A. Scope of the Method

This method allows for the quantitative determination of residues of SL-160 and its metabolites (DTPU, DTPP, ADMP and TPSA) in soil used in Soil Dissipation studies.

B. Principle of the Method

SL-160 and its metabolites are extracted from 50-g soil samples using 120-ml of acetonitrile and 30-ml of water. The samples are then shaken on the shaker for 30 minutes. After shaking, the samples are filtered through Buchner funnels into 500-ml flat bottoms and then evaporated to 10-20 ml using rotary evaporation. The sample residues are dissolved in water and extracted with methylene chloride. Methylene chloride extracts are dried using rotary evaporation. Once dried the samples are reconstituted with acetonitrile, filtered and transferred to vials for LC/MS/MS analysis. SL-160 and its metabolites are determined using LC/MS/MS. The limit of quantitation (LOQ) of the method is 0.00250 μg/g (ppm). The highest validated level in this method is 1.00 μg/g (ppm).

C. Equipment Required

Note: Some standard laboratory items and equipment may not be listed. Equipment and chemicals from other manufacturers may be used provided they are functionally equivalent.

1.	HPLC/MS/MS:	Sciex API3000 LC/MS/MS with Shimadzu LC-10AD HPLC Pumps, Shimadzu SCL-10A Controller, Perkin Elmer PE-200 Autosampler
2.	Analytical Column:	Phenomenex Prodigy 5 μ ODS (250 mm x 4.60 mm id), Catalog number: 000G-4097-E0
3.	Data System:	Analyst Version 1.1 Chromatography Software, Applied BioSystems, Inc.
4.	Labware:	500-ml flat bottom evaporation flasks 250-ml Nalgene bottles 250-ml separatory funnels Buchner funnels Glass powder funnels Class-A volumetric pipets (assorted) Hamilton syringes (assorted) Disposable Pasteur pipets 15-ml culture tubes with teflon-lined lids 7-ml culture tubes with teflon-lined lids Graduated cylinders (assorted) 2-ml chromatography vials with screw caps

Whatman #4 Filter Paper 110mm#1004 110
Whatman #4 Filter Paper 150mm#1004 150

5. Balances: Mettler AB204-S precision ± 0.1-mg for Standard preparation

Mettler SB16000, precision ± 1-g for sample Processing

Mettler PB3002-S precision ± 0.01-g for Samples and moistures

6. Rotary Evaporator; Labconco.@ rotary evaporators equipped with a Sargent Welch Direct Torr® vacuum pump

7. Platform Shaker: Eberbach, variable speed, capable of Achieving~ 200 rpm.

D. Reagents and Chemicals Required

Reagents and chemicals from other manufacturers may used provided they are suitable Substitutions. Suitability of reagents and chemicals can be proven by analyzing reagent blanks.

1. Solvents (ACS grade or equivalent):
   a. Acetonitrile, Fisher # A996-4
   b. Deionized or HPLC Grade Water, Fisher # W5-4
   c Methylene Chloride, Fisher # D151-4
   d. Hydrochloric Acid, Fisher # A144^{c}-212

2. Solid Reagents (ACS grade or equivalent)
   a. Celite 545, Fisher # C212-500
   b. Sodium Chloride, Fisher #S271-3
   c. Sodium Sulfate, Fisher # S415-10S

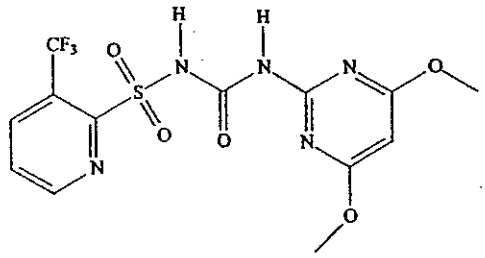
E. Standards Required

SL-160 analytical standard, CAS No. 104040-78-0, 99.89% purity, having an expiration date of December 13, 2006, was received from Midwest Research Institute, 425 Volker Boulevard, Kansas City, MO 64110.

Note: Standard information may vary depending on specific lot supplied, reassay dates, purity, etc.

