

Quantitation of the Aminoglycosides Group of Veterinary Drugs in Milk Samples

A workflow for clean, reliable aminoglycoside quantitation without the use of ion-pairing reagents



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Abstract

Veterinary drugs play a crucial role in animal husbandry, supporting disease treatment, growth promotion, and improved feed efficiency in livestock production. Among these, aminoglycosides are widely used antibiotics for the control of bacterial infections. However, their use in food-producing animals necessitates sensitive, reliable analytical methods to monitor residues in products such as milk and ensure food safety. In this study, a sensitive, robust LC/MS/MS method was developed for the quantitation of aminoglycosides in milk using an Agilent 6475 triple quadrupole LC/MS system. The method employs a straightforward sample preparation workflow involving protein precipitation and extraction with an acetonitrile/water (1:1, v/v) mixture in the presence of an edetate solution. Quantitation was achieved using pre-extracted spiked calibration points to account for matrix effects. Additionally, multiple reaction monitoring (MRM) ion ratios remained within $\pm 30\%$ across all calibration levels, confirming method reliability. Importantly, the approach uses a heptafluorobutyric acid (HFBA)-free mobile phase, minimizing ion suppression and reducing system contamination, thereby improving instrument uptime and suitability for routine analysis.

Introduction

Veterinary medicines are widely used in livestock management to treat diseases, promote growth, improve efficiency, and mitigate stress during transportation. However, their application in food-producing animals can lead to residual traces in animal-derived products such as meat, milk, and eggs, posing potential risks, including toxicity, allergic reactions, and the development of antimicrobial resistance. Consequently, reliable monitoring of veterinary drug residues is essential to ensure food safety and regulatory compliance.

Aminoglycosides represent an important class of antibiotics commonly administered to control bacterial infections in livestock. Due to their persistence and potential health implications, accurate and sensitive analytical methods are required for their quantification. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is a preferred technique for this purpose. However, conventional methods often rely on ion-pairing reagents such as heptafluorobutyric acid (HFBA) to enhance chromatographic retention and separation of these highly polar compounds.¹ Despite their effectiveness, these reagents introduce significant drawbacks, including ion suppression, contamination of the mass spectrometer, and extended system equilibration times, ultimately affecting sensitivity, reproducibility, and instrument uptime.

In this study, a robust, MS-friendly LC/MS/MS method is presented that eliminates the need for HFBA, an ion-pairing reagent. Ion-pairing reagents can contaminate LC columns and systems and are generally not suitable for LC/MS. Instead, a mobile phase comprising common modifiers, along with reversed-phase liquid chromatography (RPLC) chemistries for polar compounds, such as the Agilent InfinityLab Poroshell SB-AQ column, is employed to achieve effective retention, improved sensitivity, and reduced contamination. In reversed-phase mode, alternative stationary phase chemistries with increased polarity are available, enabling stronger interactions with polar analytes. Many of these phases are compatible with very weak mobile phases, enhancing the retention of polar compounds. The method provides a cleaner and more efficient analytical workflow while maintaining excellent chromatographic performance.

The approach is demonstrated through the analysis of a panel of commonly used veterinary antibiotics, including apramycin, erythromycin, kanamycin, spectinomycin, lincomycin, neomycin, spiramycin, tylosin, and virginiamycin M1, highlighting its suitability for routine aminoglycoside residue monitoring in milk matrix. Structures of aminoglycosides involved in the study are shown in Figure 1.

Experimental

Materials

Calibration standards were prepared using analytical standard materials provided by Analytical Technology Laboratory, Coimbatore, Tamilnadu, India.

The following other chemicals and reagents were used: ammonium formate, ammonium fluoride, and formic acid (MS-grade, Fluka, Honeywell, Muskegon, Michigan, USA), edetate salt (AR-grade, MerckDarmstadt, Darmstadt, Germany), acetonitrile (MS-grade, Biosolve, Dieuze, France), and water (MS-grade, Honeywell, Muskegon, Michigan, USA).

