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**DETERMINATION OF KASUGAMYCIN IN  
TOMATOES, POTATOES AND PEPPERS**

Analytical Method# Meth-146, Revision #4

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## DETERMINATION OF KASUGAMYCIN IN TOMATOES, POTATOES AND PEPPERS

*Reason for Revision:* To speed up the filtration step following initial extraction, a larger Büchner funnel and faster filter paper are employed.

### 1.0 PRINCIPLE

The method described herein is capable of determining kasugamycin (KSM) in tomatoes, potatoes and peppers.

Kasugamycin residues are extracted from the targeted crops (properly macerated or ground) with aqueous methanol at a pH of 4, using multiple extraction. The extracts are filtered and combined. The combined extracts are concentrated, cooled, filtered through diatomaceous earth to remove precipitates that form on cooling, then diluted with deionized water. The diluted concentrate is further purified using two separate ion exchange chromatographic cleanups. The resulting purified extract is concentrated, then submitted to HPLC analysis.

During routine analysis, determination and quantitation of kasugamycin is conducted using high pressure liquid chromatography (HPLC) employing UV detection and ion-pairing chromatography. The limit of quantitation (LOQ) for all matrices 0.04 ppm.

### 2.0 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) may be substituted for those described in this method, except where specifically noted otherwise. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

### 3.0 APPARATUS AND EQUIPMENT

Assorted laboratory glassware

Balances:	Analytical balance capable of weighing to $\pm 0.1$ mg
	Top-loading balance capable of weighing to $\pm 0.1$ g
Beakers:	Glass, 2 liter
Bottles:	Glass, 4 liter
Chromatographic columns:	Glass, 300 mm $\times$ 11 mm i.d. with 250 mL solvent reservoirs and Teflon <sup>®</sup> stopcocks

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Erlenmeyer flasks:	Glass, glass-stoppered; 1000, 500 and 250 mL
Extraction jars:	Qorpak <sup>®</sup> , 4 oz., tall, with Teflon <sup>®</sup> -lined lids
Evaporation flasks:	Round or flat bottom, glass, 125 mL and 500 mL
Evaporator:	Rotary evaporator equipped with a Dewar condenser (Labconco Corp., Kansas City, MS)
Filter (vacuum) flasks:	Glass, 500 mL
Funnels:	Büchner type, 95 and 83 mm i.d.
Graduated cylinders:	Glass; 1000, 250, 100, 50 and 25 mL
Graduated mixing cylinders:	Glass; 500, 250, 100, 50 and 25 mL
Homogenizer:	Omni Mixer Model 17105 with Generator Probe (Omni International, Waterbury, CT)
HPLC/UV system:	Thermo Separation Products P4000 quaternary gradient elution high pressure liquid chromatograph equipped with a Thermo Separation Products UV 1000 UV/vis detector.
HPLC column:	25 cm × 4.6 mm Agilent Zorbax Rx-C18, 5 micron particle size (Mac-Mod Analytical, Chaddsford, PA)
HPLC sample filter:	Nylon 66 filters, 13 mm, 0.45 $\mu$ m (Varian Sample Preparation Products, Harbor City, CA)
Microliter syringes:	Various sizes, (Hamilton Co., Reno, NV)
Pasteur pipets:	Glass, 9 inch and 5 $\frac{3}{4}$ inch, disposable
pH meter:	Beckman, $\Phi$ 34 (Beckman Electrochemistry, Fullerton, CA)
pH paper:	ColorpHast: pH 0-6, pH 5-10 pHydriion: pH 9.0-12.0
Pipets:	Glass, graduated, serological, 5 mL
Storage jars:	Qorpak, 32 oz. and 64 oz. with Teflon <sup>®</sup> -lined lids

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Syringe: Glass, 2.5 mL, Hamilton Teflon<sup>®</sup> Luer-Lok (Hamilton Co., Reno, NV)

Test (culture) tubes: Glass, 13×100 mm

Ultrasonic bath: Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ)

Volumetric flasks: Glass; 100, 50, 25 and 10 mL

#### 4.0 REAGENTS AND MATERIALS

Acetone: OmniSolv<sup>®</sup> (EM Science, Gibbstown, NJ)

Acetonitrile: HPLC Grade (Burdick and Jackson, Muskegon, MI)

Amberlite CG-50: Ion exchange resin, type 1, 100-200 mesh (Acros Organics, Fisher Scientific, Fairlawn, NJ)

Ammonium hydroxide: Certified A.C.S. Plus, 29.2% NH<sub>3</sub>, (Fisher Scientific, Fairlawn, NJ)

Butyl alcohol: OmniSolv<sup>®</sup> (EM Science, Gibbstown, NJ)

