
**Kasugamycin and Kasugamycinic Acid Soil Method as Described in
“Kasugamycin Field Dissipation in Bare Ground,”
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This method is used for the determination of Kasugamycin and Kasugamycinic acid in soil. This method has a demonstrated limit of quantitation of 0.01 ppm, equivalent to the lowest fortification level that yielded acceptable recovery. The limit of detection was taken as the concentration of the lowest calibrant or 025 ng/mL, equivalent to 0.00075 ppm in soil.

MATERIALS AND METHODS

Reference Substances

The Kasugamycin HCl H₂O (1669W-001) reference substance was provided by Arysta LifeScience of North America with a purity of 99.4% by Certificate of Analysis dated April 8, 2008, with expiration date of May 31, 2009. The Kasugamycinic acid (1669W-003) reference standard was provided by Arysta LifeScience of North America with a purity of 100% and expiration date of March 2011. The reference substance solutions were taken to be stable if stored refrigerated for approximately 12 months, based on the comparison of LCMS chromatograms of the analyses.

Statement of Analytical Reference Substances

The following standards were utilized for analysis throughout the study (see Appendix B for Certificates of Analysis):

Compound:	Kasugamycin hydrochloride hydrate
Common Name:	Kasugamycin
Chemical Name:	3- <i>O</i> -[2-amino-4-{(carboxyiminomethyl)amino}-2,3,4,6-tetra-deoxy- α - <i>D</i> -arabino-hexopyranosyl]- <i>D</i> -chiro-inositol hydrochloride hydrate
CAS No.:	19408-46-9
Purity:	99.7% (exp. December 4, 2009) (Kasugamycin represents 87.3%)

Molecular Weight: 433.8 g/mole
Lot Number: VII-1
Supplier: Arysta LifeScience North America Corporation
Date Received: April 26, 2007
Expiration Date: December 5, 2009
Storage Conditions: Frozen

Compound: **Kasugamycinic Acid**
Common Name: Kasugamycinic Acid
Chemical Name: 3-*O*-[2-amino-4-{(carboxycarbonyl)amino]-2,3,4,6-tetra-deoxy- α -*D*-arabino-hexopyranosyl]-*D*-chiro-inositol
CAS No.: 6001-03-2
Purity: 100%
Molecular Weight: 379.3 g/mole
Lot Number: KA-2
Supplier: Arysta LifeScience North America Corporation
Date Received: May 14, 2007
Expiration Date: March 31, 2011
Storage Conditions: Frozen

Reagents and Material

Ammonium Formate
Formic Acid
Sodium Bicarbonate

Solvents (HPLC grade or better)

Acetonitrile
Methanol
Water

Glassware and Miscellaneous Equipment

Balance(s)
Bottle, Glass centrifuge, 250 mL
Bottle, Amber Glass

Centrifuge, Mistral 3000E
Centrifuge, Eppendorf 5415C
Graduated cylinder, various sizes
Microfilterfuge tubes, Rainin (0.45 μm , Catalog no. 7016-022)
Pasteur pipettes
Sonicator, Branson 2210
Syringes, microliter, various sizes
Vacuum evaporator, Büchi Model RE111 with temperature controlled bath,
Brinkmann Instruments, Burlingame, CA
Vials, amber (2 mL capacity) with Teflon[®]-lined crimp cap, Chromacol, Inc.,
Trumbull, CT
Volumetric flask, various sizes
Volumetric pipette, various sizes
Wrist Action Shaker

ANALYTICAL PROCEDURES

Preparation of Standards

Stock standard solution of Kasugamycin (lot # VII-1) were prepared at 700 $\mu\text{g}/\text{mL}$ in methanol with ammonium acetate (114.85 mg Kasugamycin HCl H_2O dissolved in 100.19 mL methanol plus 43 mL 5% aqueous ammonium acetate). A 1mg/mL stock solution of Kasugamycin was prepared by dilution in water (11.69 mg was diluted to 10.19 mL with water). Stock standard of Kasugamycinic acid (lot KA-2, 100% purity) was prepared by dilution of 10.67 mg in 10.31 mL water. Similar preparations of stock solutions were prepared during the study. The stock standard solutions were stored refrigerated when not in use. Working solutions were made by diluting the stock standard to prepare calibration standard solutions, as described below.

Preparation of Fortification Standards

Mixed Kasugamycin and Kasugamycinic acid fortification standards were prepared by dilution with water as follows: 0.250 mL of 1 mg/mL Kasugamycin plus 0.250 mL of Kasugamycinic acid were diluted to 25 mL with water to generate a 10 $\mu\text{g}/\text{mL}$ mixed fortification solution. A 10-fold dilution of 10 $\mu\text{g}/\text{mL}$ mixed fortification solution was prepared to generate a 1 $\mu\text{g}/\text{mL}$ fortification solution. Similar dilutions were prepared

throughout the study to generate the necessary mixed fortification solutions. Dilutions were prepared using Hamilton syringes, volumetric flasks and volumetric pipettes. The fortification standards were stored at refrigerated when not in use.

