Aminoglycosides in Milk Using Agilent Bond Elut Plexa SPE, Agilent Poroshell 120, and LC/Tandem MS

Application Note

Food Testing & Agriculture

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Abstract

A method for the simultaneous determination of aminoglycoside residues of spectinomycin, hygromycin B, streptomycin, dihydrostreptomycin, amikacin, kanamycin, apramycin, tobramycin, gentamicin, and neomycin in milk was developed and validated. The analytes were extracted and cleaned with Agilent Bond Elut Plexa solid phase extraction (SPE) and quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/Tandem MS) operating in the positive ion multiple-reaction-monitoring mode. The method provided ng/g level of limit of detection for all aminoglycoside residues in milk. The dynamic calibration ranges for these compounds were obtained from 10 to 500 ng/g. The overall recoveries ranged from 67 to 107%, with RSD values between 1.7 and 10.1%.

Introduction

Aminoglycosides (AGs) are a class of broad-spectrum antibiotics that have bacterial activity against some aerobic gram-positive and gram-negative organisms. AGs have been extensively employed in animal husbandry for the treatment of bacterial infections or growth promotion. Due to their toxicity, and possible antibiotic resistance, considerable attention has been paid to the potential human health risk. The European Union (EU), China, USA, Japan, and other countries have issued strict maximum residue levels (MRLs) for AGs in various animal-origin foods [1, 2].

The objective of this work was to develop a multiresidue method that would be simple and fast for routine regulatory analysis of aminoglycoside residues in milk. The method relies on a simple SPE step using a polymer sorbent (Bond Elut Plexa). Table 1 shows details of the aminoglycosides.

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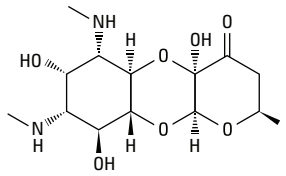
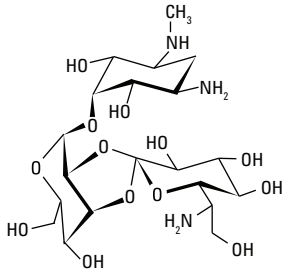
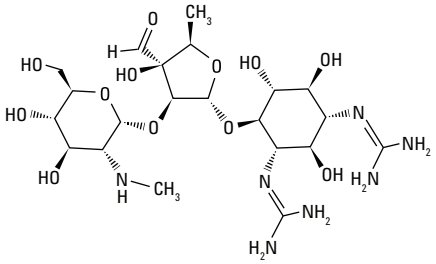
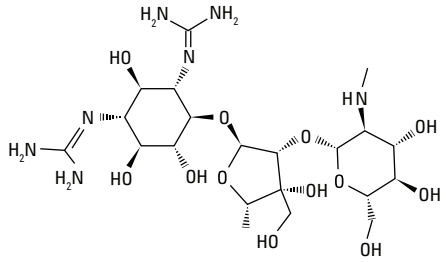
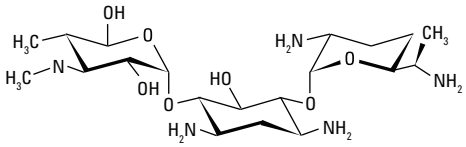
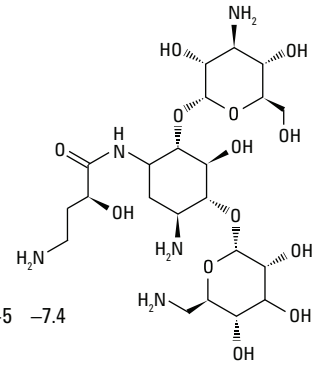
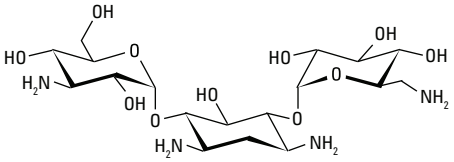
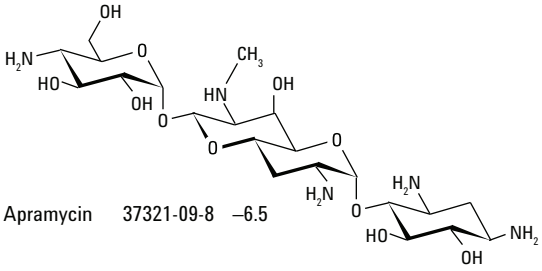
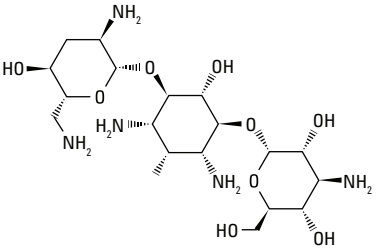
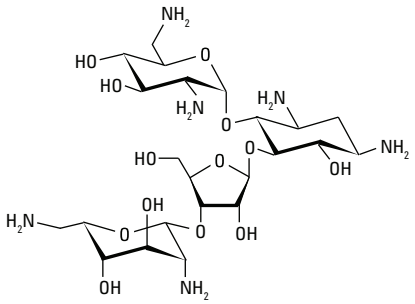
Table 1. Aminoglycoside compounds used in this study.