Celite 545: Fisher Scientific, Fairlawn, NJ

1-Decanesulfonic acid (Na): Sigma Chemical, St. Louis, MO

Dowex<sup>®</sup> 50WX8 Ion exchange resin, 100-200 mesh, H form (Supelco, Bellefonte, PA)

Filter paper: Whatman #2, 9.0 cm (VWR Scientific, Bridgeport, NJ)  
Whatman #3, 7.0 cm (VWR Scientific, Bridgeport, NJ)

Glass wool VWR Scientific, Bridgeport, NJ

Hydrochloric acid: 36.5-38.0%, "Baker Analyzed<sup>®</sup>" (J.T. Baker Chemical Company, Phillipsburg, NJ)

Kasugamycin (KSM): As the hydrochloride salt, analytical grade

Methanol: OmniSolv<sup>®</sup> (EM Science, Gibbstown, NJ)

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Phosphoric acid:	O-phosphoric acid, 85% HPLC grade (Fisher Scientific, Fairlawn, NJ)
Sodium hydroxide:	"Baker Analyzed <sup>®</sup> " (J.T. Baker Chemical Company, Phillipsburg, NJ)
Water:	Deionized (DI) water (Polymetrics System, Morse Laboratories, Inc.)

HPLC Grade water (Fisher Scientific, Fairlawn, NJ)

- 4.1 Reagents and Materials to be Prepared (including typical preparation instructions)
- 4.1.1 Methanol:DI water (7:3, v/v) solution: To a 250 mL graduated mixing cylinder, add 75 mL of DI water. Bring to a final volume of 250 mL with methanol. Mix well.
- 4.1.2 Acetone:DI water (2:8, v/v): To a 250 mL graduated mixing cylinder, add 50 mL of acetone. Bring to a final volume of 250 mL with DI water. Mix well.
- 4.1.3 Hydrochloric acid (6N): To a 100 mL graduated mixing cylinder, add 48.5 mL of DI water. Slowly add 51.5 mL of concentrated HCl (36%). Stopper and carefully mix well.
- 4.1.4 Hydrochloric acid (2N): To a 4 liter bottle, add 3312 mL of DI water. Slowly add 688 mL of concentrated HCl (36%). Cap and carefully mix well.
- 4.1.5 Hydrochloric acid (1N): To a 100 mL graduated mixing cylinder, add 91.9 mL of DI water. Slowly add 8.1 mL of concentrated HCl (36%). Stopper and carefully mix well.
- 4.1.6 Hydrochloric acid (0.2N): To a 100 mL graduated mixing cylinder, add ~90 mL of DI water. Slowly add 1.6 mL of concentrated HCl (36%). Stopper and carefully mix well. Bring to a final volume of 100 mL with DI water. Stopper and carefully mix well.
- 4.1.7 Ammonia solution (2N): To a 4 liter bottle, add 1733 mL of DI water. Slowly add 267 mL of concentrated NH<sub>4</sub>OH solution (14.8N). Cap and carefully mix well.
- 4.1.8 Ammonia solution (1.0%, v/v): To a 250 mL graduated mixing cylinder, add ~200 mL of DI water. Add 8.6 mL of concentrated NH<sub>4</sub>OH solution (14.8N). Bring to a final volume of 250 mL with DI water. Stopper and carefully mix well.
- 4.1.9 Ammonia solution (0.5%, v/v): To a 500 mL graduated mixing cylinder, add ~400 mL of DI water. Add 8.6 mL of concentrated NH<sub>4</sub>OH solution (14.8N). Bring to a final volume of 500 mL with DI water. Stopper and carefully mix well.