Common Name:	Flazasulfuron (SL-160)
Chemical Name:	1-(4,6-dimethoxypyrimidin-2-yl)-3-(3-trifluoromethyl-2-pyridylsulphonyl)urea
CAS Number:	104040-78-0
Molecular Formula:	$C_{13}H_{12}F_3N_5O_5S$
Molecular Weight:	407.3
Source:	ISK Biosciences Corporation
Lot No.:	Y-920205
Purity:	99.89%
Expiration Date:	12/13/2006

Structure:



SL-160

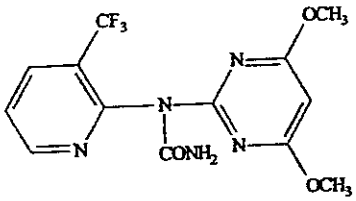
DTPU analytical standard, 98.4% purity, having an expiration date of April 30, 2007, was received from Ishihara Sangyo Kaisha, Ltd, 2-3-1 Nishi-Shibukawa, Kusatsu, Shiga, Japan 5250025.

Note: Standard information may vary depending on specific lot supplied, reassay dates, purity, etc.

Common Name:	DTPU
Chemical Name:	1-(4,6-dimethoxypyrimidin-2-yl)-1-(3-trifluoromethyl-2-pyridyl)urea
CAS Number:	Not Available
Molecular Formula:	$C_{13}H_{12}F_3N_5O_3$

Molecular Weight:	343.26
Source:	ISK Biosciences Corporation
Lot No.:	0205
Purity:	98.4%
Expiration Date:	4/30/2007

Structure:



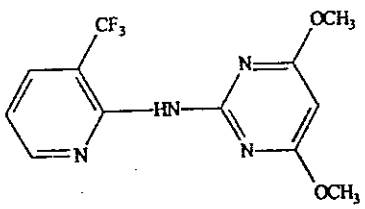
DTPU

DTPP analytical standard, 98.5% purity, having an expiration date of May 7, 2007, was received from Ishihara Sangyo Kaisha, Ltd, 2-3-1 Nishi-Shibukawa, Kusatsu, Shiga, Japan 5250025.

Note: Standard information may vary depending on specific lot supplied, reassay dates, purity, etc.

Common Name:	DTPP
Chemical Name:	4,6-dimethoxy-2-(3-trifluoromethyl-2-pyridylamino) pyrimidine
CAS Number:	Not Available
Molecular Formula:	$C_{12}H_{11}F_3N_4O_2$
Molecular Weight:	300.24
Source:	ISK Biosciences Corporation
Lot No.:	0205
Purity:	98.5%
Expiration Date:	5/7/2007

Structure:



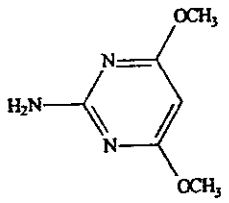
DTPP

ADMP analytical standard, 99.3% purity, was received from Ishihara Sangyo Kaisha, Ltd, 2-3-1 Nishi-Shibukawa, Kusatsu, Shiga, Japan 5250025.

Note: Standard information may vary depending on specific lot supplied, reassay dates, purity, etc.

Common Name:	ADMP
Chemical Name:	2-amino-4,6-dimethoxypyrimidin
CAS Number:	Not Available
Molecular Formula:	$C_6H_9N_3O_2$
Molecular Weight:	155.15
Source:	ISK Biosciences Corporation
Lot No.:	Y-Ba. 79
Purity:	99.3%
Expiration Date:	not given

Structure:



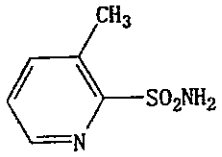
ADMP

TPSA analytical standard, 99.6% purity, having an expiration date of May 7, 2007, was received from Ishihara Sangyo Kaisha, Ltd, 2-3-1 Nishi-Shibukawa, Kusatsu, Shiga, Japan 5250025.

Note: Standard information may vary depending on specific lot supplied, reassay dates, purity, etc.