Compound	CAS no.	LogP
 Spectinomycin	1695-77-8	–2.3
 Hygromycin B	31282-04-9	NA
 Streptomycin	57-92-1	–6.4
 Dihydrostreptomycin	128-46-1	NA
 Gentamicin	1403-66-3	NA

Compound	CAS no.	LogP
 Amikacin	37517-28-5	–7.4
 Kanamycin	59-01-8	–6.3
 Apramycin	37321-09-8	–6.5
 Tobramycin	32986-56-4	–5.8
 Neomycin	1404-04-2	–7.8

Materials and Methods

Reagents and chemicals

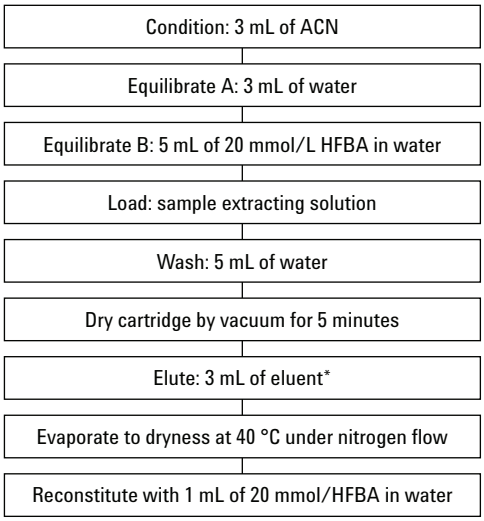
All reagents were MS, HPLC, or analytical grade. Acetonitrile and water were from Honeywell International, Inc. The standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Bovine milk was purchased from a local supermarket. Standard solutions (1.0 mg/mL) were made in water individually and stored in a freezer at 4 °C. A combined working solution (10 µg/mL) was made in acetonitrile:water (10:90) and also stored at 4 °C. The spiked solutions were then made weekly by appropriately diluting the combined working solution in water.

Sample preparation

Bovine milk (5 g) was weighed into a polypropylene centrifuge tube. Ten mL of extracting solution (5% trichloroacetic acid, 0.6 mmol/L Na_2EDTA, and 15 mmol/L KH_2PO_4) was added to the tube. The mixture was shaken thoroughly for 5 minutes and then centrifuged at 4,000 rpm for 5 minutes at 4 °C. The supernatant was transferred to another tube. The same extraction procedure was repeated with 5 mL of extracting solution, and the supernatant was combined into the same tube. A 5 mL volume of 0.2 mol/L heptafluorobutyric acid (HFBA) in water was added to the extracts. After vortex-mixing for 1 minute and centrifuging at 4,000 rpm for 5 minutes, the supernatant was adjusted to pH 4.0 ± 0.5 with 5 mol/L NaOH in water. The sample extracting solution was then ready for the SPE procedure.