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- 4.1.10 Sodium hydroxide (2N): To a 4 liter bottle, add 2000 mL of DI water. Slowly add 320 g of NaOH pellets. Add pellets in batches, swirling each addition to aid in dissolving. Once all pellets have been added and are in solution, add a final 2000 mL DI water. Cap and carefully mix well. Allow reagent to cool to room temperature prior to use.
- 4.1.11 Amberlite CG-50 [NH<sub>4</sub><sup>+</sup>:H<sup>+</sup> (7:3, v/v)]: To a 250 mL Erlenmeyer flask, add 70 mL of settled, conditioned CG-50 (NH<sub>4</sub><sup>+</sup>) resin and 30 mL of settled, conditioned CG-50 (H<sup>+</sup>) resin. Stopper and mix well prior to use.
- 4.1.12 HPLC mobile phases:
- (A) *Acetonitrile:water:sodium decane sulfonate (18.5:81.5:0.1, v/v/w), pH 3.5*: In a 1-liter mobile phase reservoir, weigh 1.0 g of 1-decanesulfonic acid (Na salt). Add 815 mL of HPLC grade water and 185 mL of HPLC grade acetonitrile. Stir, using a glass rod to help break up the clumps of salt, or sonicate the solution to dissolve the salt. Using a pH meter, adjust the pH of the solution to 3.5 using dilute H<sub>3</sub>PO<sub>4</sub> (approximately 1 to 10 dilution of HPLC grade o-phosphoric acid).
- (B) *Acetonitrile:water:sodium decane sulfonate (50:50:0.1, v/v/w), pH 3.5*: In a 1-liter mobile phase reservoir, weigh 1.0 g of 1-decanesulfonic acid (Na salt). Add 500 mL of HPLC grade water and 500 mL of HPLC grade acetonitrile. Stir, using a glass rod to help break up the clumps of salt, or sonicate the solution to dissolve the salt. Using a pH meter, adjust the pH of the solution to 3.5 using dilute H<sub>3</sub>PO<sub>4</sub> (approximately 1 to 10 dilution of HPLC grade o-phosphoric acid).
- 4.1.13 0.04% H<sub>3</sub>PO<sub>4</sub>: To a 10 mL volumetric flask, add ~ 5 mL of HPLC grade water. Add 1.0 mL of concentrated (85%) HPLC grade phosphoric acid. Bring to a final volume of 10 mL with HPLC grade water. Stopper and mix well. Remove 0.47 mL of the 1 to 10 dilution and transfer to a 100 mL volumetric flask containing ~50 mL HPLC grade water. Bring to a final volume of 100 mL with HPLC grade water. Stopper and mix well. Larger volumes using the same proportions may be prepared. Store refrigerated. Prepare weekly.

## 5.0 STANDARD PREPARATION

### 5.1 Stock Standard Solutions

28.5 mg of KSM-HCl (correcting for purity), equivalent to 25.0 mg of KSM as free base, is accurately weighed and quantitatively transferred to a 25 mL volumetric flask with HPLC grade water. The solution is brought to a final volume of 25 mL with HPLC grade water. The resulting concentration is 1000 µg/mL as KSM free base. This solution is stored at 1 to 8°C when not in use.

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## 5.2 Fortification Solutions (as free base)

Typically the following concentrations of fortification solutions are prepared. All solutions are stored at 1 to 8°C when not in use.

100 µg/mL: Transfer 2.5 mL of 1000 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in HPLC grade water. Mix well.

10 µg/mL: Transfer 2.5 mL of 100 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in HPLC grade water. Mix well.

1 µg/mL: Transfer 2.5 mL of 10 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume in HPLC grade water. Mix well.

## 5.3 HPLC (Calibration) Standard Solutions (as free base)

All standard solutions prepared in this section are to be stored at 1 to 8°C when not in use. Typically the following concentrations of HPLC standard solutions are prepared:

0.06 µg/mL: Transfer 60 µL of 100 µg/mL standard solution to a 100 mL volumetric flask. Bring to volume with HPLC grade water. Mix well.

0.1 µg/mL: Transfer 50 µL of 100 µg/mL standard solution to a 50 mL volumetric flask. Bring to volume with HPLC grade water. Mix well.

0.5 µg/mL: Transfer 250 µL of 100 µg/mL standard solution to a 50 mL volumetric flask. Bring to volume with HPLC grade water. Mix well.

1.0 µg/mL: Transfer 250 µL of 100 µg/mL standard solution to a 25 mL volumetric flask. Bring to volume with HPLC grade water. Mix well.

## 6.0 SAMPLE FORTIFICATION

**Note:** All samples must be kept in the frozen state until the addition of the extraction solvent.

1. Weigh 10.0 grams of frozen ground sample into a 4 oz. Qorpak jar. Cap and keep the sample frozen in a suitable storage container until fortified.
2. Fortify the sample with the appropriate amount of standard solution. Use a volume of fortification solution  $\leq 2.0$  mL.
3. Proceed with Step 7.0.1.