Common Name:	TPSA
Chemical Name:	3-trifluoromethyl-2-pyridinesulphonamide
CAS Number:	Not Available
Molecular Formula:	$C_6H_5F_3N_2O_2S$
Molecular Weight:	226.18
Source:	ISK Biosciences Corporation
Lot No.:	0205
Purity:	99.6%
Expiration Date:	5/7/2007

Structure:



TPSA

F. Preparation of Solutions

NOTE: Solution concentrations and volumes may be changed accordingly as necessary.

1. LC/MS/MS Standard Solutions

a. Stock Standard Solutions (~1.00 mg/ml)

Accurately weigh, to the nearest 0.1-mg, approximately 25-mg of SL-160 analytical reference standard into a 25-ml Class A volumetric flask. Adjust the volume to the calibrated mark using acetonitrile and mix to completely dissolve the test material. The final concentration of the solution should be ~1.0 mg/ml. Repeat for each metabolite.

b. Intermediate Standard and Lab Fortification Solutions

Dilute appropriate volumes of the stock solutions with acetonitrile (F.1.a) to obtain mixed intermediate solutions with concentrations of 1.00 µg/ml and 100 ng/ml of SL-160, DTPU, DTPP and ADMP and concentrations of 20.0 µg/ml and 2.00 µg/ml of TPSA.

c. LC/MS/MS Standard Solutions for Calibration Curve

Aliquot appropriate volumes of the 100 ng/ml/ 2.00 µg/ml intermediate solution (F.1.b) into Class A volumetric flasks and bring to volume with acetonitrile to obtain solutions with the following concentrations of SL-160, DTPU, DTPP and ADMP: 20.0, 10.0, 5.00, 2.00, 1.00 and 0.500 ng/ml, and TPSA at concentrations of 400, 200, 100, 40.0 , 20.0 and 10.0 ng/ml.

The LC/MS/MS standards are considered stable for at least 3 months, if stored in amber bottles, and kept refrigerated when not in use.

2. Fortification Solutions

a. Fortification Solutions

Accurately weigh, to the nearest 0.1-mg, approximately 25-mg of SL-160 analytical reference standard into a 25-ml Class A volumetric flask. Adjust the volume to the calibrated mark using acetonitrile and mix to completely dissolve the test material. The final concentration of the solution should be ~1.0 mg/ml. Repeat for all the metabolites.

Dilute appropriate volumes of the stock solution with acetonitrile to obtain individual fortification solutions with all five compounds at appropriate concentrations to achieve the desired fortification level. Fortification solutions are stable for at least 3 months if stored in amber bottles and kept refrigerated when not in use.

3. Mobile Phase A

For every 1-L of mobile phase, mix together 1.0 ml of Acetic Acid into 999-ml acetonitrile. Mix well and using vacuum filtration, filter the mobile phase through a 0.45 µm filter. Sonicate for 5 minutes and purge with helium to degas. Mobile phase should be stored at room temperature and is stable for one month.

4. Mobile Phase B

For every 1-L of mobile phase, mix together 1.0 ml of Acetic Acid into 999-ml water. Mix well and using vacuum filtration, filter the mobile phase through a 0.45 µm filter. Sonicate for 5 minutes and purge with helium to degas. Mobile phase should be stored at room temperature and is stable for one month.

G. Typical LC/MS/MS Conditions

LC/MS/MS conditions may vary slightly to optimize for sensitivity or resolution of the analytes. Exact conditions will be documented with each analytical set.

Mobile Phase A:	0.1% Acetic Acid in Acetonitrile
Mobile Phase B:	0.1% Acetic Acid in Water

Gradient Program:

Time (minutes)	A (%)	B (%)
0.0	60	40
6.0	60	40
6.1	90	10
9.0	90	10
9.1	60	40
13.0	60	40

Flow Rate: 1.00 ml/min with 2:1 split

Column Temperature: 45.0 °C

Retention Time:

SL-160	~5.8 min
DTPU	~4.1 min
DTPP	~6.0 min
ADMP	~3.1 min
TPSA	~3.1 min

Run Time: 15.0 min

Injection Volume: 10 µL

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

Interface:	TurboIon Spray (ESI)
Polarity:	Positive
Scan Type:	MRM Monitoring with Unit resolution
Nebulizer Gas (NEB):	7
Curtain Gas (CUR):	6
Collision Gas (CAD):	9
Entrance Potential (EP):	-10
Ionspray Voltage (IS):	4000
Temperature (TEM):	500