Solid phase extraction

Figure 1 shows the SPE procedure. Bond Elut Plexa cartridges were preconditioned with 3 mL of acetonitrile (ACN) and then equilibrated with 3 mL of water and 5 mL of 20 mmol/L HFBA in water. The sample extracting solution was then loaded onto a cartridge and passed through under gravity (approximately 1 mL/min). The cartridges were washed with 5 mL of water. A full vacuum was applied to the cartridge for 5 minutes to completely dry the resin. The compounds were eluted with 3 mL ACN:0.2 mol/L HFBA in water at a rate of 1 mL/min. The eluent was dried under nitrogen at 40 °C. The residue was reconstituted in 1 mL of 20 mmol/L HFBA in water. The sample was then vortex mixed and ultrasonicated to completely dissolve the residue and filtered through a 0.22 µm membrane. Then the sample was finally transferred to a 2 mL chromatography vial for analysis.



*Eluent: ACN:0.2 mol/L HFBA in water (8:2).

Figure 1. Milk clean-up and enrichment – Agilent Bond Elut Plexa SPE procedure.

Conditions

Column:	Agilent Poroshell 120 SB-C18, 2.1 × 100 mm, 2.7 µm (p/n 685775-902)		
Sample prep:	Agilent Bond Elut Plexa cartridges, 500 mg, 6 mL (p/n 12259506)		
Mobile phase:	A: Water:acetonitrile (950:50, 20 mmol/L HFBA), B: acetonitrile:water (800:200, 20 mmol/L HFBA)		
Injection volume:	20 µL		
Flow rate:	0.3 mL/min		
Gradient:	Time (min)	%A	%B
	0	85	15
	3	85	15
	9.5	25	75
	9.55	85	15
	10	85	15
Temperature:	Ambient		
Manifold:	Agilent Vac Elut 20 Manifold (p/n 12234101)		
Instrument:	Agilent 1200 Infinity Series Agilent 6460 Triple Quadrupole LC/MS/MS system		

MS conditions

The AGs were monitored in positive mode. Table 2 shows the multiple-reaction-monitoring details.

MS source parameters

Gas temperature:	350 °C
Gas flow:	5 L/min
Nebulizer:	45 psi
Sheath gas temperature:	400 °C
Sheath gas flow:	11 L/min
Nozzle voltage:	Positive, 0 V
Capillary:	Positive, 3,500 V

Results and Discussion

Linearity and limit of detection

Solutions used to create external calibration curves were prepared by using a combined working solution to spike matrix blanks (0.01, 0.02, 0.05, 0.1, and 0.5 mg/kg). Matrix blanks were created by taking milk through the entire procedure, including pretreatment and SPE procedures. The limits of detection (LODs) were chosen as the concentration of each compound that gave a signal-to-noise (S/N) ratio greater than 3:1. The results for the calibration curves and LODs are shown in Table 3.

Table 2. Masses monitored by multiple-reaction monitoring.

Compound	Precursor ion	Product ion	Fragmentor (V)	Collision energy (V)
Spectinomycin	351.2	333.2	170	15
		207.1	170	18
Hygromycin B	528.3	177.1	170	25
		352	170	20
Streptomycin	582.4	263.2	180	30
		245.8	180	35
Dihydrostreptomycin	584.4	263.3	180	30
		246.2	180	40
Amikacin	586.4	163.1	170	30
		425.2	170	15
Kanamycin	485.3	163.1	150	20
		324.2	150	10
Apramycin	540.3	217.1	140	25
		378.2	140	12
Tobramycin	468.3	163.2	125	20
		324.2	125	8
Gentamicin	478.3	322.3	125	8
		157.2	125	15
Neomycin	615.3	161.1	175	30
		293.1	175	20

Table 3. Linearity and LODs of aminoglycosides in milk.

Compound	Regression equation	R^2	LOD in milk (ng/g)
Spectinomycin	Y = 13790.2955x + 39429.2957	0.996	0.1
Hygromycin B	Y = 891.7225x – 1190.5976	0.998	0.2
Streptomycin	Y = 1197.4506x – 2934.9645	0.994	2
Dihydrostreptomycin	Y = 2240.1902x – 1236.0908	0.998	0.2
Amikacin	Y = 1393.7561x – 1742.7928	0.999	0.2
Kanamycin	Y = 1107.6982x – 1720.5534	0.998	0.2
Apramycin	Y = 288.9123x – 207.5481	0.999	0.5
Tobramycin	Y = 804.6063x – 1295.9858	0.999	0.2
Gentamicin	Y = 1494.7223x – 894.2355	0.999	0.2
Neomycin	Y = 183.7889x – 240.7272	0.994	0.5

Recovery and reproducibility

The recovery and repeatability for the method were determined at three levels; milk spiked to concentrations of 0.01, 0.02, and 0.05 mg/kg. The analysis was performed with six replicates at each level. Table 4 shows the recovery and reproducibility data. Figure 2 shows the chromatograms of spiked bovine milk extracts (0.02 mg/kg).