## 7.0 SAMPLE EXTRACTION

**Note:** All samples must be kept in the frozen state until the addition of the extraction solvent.

1. Weigh 10.0 grams of frozen ground sample into a 4 oz. Qorpak jar. Cap and keep the sample frozen in a suitable storage container. As applicable, fortify appropriate samples at this point. Add 50 mL of methanol:deionized water (7:3, v/v)
2. Adjust pH to 4 with 6N HCl using pH paper.
3. Place the extraction jar in an ice bath and blend the contents using a high speed homogenizer at high speed for 3 minutes.
4. Vacuum filter the extract through a Büchner funnel (95 mm i.d.), fitted with Whatman #2 filter paper, into a 500 mL side-arm flask placed in an ice bath. Return the filter cake to the Qorpak extraction jar.
5. Add 50 mL of methanol:deionized water (7:3, v/v) to the returned sample, adjust the pH to 4 with 1N HCl and reblend for 2 minutes in an ice bath at high speed.
6. Filter the sample through the same filter paper and into the same flask used in Step 4, thus combining both extracts. Wash the filter cake with 25 mL of methanol:deionized water (7:3, v/v) and combine with the extracts. Discard the filter cake.
7. Quantitatively transfer the filtrate to a 500 mL evaporation flask and add 5 mL n-butanol.
8. Concentrate the extract to approximately 30 mL on a rotary evaporator at  $\leq 40^{\circ}\text{C}$ .
9. Place the evaporation flask containing the concentrate in an ice bath for ~15 minutes. A precipitate will form.
10. Vacuum filter the resulting suspension through a Büchner funnel (83 mm i.d.), containing a ~7 g layer of celite 545 on Whatman #3 filter paper, into a 500 mL side-arm flask. Add 20 mL of DI water to the evaporation flask, sonicate to assist in rinsing, and pass the rinsate through the celite layer. Repeat this 20 mL DI water rinse of the flask and combine with the original filtrate.
11. Quantitatively transfer the filtrate to a 100 mL graduated mixing cylinder. Bring to a final volume of 80 mL with deionized water and invert to mix.

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12. Remove a 40 mL aliquot and proceed with ion exchange (I) column cleanup (Subsection 8.1).

## 8.0 ION EXCHANGE COLUMN CLEANUPS

### 8.1 Ion Exchange (I) Column Cleanup (Dowex 50WX8)

- Notes: 1) See Appendix II for appropriate preparation (conditioning) of this resin. Once the resin has been placed in the chromatographic column, do not let it go dry.
- 2) All elutions are conducted at a flow rate of ~2-3 mL/min.
1. Prepare a chromatographic column (300 mm × 11 mm i.d., with solvent reservoir) by placing a glass wool plug (~1 cm) at the bottom. Open stopcock fully.
  2. Add Dowex 50WX8 (100-200, H form) ion exchange resin to a 25 mL graduated cylinder until 5 mL (settled) of resin have been added. (Conditioned resin is stored as a slurry and thus facilitates this transfer.) Using a pasteur pipet, draw off excess water to a final volume of 10 mL.
  3. Transfer the resin and water, as a slurry, to the chromatographic column.
  4. Rinse the graduated cylinder with 10 mL of deionized water. Transfer the rinse and any remaining resin to the column. Place a plug of glasswool on top of the resin (**do not** compress resin). Stop elution when water has drained within ~0.5 cm of the top of the glasswool.
  5. Rinse the resin with 25 mL deionized water. Stop elution when water has drained to within ~0.5 cm of the top of the glasswool. Discard eluate.
  6. Pass the aqueous sample concentrate (40 mL) from Step 7.0.12 through the column. Stop elution when concentrate has drained to within ~0.5 cm of the top of the glasswool. Discard eluate.
  7. Wash the column with 15 mL of acetone:deionized water (2:8, v/v) followed by 15 mL of deionized water. Stop elution when last wash solvent has drained to within ~0.5 cm of the top of the glasswool. Discard eluate.
  8. Place a 125 mL evaporation flask under the column and elute kasugamycin from the column with 30 mL\* of 0.5% ammonia solution.

**\*Note:** The elution volume is matrix specific and must be determined for each. The elution volume must be determined for each batch of conditioned resin prepared as well. See Appendix IV for determination of elution volume.

9. Concentrate the eluate to approximately 5 mL on a rotary evaporator at  $\leq 40^{\circ}\text{C}$ .
10. Adjust the pH of the concentrate to 6-7 with 0.2N HCl using pH paper.
11. Quantitatively transfer the solution (using small DI water rinses with sonication) to a 25 mL graduated cylinder and bring to a final volume of 20 mL with deionized water. Proceed to ion exchange (II) column cleanup (Subsection 8.2)

#### 8.2 Ion Exchange (II) Column Cleanup (Amberlite CG-50)

- Notes:**
- 1) See Appendix III for appropriate preparation (conditioning) of this resin. Once the resin has been placed in the chromatographic column, do not let it go dry.
  - 2) All elutions are conducted at a flow rate of  $\sim 2\text{-}3$  mL/min.
1. Prepare a chromatographic column (300 mm  $\times$  11 mm i.d., with solvent reservoir) by placing a glass wool plug ( $\sim 1$  cm) at the bottom. Open stopcock fully.
  2. Add Amberlite CG-50 [ $\text{NH}_4^+:\text{H}^+$  (7:3, v/v)] mixed ion exchange resin to a 25 mL graduated cylinder until 5 mL (settled) of resin have been added. (Conditioned resin is stored as a slurry and thus facilitates this transfer.) Using a pasteur pipet, draw off excess water to a final volume of 10 mL.
  3. Transfer the resin and water, as a slurry, to the chromatographic column.
  4. Rinse the graduated cylinder with 10 mL of deionized water. Transfer the rinse and any remaining resin to the column. Place a plug of glasswool on top of the resin (**do not** compress resin). Stop elution when water has drained within  $\sim 0.5$  cm of the top of the glasswool.
  5. Rinse the resin with 30 mL deionized water. Stop elution when water has drained to within  $\sim 0.5$  cm of the top of the glasswool. Discard eluate.
  6. Pass the aqueous sample concentrate (20 mL) from Step 8.1.11 through the column. Stop elution when concentrate has drained to within  $\sim 0.5$  cm of the top of the glasswool. Discard eluate.