	SL-160	DTPU	DTPP	ADMP	TPSA
Declustering Potential (DP)	51	46	51	36	56
Focusing Potential (FP)	300	50	50	190	300
Collision Energy (CE)	27	19	31	25	35
Collision Cell Exit Potential (CXP)	10	16	18	6	12

Ions Monitored:	(SL-160)	*m/z* 408 (quantitation)
		m/z 181.7 (confirmation)
	(DTPU)	*m/z* 344 (quantitation)
		m/z 301 (confirmation)
	(DTPP)	*m/z* 301 (quantitation)
		m/z 281 (confirmation)
	(ADMP)	*m/z* 156 (quantitation)
		m/z 99 (confirmation)
	(TPSA)	*m/z* 227 (quantitation)
		m/z 146 (confirmation)

H. Linearity Check

Inject 10.0 µL of each calibration standard and plot the peak response (area or height) for each component versus its concentration to demonstrate linearity of response. The injection volume must remain constant during LC/MS/MS analysis of any analytical set. Quadratic regression will be used for all calibration curves. Significant departure from linearity (a correlation coefficient of less than 0.990) indicates instrumental or operational difficulties that must be corrected before proceeding.

I. Preparation of Samples

Samples from the field are taken in cores. Each sample will be processed before extraction can take place to insure the sample is homogeneous and the particle size is usable. Once processed, a subsample will be weighed out into 250-ml Nalgene bottles.

J. Analysis Procedure

1. Prior to starting the extraction, wash and rinse all glassware with soapy water and acetone thoroughly.

   Soil samples are removed from the freezer and allowed to thaw. Once thawed, a 50-g sample is weighed out into 250-ml Nalgene bottles. Lab fortification samples are prepared at this time by weighing out the control two extra times and spiking the extra control soil samples at levels ranging from 0.500 to 5.00 µg/sample.
   Note: The LOQ is 0.00250 µg/g (ppm) for all compounds.

2. After allowing the spiking solvent to dry, add 120-ml of acetonitrile and 30-ml of water to each sample.

3. Cap the samples securely invert/shake to check for leaks

4. Place the sample bottles on a platform shaker (~200 rpm) for approximately 30 min.

Note: The bottles should be positioned on their sides for maximum shaking efficiency.

5. Remove the samples from the shaker and allow them to settle.

6. Filter each sample using a Buchner funnel across a Whatman #4 filter, 110 mm, with 5 grams of Celite into a properly labeled 500-ml flat bottom evaporation flask.

7. Rinse the filter cake with 50 ml of acetonitrile and allow to suction dry. Discard the filter cake.

8. Evaporate the each sample to a volume of 10-20 ml using rotary evaporation equipped with a water bath at approximately 40°C.

9. Dissolve the remaining 10-20 ml sample in 50 ml of water and transfer each sample into a properly labeled 250-ml separatory funnel.

10. Rinse each 500-ml flat bottom with an additional 30 ml of water, swirl the water around the flat bottom, and add the 30 ml to the appropriate separatory funnel.

11. Rinse each 500-ml flat bottom with 25 ml of acetonitrile, swirl the acetonitrile around the flat bottom, and add the 25 ml to the appropriate separatory funnel.

12. To each separatory funnel add ~ 1.5 grams of sodium chloride. Cap and shake separatory funnel until dissolved.

13. Add 80 ml of methylene chloride to each separatory funnel. Cap, shake and vent for about 1 minute. Allow the layers to separate.

14. Drain the bottom organic layer through a glass powder funnel filled with pre-rinsed sodium sulfate supported by a Whatman #4 filter paper, 150 mm in diameter, into a properly labeled 500-ml flat bottom flask.

15. Add 40 ml of methylene chloride to the separatory funnels. Cap, shake and vent for another minute. Allow layers to separate.

16. Drain the bottom organic layer through sodium sulfate into the 500-ml flat bottom flask, combining the organic layers.

17. To each sample, add 4 ml of 6 N Hydrochloric Acid and swirl.

18. Add 50 ml of methylene chloride to the separatory funnel. Cap, shake and vent for about a minute. Allow the layers to separate.