Table 4. Recoveries and reproducibility of aminoglycosides in milk.

Compound	Spiked level (mg/kg)	Recovery (%)	RSD (n = 6, %)
Spectinomycin	0.01	78.7	3.8
	0.02	82.5	5.6
	0.1	87.3	4.1
Hydromycin B	0.01	73.1	8.7
	0.02	69.7	6.3
	0.1	77.3	5.9
Streptomycin	0.01	78.1	7.7
	0.02	66.5	10.1
	0.1	71.8	7.1
Dihydrostreptomycin	0.01	84.2	2.1
	0.02	88.2	3.1
	0.1	91.5	5.4
Amikacin	0.01	102.3	2.4
	0.02	97.2	2.7
	0.1	99.4	3.6
Kanamycin	0.01	98.7	4.5
	0.02	92.1	3.9
	0.1	93.6	6.8
Apramycin	0.01	97.1	4.8
	0.02	101.9	6.6
	0.1	89.6	7.1
Tobramycin	0.01	92.5	2.9
	0.02	98.5	4.9
	0.1	94.8	1.7
Gentamicin	0.01	107.3	3.9
	0.02	101.4	3.1
	0.1	105.8	4.5
Neomycin	0.01	88.2	6.7
	0.02	97.4	7.2
	0.1	87.6	5.4

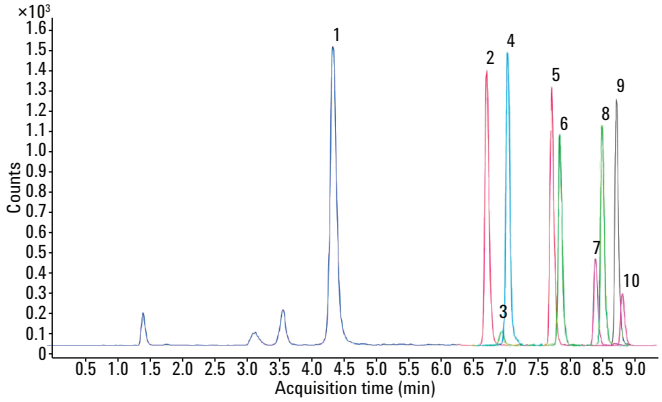


Figure 2. Chromatogram of 0.02 mg/kg spiked milk sample extract. 1. spectinomycin, 2. hygromycin B, 3. streptomycin, 4. dihydrostreptomycin, 5. amikacin, 6. kanamycin, 7. apramycin, 8. tobramycin, 9. gentamicin, and 10. neomycin.

Conclusions

LC/MS/MS is a reliable and powerful technique for the simultaneous quantification and confirmation of aminoglycosides in milk. The results of this application note show that Agilent Bond Elut Plexa can be used as an effective method for purification and enrichment of multiple aminoglycosides in a complex matrix such as milk. The recovery and reproducibility results based on matrix spiked standards are acceptable for aminoglycoside residue determination in milk under international regulations. The impurities and matrix effects are minimal and do not interfere with the quantification of any target compound. The limits of quantitation are significantly lower than the MRLs [3].

References

1. W. Zhu, J. Yang, W. Wei, Y. L, S. Zhang. *J. Chromatography A*, 1207, 29 (2008).

2. A. Kaufmann, P. Butcher, K. Maden. *Anal. Chim. Acta,* 711, 46 (2012).

3. Anon. GB/T 21323-2007 Determination of aminoglycosides residues in animal tissues - HPLC-MS/MS method. China Standard. General Administration of Quality Supervision, Inspection and Quarantine, Beijing, China.

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