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7. Wash the column with 20 mL of acetone:deionized water (2:8, v/v). Stop elution when last wash solvent has drained to within ~0.5 cm of the top of the glasswool. Discard eluate.
8. Place a 125 mL evaporation flask under the column and elute kasugamycin from the column with 30 mL\* of 1.0% ammonia solution.

**\*Note:** The elution volume is matrix specific and must be determined for each. The elution volume must be determined for each batch of conditioned resin prepared as well. See Appendix V for determination of elution volume.

9. Concentrate the eluate to dryness on a rotary evaporator at  $\leq 40^{\circ}\text{C}$ .
10. Redissolve the residue in 2.0 mL of 0.04% phosphoric acid. Sonicate to help dissolve the residue. Check pH with narrow range pH paper. The resulting pH must be  $< 7$  to prevent retention time shifts during HPLC analysis.
11. Transfer the solution to a  $13 \times 100$  mm test tube. Mark the meniscus. Submit to HPLC analysis. The concentration of the HPLC-ready extract is  $1.0 \text{ mL} = 2.5 \text{ g}$ .

## 9.0 HIGH PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS

Note: The column and conditions stated in the method have been satisfactory for the matrices being analyzed. The specific column packing, mobile phase, column temperature and flow rate listed are typical conditions for this analysis. Alternate columns may be used depending on the need to resolve analyte and/or interfering responses. Specific conditions used will be noted on each chromatographic run and will not otherwise be documented.

### 9.1 Operating Conditions

Instrument: Thermo Separation Products P4000 quaternary gradient elution high pressure liquid chromatograph equipped with a Thermo Separation Products UV 1000 UV/vis detector.

HPLC Column: 25 cm  $\times$  4.6 mm Agilent Zorbax Rx-C18, 5 micron particle size

UV Detector  
Wavelength: 230 nm

Column  
Temperature: 35  $^{\circ}\text{C}$

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Flow Rate: 1.0 mL/min.

Mobile Phases: (A) Acetonitrile:water:sodium decane sulfonate (18.5:81.5:0.1, v/v/w), pH 3.5

(B) Acetonitrile:water:sodium decane sulfonate (50:50:0.1, v/v/w), pH 3.5

Gradient:

<u>Time (min)</u>	<u>Mobile phase A</u>	<u>Mobile phase B</u>
0-10.0	100%	0%
10.2-15.0	0%	100%
15.2-21.0	100%	0%

Injection Volume: 20  $\mu$ L

Retention Time: ~ 8.4 minutes

## 9.2 Sample Analysis

Prepare a four-point standard curve by injecting constant volumes of calibration standard solutions. Use constant volume injections for sample extracts as well. Sample responses not bracketed by the standard curve require dilution and reinjection. Inject a curve check standard every 4-5 sample injections.

## 10.0 CALCULATIONS

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. The regression functions are used to calculate a best fit line (from a set of standard concentrations in  $\mu$ g/mL versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line.

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y = peak response

x =  $\mu$ g/mL found for peak of interest

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m = slope

b = y-intercept

The calculations for ppm found and percent recovery (for fortified samples) are:

1. The amount of analyte (in ppm) found in the sample is calculated according to the following equation:

$$\text{ppm} = \mu\text{g/mL} \times \frac{\text{HPLC final vol. (mL)}}{\text{sample wt. (g)}} \times \frac{\text{mL ext. solv.}}{\text{mL aliq. 1}} \times \frac{\text{mL filtrate}}{\text{mL aliq. 2}} \times \text{HPLC dil. factor}$$

where:

$\mu\text{g/mL}$ found	=	$\mu\text{g/mL}$ of analyte found
sample wt. (g)	=	gram weight of sample extracted (typically 10.0 g)
mL ext. solv.	=	volume of extraction solvent (typically a combined 100 mL)
mL aliq. 1	=	volume of sample extract taken through procedure (typically 100 mL)
mL filtrate	=	volume of diluted filtrate following precipitation step (typically 80 mL)
mL aliq. 2	=	volume of diluted filtrate taken through remainder of procedure (ion exchange column cleanups, typically 40 mL).
HPLC final vol.	=	volume of final extract submitted to HPLC (typically 2.0 mL)
HPLC dilution factor	=	dilution of sample extract required to produce an analyte response bracketed by standards