19. Drain the bottom organic layer through sodium sulfate into the 500-ml flat bottom flask, combining the organic extracts.

20. Repeat steps 18 and 19 a second time.

21. Rinse the sodium sulfate with approximately 30 ml of methylene chloride.

22. Evaporate each sample collected in the 500-ml flat bottom to dryness using rotary evaporation with a water bath set at 40 °C.

23. Dissolve the sample residue in 15-ml acetonitrile and transfer the residues to a 7-ml test tube with a Teflon lined cap.

24. Filter the sample extract into a pre-labeled HPLC vial using a 0.45 µm filter and seal pending analysis by LC/MS/MS.

25. For higher levels, further dilution may be necessary using acetonitrile such that the concentration of analyte in the extract falls within the standard curve range.

Note: Step 10 may be omitted for some soil types. The remaining extract will be retained until LC/MS/MS analysis is completed. When the analyst accepts the results, the extract may be disposed of.

K. Recovery Criteria

The ability of the analyst to perform these procedures satisfactorily must be demonstrated by recovery tests before analysis of study samples is attempted. This is accomplished by conducting a method validation. In addition, a minimum of 10% fortified samples must be run concurrently with each batch of study samples to demonstrate that the overall operation of the procedure for that batch of samples is satisfactory. Recovery samples (also referred to as spikes or fortification samples) must yield recoveries generally ranging from 70 to 120%.

L. Retention Time Criteria

The retention time of the analyte in the fortified samples and actual field samples must be within ± 0.2 minutes of the retention time of the nearest standard in the analytical set to be accepted as the analyte.

M. Automated LC/MS/MS Analysis

Begin the automatic LC/MS/MS analytical set with at least two standards, followed by unknown (study) samples or fortified samples and standards arranged on the autosampler tray so that no more than four unknowns or fortified samples are injected without a standard injection. At least two standards should be run following the last injected sample. If a sample peak response exceeds the peak response of the most concentrated standard in the standard curve by more than 10%,

dilute the sample further so that its peak response falls within the standard curve and re-inject the diluted sample on LC/MS/MS. Record the dilution factor for use in calculations as described in Section N.

N. Calculations

Calculate the concentration of analyte as follows:

1. Through the Analyst Version 1.1 Chromatography Software, the concentrations of the standards injected and their corresponding peak responses are compiled.

2. Analyst Version 1.1 calculates a standard calibration curve using linear or quadratic regression and a correlation coefficient (r) based on the standard concentrations and their respective peak responses.

3. From the standard calibration curve, SL-160 concentrations (µg/sample), as well as its metabolites, in unknown samples are determined using the following equation:

$$\mu g/g = \frac{\left(\begin{matrix}ng/ml\ from\\ the\ curve\end{matrix}\right)\left(\begin{matrix}final\\ volume\ in\ ml\end{matrix}\right)\left(\begin{matrix}dilution\\ factor\end{matrix}\right)}{(50g)(1000\,ng/\mu g)}$$

$$dilution\ factor = \frac{final\ volume\ after\ dilution\ (ml)}{initial\ volume\ (ml)}$$

$$\%Recovery = \frac{(measured\ concentration)}{(theoretical\ concentration)} \times 100$$

4. An example calculation for SL-160 in method validation set 116MV01A, sample 116MV01A-4 Mid Spike A at 0.101 µg/g, is as follows:

standard curve equation: $y = 55\,(x^2) + 2.99\,E4\,(x) - 3.1\,E4$
where x *= SL-160 concentration in ng/ml and*
y *= peak response = 542809.4*
SL-160 concentration from the curve = 16.6 ng/ml

$$Dilution\ factor = \frac{300\ ml}{15\ ml} = 20$$

$$\mu g/sample = \frac{(16.6\ ng/ml\ SL\text{-}160)\,(15\ ml)\,(20)}{(50\ g)(1000\ ng/\mu g)} = 0.0996\ \mu g/g$$

$$\%\ recovery = \frac{0.0996\ \mu g/g}{0.101\ \mu g/g} \times 100 = 98.6\%$$