Fortification Procedure

Fortification of untreated soil (50 g) with Kasugamycin and Kasugamycinic acid was performed to analyze method percent recoveries for method validation and for sample set analysis, as shown below:

Method Validation Fortification Level (ppm)	Mixed Stock Standard
0.01	500 μ L of 1.0 μ g/mL mixed stock
0.10	500 μ L of 10 μ g/mL mixed stock

Preparation of Linearity Standards

Dilutions of the 10 μ g/mL fortification standard was used to prepare the following linearity standards. All mixed calibrant dilutions were prepared with water. Standards were prepared using volumetric flasks and Hamilton syringes. Mixed calibrants were stored refrigerated when not in use.

Concentration of Kasugamycin and Kasugamycinic Acid (ng/mL)	Mixed Standard Solution Used	Volume of Mixed Standard added	Final Volume (mL)
200	10 µg/mL	500 µL	25
100	10 µg/mL	250 µL	25
50	10 µg/mL	125 µL	25
25	1 µg/mL	625 µL	25
10	1 µg/mL	250 µL	25
5	1 µg/mL	125 µL	25
2.5	0.1 µg/mL	625 µL	25
1	0.1 µg/mL	250 µL	25

EXTRACTION METHOD

Kasugamycin (KSM) Extraction

1. Weigh 50.0 grams of soil into 250 mL centrifuge bottle. Fortify, as necessary.
2. Add 50 mL 0.05 M Formic acid in HPLC water to soil. (Preparation of 0.05M Formic Acid in Water: 480µL in 250mL HPLC water)
3. Extract by shaking on wrist action shaker for 1 hour.
4. Centrifuge at 2500 rpm for 15 minutes.
5. Carefully transfer the supernatant to an amber bottle with Teflon lined cap.
6. Repeat steps 2 through 5, combining extracts in bottle.
7. Repeat steps 2 through 5, combining extracts in bottle. Mix by shaking.
8. Microfilterfuge an aliquot of the extract and transfer filtrate to GC vial for LCMS analysis. Dilute samples, if necessary.
9. Aliquot unfiltered final extract to 16mL amber bottles for back up. Dispose remaining final extract.

Kasugamycinic Acid (KSMA) Extraction

10. Add 50mL basified water (49 mL HPLC water + 1 mL saturated NaHCO₃) to Soil.

11. Extract by shaking on wrist action shaker for 1 hour.
12. Centrifuge at 2500 rpm for 15 minutes.
13. Carefully transfer the supernatant to an amber bottle with Teflon lined cap
14. Add 50 mL HPLC water to soil.
15. Repeat steps #11-13, combining extracts in bottle. Shake to mix well.
16. Aliquot about 15mL of final extract into centrifuge tubes, centrifuge at 1700 rpm for 10 minutes. (This step maybe skipped if extract is not muddy and able to be microfilterfuged)
17. Aliquot final extract to 16mL amber bottles for back up. Dispose remaining final extract.

Kasugamycin + Kasugamycinic Acid combined concentrate (for Kasugamycinic Acid analysis)

18. Aliquot 1.5mL of Kasugamycin unfiltered final extract from step # 7 or 9, and 1.0mL of Kasugamycinic Acid extract prepared from step #16 or #17 into 10mL pear-shape concentrating flask, add few drops of MeOH.
19. Roto-evaporate to half of the volume (1.25mL) at ~35 °C. Adjust to 1.25mL with HPLC water as needed, mix well.
20. Microfilterfuge KA + KM concentrated extract and aliquot to GC vials for KA LCMS analysis, store remaining final extract to GC vials for back up.

LC-MS/MS Analysis of Kasugamycin:

SCIEX API3000 Components (HPLC/Turbo Ion Spray Mode) or equivalent:

LC Pump	Agilent 1100 Series Binary Pump, Model G1312A
Autosampler	Agilent 1100 Series Autosampler, Model G1313A or G1329A
Vacuum Degasser	Agilent 1100 Series Vacuum Degasser, Model G1379A

Column: Spherex 3 μ ODS (C18) 150 x 3.20mm

Injection Volume: 20 μ L

Solvent System and Gradient Program:

Solvent A = Water (0.5% formic acid)

Solvent B = Acetonitrile (0.5% formic acid)

Solvent Program:	<u>Minutes</u>	<u>Flow Rate</u>	<u>Solvent A</u>	<u>Solvent B</u>
	0	600 µL/min.	0	100
	5.0	600 µL/min.	0	100
	10.0	600 µL/min.	100	0
	15.0	600 µL/min.	100	0
	16.0	600 µL/min.	0	100
	17.0	900 µL/min.	0	100
	22.0	600 µL/min.	0	100