2. The percent recovery for fortified control samples is calculated as follows:

$$\% \text{ Rec.} = \frac{\text{ppm found in fortified control (spike)} - \text{ppm found in control}}{\text{fortification level (ppm) added}} \times 100$$

## 11.0 REFERENCE

1. *Method for the Determination of Kasugamycin Residues in Tomatoes, Potatoes, and Sweet Peppers by High-Performance Liquid Chromatography*, Report No. TR167-2001-02, dated April 25, 2001, Hokko Chemical Industry Co., Ltd., Tokyo, Japan

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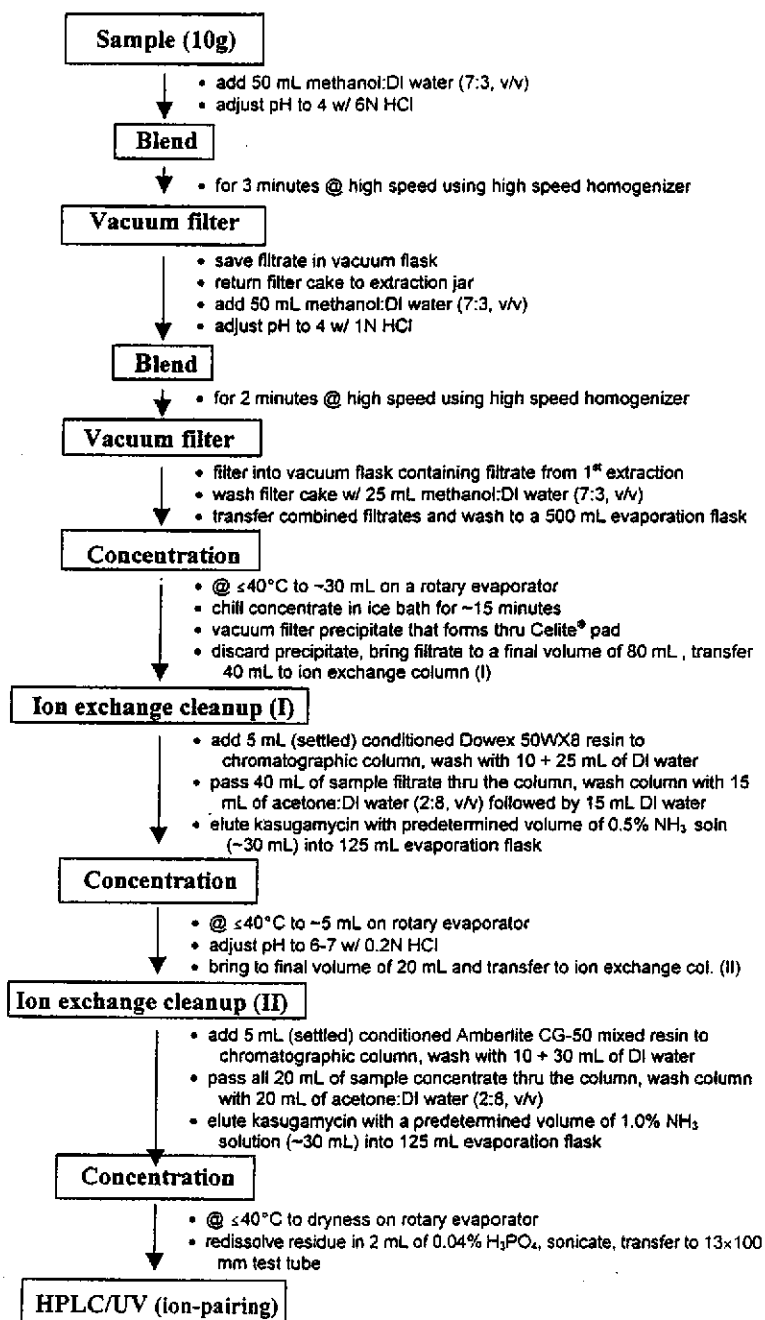
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**APPENDIX I**

Flowchart for Kasugamycin Crop Analysis

## FLOWCHART FOR THE DETERMINATION OF KASUGAMYCIN IN CROPS



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**APPENDIX II**

Conditioning (Preparation) of Ion Exchange Resin (I)

**Conditioning (Preparation) of Ion Exchange Resin (I): Dowex 50WX8 (NH<sub>4</sub><sup>+</sup>)**

1. Pour approximately 200 mL of dry Dowex 50WX8 resin into a 2 liter beaker. Add approximately 600 mL of 2N HCl. Stir with a glass rod. Allow resin to swell and settle (5-10 minutes), decant supernatant and discard.

**Note:** Allow ample time and mixing for the resin beads to swell completely. Saturated resin beads will swell to approximately **2 times** the starting volume, in this case the saturated volume will be ~400 mL.