Equipment

Analysis was performed on an Agilent 1290 Infinity II LC system coupled with an Agilent 6475 triple quadrupole LC/MS. The LC system was equipped with the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117A)

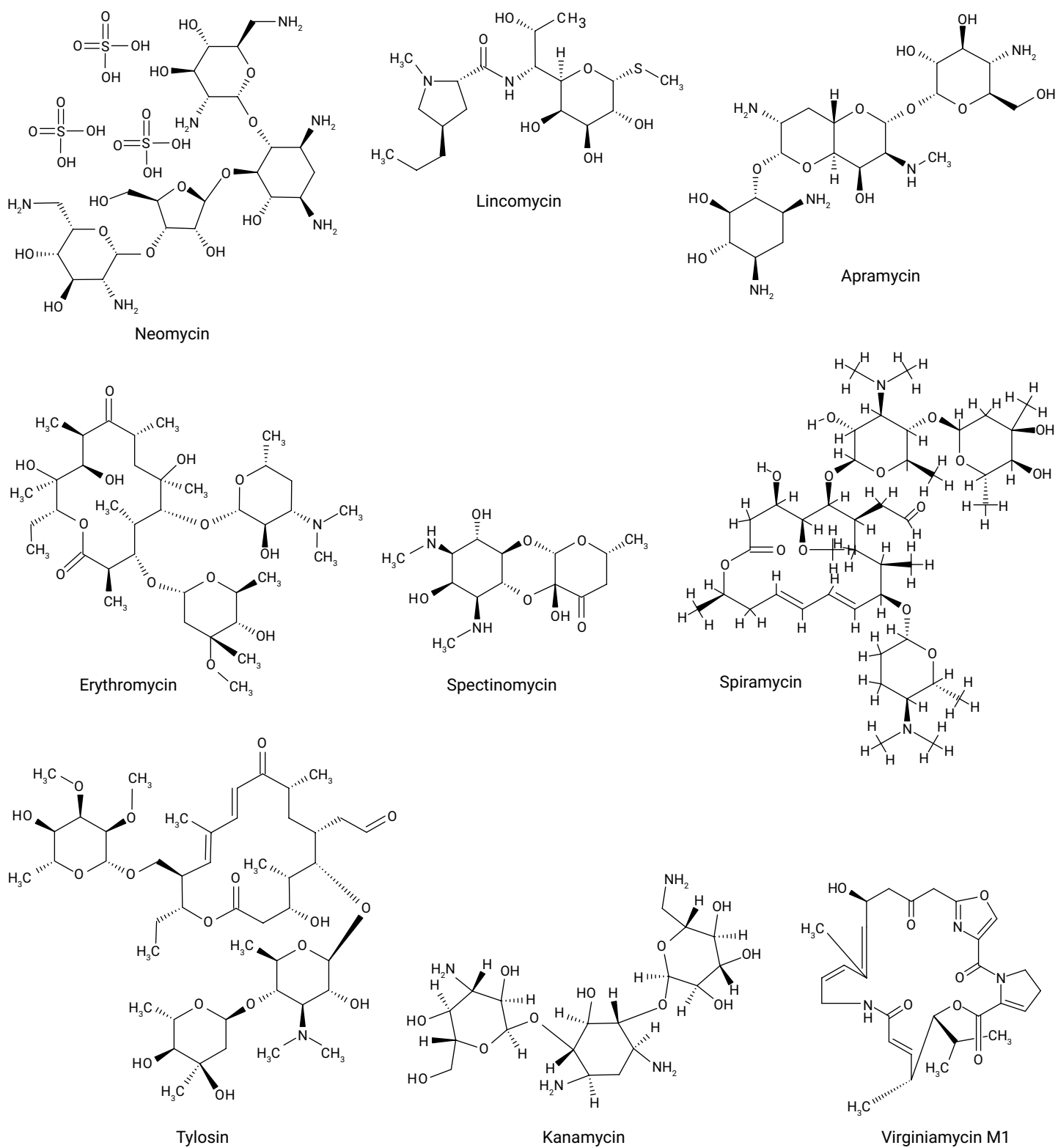


Figure 1. Structures of aminoglycosides involved in this study.

Method

The conditions used for the LC/MS/MS method are summarized in Tables 1, 2, and 3.

A selective LC/MS/MS method employing multiple reaction monitoring (MRM) was established on a triple quadrupole system. Ionization was achieved using electrospray ionization in positive polarity.