Period 1 settings: Experiment 1:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	EP	CXP
380.40	200.30	200.00	17.00	3.50	15.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	EP	CXP
380.40	112.10	200.00	27.00	6.00	19.00

Representative Mass Spectrometer Settings

	Period 1
Scan Type:	API 3000
Polarity:	MRM
Ion Source:	Positive
NEB gas flow rate:	Turbo Spray
CUR gas:	13.0 (arb)
CAD gas:	11.0
Turbo IonSpray (L/min):	8.0
IS (V):	6.0
TEMP (°C):	5000.0
DP (V):	450.0
FP (V):	35.0
	160.0

Retention Time: Kasugamycin at ~ 9.7 minutes

LC-MS/MS Analysis of Kasugamycinic Acid:

SCIEX API3000 Components (HPLC/Turbo Ion Spray Mode) or equivalent:

LC Pump	Agilent 1100 Series Binary Pump, Model G1312A
Autosampler	Agilent 1100 Series Autosampler, Model G1313A or G1329A
Vacuum Degasser	Agilent 1100 Series Vacuum Degasser, Model G1379A

Column: Luna NH2 100A 250 x 4.60mm

Injection Volume: 50 µL

Solvent System and Gradient Program:

Solvent A = 96:2:2 (Acetonitrile:Water: 0.5mM pH 3 Ammonium formate buffer)

Solvent B = 2:96:2 (Acetonitrile:Water: 0.5mM pH 3 Ammonium formate buffer)

Flow Rate: 0.800 mL/minute

Solvent Program:	Minutes	Solvent A	Solvent B
	0	100	0
	19.0	25	75
	19.5	100	0
	26.0	100	0

Period 1 settings: Experiment 1:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE (Collision Energy)	CXP (Collision cell Exit Potential)
379.10	160.90	100	-27.00	-12.00
Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE (Collision Energy)	CXP (Collision cell Exit Potential)
379.10	179.00	100	-30.00	-13.00

Representative Mass Spectrometer Settings

	Period 1
	API 3000
Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray

NEB Gas Flow Rate:	10.0 (arb)
CUR gas:	13.0
CAD gas:	8.0
Turbo IonSpray (L/min):	6.0
IS (V):	-2500.0
TEMP (°C):	500.0
DP (V):	-35.0
FP (V):	-170.0
EP (V):	-14.0

Retention Time: Kasugamycinic Acid at ~ 20 minutes.

Separation of Kasugamycin and Kasugamycinic acid was achieved by high performance liquid chromatography. The analytes were identified by the coincidence of their retention times with that of the respective reference standards.

Methods of Calculation:

Preparation of Stock Standards

$$\text{Volume of solvent (mL)} = \frac{(W) \times (P)}{(FC)}$$

where W = Milligrams of neat standard
 P = Chemical purity of neat standard
 FC = Final Concentration (mg/mL)

The Kasugamycin and Kasugamycinic acid quantitation was conducted by peak area relative to external calibration curves. A calibrant peak area (y) relative to the concentration of the calibrant in ng/mL (x) yielded a linearity curve, where $y = mx + b$ was plotted.

The residue of Kasugamycin was calculated as follows:

$$\text{ppm KSM (ppm)} = \frac{\text{ng/mL KSM} \times \text{Dilution Factor} \times \text{Final vol. (mL)}}{\text{Sample Wt. (g)}} \times 0.001 \mu\text{g/ng}$$

$$\% \text{ Recovery} = \frac{\text{KSM Residue Detected (ppm)} - \text{KSM Residue in control}}{\text{KSM Fortification Level (ppm)}} \times 100$$

Similar calculations were conducted for Kasugamycinic acid, using the corresponding calibration curves.

To demonstrate validity of the analytical method for acceptable recovery (70-120%) of the KSM and KSMA from soil, method validation sets were conducted with replicates of fortified control soil samples at two different fortification levels (0.01 ppm and 0.1 ppm). Residues of both analytes in treated samples were calculated as shown above, with no control residues subtracted.

An example calculation for the KSM residue in soil (Fort 1 at 0.01 ppm in CA soil method validation) is shown below:

Linear regression analysis of the KSM standards gave a curve with the equation $x = (y - 4,093) \div 4,192$ ($r^2 = 0.9997$). The ng/mL KSM injected determined by this curve was:

$$\text{ng/mL KSM} = [(18,444 - 4,093) \div 4,192] = 0.342 \text{ ng/mL}$$

$$\text{KSM ppm} = \frac{3.42 \text{ ng/mL} \times 150 \text{ mL}}{50\text{g}} \times 0.001 = 0.0103 \text{ ppm}$$

$$\text{Percent KSM Recovery} = \frac{0.0103\text{ppm} - 0.0000 \text{ ppm}}{0.01 \text{ ppm}} \times 100 = 103\%$$