2. Add 2N HCl to the resin slurry from Step 1 in a volume approximately equal to the volume of the slurry (referred to as "slurry volume"). Stir with a glass rod. Allow the resin to settle, decant the supernatant and discard. Repeat this step one additional time.
3. Wash the resin with DI water by adding a one slurry volume of DI water to the slurry. Mix and allow the slurry to settle. Decant supernatant and discard. Repeat this step until the pH of the supernatant is 4-5. Check pH with appropriate narrow range pH paper (pH 0-6). It will take approximately 5-10 volume exchanges to achieve the target pH.

**Note:** Rinse glass rod with DI water between pH checks, do not contaminate the supernatant with dye from the paper.

4. Wash the resin with one slurry volume of 2N NaOH **three** times. Each time, stir and allow the resin to settle. Decant and discard the supernatant between washes.
5. Wash the resin with DI water as in Step 3. Repeat the process until the pH of the supernatant is below 10. Check pH with appropriate narrow range pH paper (pH 5-10).
6. Wash the resin with one slurry volume of 2N HCl **three** times. Each time, stir and allow the resin to settle. Decant and discard the supernatant between washes.
7. Wash the resin with DI water as in Step 3. Repeat the process until the pH of the supernatant is 4-5. Check pH with appropriate narrow range pH paper (pH 0-6).
8. Wash the resin with one slurry volume of 2N ammonia solution **three** times. Each time, stir and allow the resin to settle. Decant and discard the supernatant between washes.
9. Wash the resin with DI water as in Step 3. Repeat the process until the pH of the supernatant is below 9.5. Check pH with appropriate narrow range pH paper (pH 5-10).
10. Transfer the conditioned resin to a 32 oz. Qorpak jar, rinse the beaker with small amounts of DI water to facilitate the transfer. Cap with a Teflon-lined lid and store refrigerated, under some water (the water used to assist in the transfer).

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**APPENDIX III**

Conditioning (Preparation) of Ion Exchange Resin (II)

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**Conditioning (Preparation) of Ion Exchange Resin (II): Amberlite CG-50 (H<sup>+</sup>) and Amberlite CG-50 (NH<sub>4</sub><sup>+</sup>)**

**Amberlite CG-50 (H<sup>+</sup>):**

1. Pour approximately 200 mL of dry Amberlite CG-50 resin into a 2 liter beaker. Add approximately 600 mL of 2N NaOH. Stir with a glass rod. Allow resin to swell and settle (5-10 minutes), decant supernatant and discard.

**Note:** Allow ample time and mixing for the resin beads to swell completely. Saturated resin beads will swell to approximately 2½ times the starting volume, in this case the saturated volume will be ~500 mL.

2. Add 2N NaOH to the resin slurry from Step 1 in a volume approximately equal to the volume of the slurry (referred to as "slurry volume"). Stir with a glass rod. Allow the resin to settle, decant the supernatant and discard. Repeat this step one additional time.
3. Wash the resin with DI water by adding a one slurry volume of DI water to the slurry. Mix and allow the slurry to settle. Decant supernatant and discard. Repeat this step until the pH of the supernatant is below 11. Check pH with appropriate narrow range pH paper (pH 9.0-12.0). It will take approximately 5-10 volume exchanges to achieve the target pH.  
**Note:** Rinse glass rod with DI water between pH checks, do not contaminate the supernatant with dye from the paper.
4. Wash the resin with one slurry volume of 2N HCl three times. Each time, stir and allow the resin to settle. Decant and discard the supernatant between washes.
5. Wash the resin with DI water as in Step 3. Repeat the process until the pH of the supernatant is above 2. Check pH with appropriate narrow range pH paper (pH 0-6).
6. Wash the resin with one slurry volume of 2N NaOH three times. Each time, stir and allow the resin to settle. Decant and discard the supernatant between washes.
7. Wash the resin with DI water as in Step 3. Repeat the process until the pH of the supernatant below 11. Check pH with appropriate narrow range pH paper (pH 9.0-12.0).
8. Repeat Steps 4 and 5.
9. Transfer the conditioned resin to a 64 oz. Qorpak jar, rinse the beaker with small amounts of DI water to facilitate the transfer. Cap with a Teflon-lined lid and store refrigerated, under some water (the water used to assist in the transfer).

Amberlite CG-50 (NH<sub>4</sub><sup>+</sup>):

1. Transfer approximately 350 mL of conditioned CG-50 (H<sup>+</sup>) resin into a 2 liter beaker.
2. Wash the resin with one slurry volume of 2N ammonia solution **three** times. Each time, stir and allow the resin to settle. Decant and discard the supernatant between washes.
3. Wash the resin with DI water by adding a one slurry volume of DI water to the slurry. Mix and allow the slurry to settle. Decant supernatant and discard. Repeat this step until the pH of the supernatant is below 11. Check pH with appropriate narrow range pH paper (pH 9.0-12.0). It will take approximately 5-10 volume exchanges to achieve the target pH.

