{fortification amount } (\mu g/L)} \times 100 \qquad \text{[Eq. 7]}$$

10.0 QUALITY CONTROL PROCEDURES

10.1 LC-MS/MS Analysis

These values are used to establish the fortification recoveries. The calibration plot is not forced through zero.

There are a minimum of six calibration standards that bracket the concentration range with the lowest standard corresponding to 70% or less of the LOQ. Sample extracts containing analyte levels above the calibration curve are diluted accordingly to fit the calibrated range. Calibration standards are injected at the beginning and end of an analytical set. Standards are also injected periodically throughout the set. No more than six sample injections are made without a standard injection.

10.1.1 Acceptance Criteria

The acceptance criterion for linearity is that the correlation coefficient (r) of the calibration curve must be ≥0.990 (or consequently, the coefficient of determination (r^2) must be ≥0.980).

The acceptance criteria for QC recovery are recoveries between 70 – 120%.

The acceptance criteria for accuracy is the lowest calibration standard (at or below the LOQ) will have an RPD value of ≤ 25% and ≤ 20% for all other calibration standards.

The acceptance criterion for specificity for the control samples (unfortified, blank matrix samples) is that the concentration will be ≤ 20% of the LOQ as estimated from peak area response. Control sample contributions must not be subtracted from the response of the fortified or unknown samples.

11.0 METHOD FLOW CHART

Analysis of SL-160, DTPU, DTPP, and TPSA in Water by LC-MS/MS

Measure a 10 mL sub-sample of water into a clear vial

↓

Fortify as necessary

↓

Take a aliquot of sample to an aliquot of acetonitrile in a ratio of 9:1
(e.g. 4.5 mL sample + 0.5 mL acetonitrile)

Aliquot Volume: _____ Volume of Acetonitrile Added:_____

↓

Shake by hand for approximately 5 seconds

↓

Filter the extract through a 0.45-µm PTFE filter

↓

Vial and load samples onto LC-MS/MS

If further dilution is necessary,
dilute with Acetonitrile: HPLC grade water (10:90, v/v)

Initial: _____ Date: _____