**Note:** Rinse glass rod with DI water between pH checks, do not contaminate the supernatant with dye from the paper.

4. Transfer the conditioned resin to a 32 oz. Qorpak jar, rinse the beaker with small amounts of DI water to facilitate the transfer. Cap with a Teflon-lined lid and store refrigerated, under some water (the water used to assist in the transfer).

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**APPENDIX IV**

**Determination of Elution Volume for Ion Exchange Column (I)**

**Determination of Elution Volume for Ion Exchange Column (I)**

1. Prepare a control sample extract as per Section 7.0 of this method. Fortify the 40 mL aliquot resulting from Step 7.0.12 with kasugamycin (free base) at 10 ppm (add 500  $\mu$ L of a 100  $\mu$ g/mL standard solution).
2. Prepare the Dowex 50WX8 ion exchange column as per Subsection 8.1, Steps 1-5.
3. Pass the fortified aqueous sample extract (40 mL) through the column. Stop elution when sample has drained to within  $\sim$ 0.5 cm of the top of the glasswool. Discard eluate.
4. Wash the column with 15 mL of acetone:deionized water (2:8, v/v) followed by 15 mL of deionized water. Stop elution when last wash solvent has drained to within  $\sim$ 0.5 cm of the top of the glass wool. Discard eluate.
5. Elute kasugamycin from the column with 50 mL of 0.5% ammonia solution in 5 mL portions, collecting each 5 mL portion in a separate 125 mL evaporation flask. Check and record the pH of each 5 mL elution at the beginning and the end of the elution with narrow range (pH 5-10) pH paper. Kasugamycin elutes at pHs  $\geq$  8.
6. Evaporate each eluate to dryness on a rotary evaporator at  $\leq$ 40°C.
7. Redissolve the residue in 2.0 mL of 0.04% phosphoric acid. Sonicate to help dissolve the residue. Check pH with narrow range pH paper. The resulting pH must be  $<$ 7.
8. Transfer the solution to 13  $\times$  100 mm test tube and mark the meniscus. Submit to HPLC analysis. The concentration of the HPLC-ready extract is 1.0 mL = 2.5 g.

Results:

Kasugamycin generally elutes in fractions 1-4. Fractions 1-6 (two clean fractions following analyte elution) are collected and combined for analysis to ensure total analyte recovery and to compensate for any sample to sample co-extractive variation which may affect ion exchangeability.

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**APPENDIX V**

Determination of Elution Volume for Ion Exchange Column (II)

**Determination of Elution Volume for Ion Exchange Column (II)**

1. Prepare a control sample extract as per Section 7.0 and Subsection 8.1 of this method. Fortify the 20 mL aliquot resulting from Step 8.1.11 with kasugamycin (free base) at 10 ppm (add 500  $\mu$ L of a 100  $\mu$ g/mL standard solution).
2. Prepare the Amberlite CG-50 [ $\text{NH}_4^+:\text{H}^+$  (7:3, v/v)] ion exchange column as per Subsection 8.2, Steps 1-5.
3. Pass the fortified aqueous sample extract (20 mL) through the column. Stop elution when sample has drained to within  $\sim$ 0.5 cm of the top of the glasswool. Discard eluate.
4. Wash the column with 20 mL of acetone:deionized water (2:8, v/v). Stop elution when last wash solvent has drained to within  $\sim$ 0.5 cm of the top of the glass wool. Discard eluate.
5. Elute kasugamycin from the column with 50 mL of 1.0% ammonia solution in 5 mL portions, collecting each 5 mL portion in a separate 125 mL evaporation flask. Check and record the pH of each 5 mL elution at the beginning and the end of the elution with narrow range (pH 5-10) pH paper. Kasugamycin elutes at pHs  $\geq$  10.
6. Evaporate each eluate to dryness on a rotary evaporator at  $\leq$ 40°C.
7. Redissolve the residue in 2.0 mL of 0.04% phosphoric acid. Sonicate to help dissolve the residue. Check pH with narrow range pH paper. The resulting pH must be  $<$ 7.
8. Transfer the solution to 13  $\times$  100 mm test tube and mark the meniscus. Submit to HPLC analysis. The concentration of the HPLC-ready extract is 1.0 mL = 2.5 g.

Results:

Kasugamycin generally elutes in fractions 3 and 4. Fractions 1-6 (two clean fractions prior to analyte elution and two clean fractions following analyte elution) are collected and combined for analysis to ensure total analyte recovery and to compensate for any sample to sample co-extractive variation which may affect ion exchangeability.