Preparation of standards and samples

Preparation of standards: The individual standard stock solution (100 µg/mL) was prepared by dissolving 5 mg of the analytical standard in diluent and bringing the volume to 50 mL in a volumetric flask. A 0.1 mL aliquot of the stock solution was diluted to 10 mL to prepare a 1 ppm working standard solution. Seven linearity levels were prepared by serial dilution of this working standard solution.

Matrix-based calibration samples were prepared by spiking 1 mL milk samples with working standard solution. For apramycin, kanamycin, and spectinomycin, the spiking procedure for the calibration levels is shown in Table 4. For all other analytes, the spiking procedure is shown in Table 5.

Table 3. MS Source parameters.

Ionization Source	Agilent Jet Stream Technology Ion Source (AJS) (p/n G1958B)	
	Parameter ID	Positive Value
1	Gas temperature	340 °C
2	Gas flow	10 L/min
3	Nebulizer	45 psi
4	Capillary voltage	3,700 V
5	Sheath gas temperature	390 °C
6	Sheath gas flow	11 L/min
7	Nozzle voltage	100 V

Table 1. Chromatography conditions.

Parameter	Value
Column	Agilent InfinityLab Poroshell SB-Aq, 4.6 × 150 mm, 2.7 µm (p/n 683975-914)
Mobile Phase A	4.5 mM ammonium formate, 0.5 mM ammonium fluoride, and 0.1% formic acid in water
Mobile Phase B	Acetonitrile with 0.1% formic acid
Flow Rate	0.5 mL/min
Injection Volume	20 µL
Sample Cooler Temperature	8 °C
Column Temperature	30 °C
Needle Wash	Acetonitrile/water (50:50, v/v)
Diluent	Acetonitrile/water (50:50, v/v)
Elution	Gradient
Acquisition Time	12.0 minutes
Gradient Program	Time (min) %A %B
	1 Start cond. 90 10
	2 1 90 10
	3 5 40 60
	4 10 10 90
	5 12 10 90
Post-time: 2.0 minutes	

Table 2. MRM parameters.

Compound Name	Precursor (m/z)	Product (m/z)	Fragmentor (V)	Cell Accelerator Voltage (CAV)(V)	Collision Energy (CE)(V)	Polarity
Apramycin	540.2	217.1	131	4	26	+
Apramycin	540.2	199	131	4	26	+
Apramycin	540.2	67.9	131	4	80	+
Erythromycin	735.5	577.4	171	5	19	+
Erythromycin	735.5	158.1	171	5	32	+
Erythromycin	734.5	576.3	171	5	16	+
Kanamycin	485.3	324.2	117	5	14	+
Kanamycin	485.3	163.1	117	5	24	+
Kanamycin	485.3	102	117	5	48	+
Lincomycin	407.3	359.2	130	5	20	+
Lincomycin	407.3	126.1	130	5	30	+
Lincomycin	407.3	42	130	5	80	+
Neomycin	615.3	293.1	157	5	24	+
Neomycin	615.3	203.1	157	5	34	+
Neomycin	615.3	163	157	5	38	+
Spectinomycin	351.2	333.2	145	5	18	+
Spectinomycin	351.2	207.1	145	5	24	+
Spectinomycin	351.2	100	145	5	28	+
Spiramycin	843.6	174.1	140	4	40	+
Spiramycin	843.6	101	140	4	45	+
Spiramycin	843.6	43	140	4	80	+
Tylosin	916.5	174.1	228	5	44	+
Tylosin	916.5	101	228	5	56	+
Tylosin	916.5	83	228	5	60	+
Virginiamycin M1	526.3	508.2	110	5	12	+
Virginiamycin M1	526.3	133.1	110	5	32	+
Virginiamycin M1	526.3	109.1	110	5	36	+

Sample preparation: Sample preparation was performed as follows:

1. Mix spiked samples with 0.1 mL of 0.1 M edetate sodium solution (EDTA sodium salt).
2. Allow the mixture to stand for 10 minutes.
3. Extract analytes using 1 mL of 5% trichloroacetic acid in a 50:50 acetonitrile/water solution.
4. Vortex the samples for 1 minute to precipitate proteins.
5. Centrifuge the contents at 9,000 rpm for 10 minutes.
6. Filter 1 mL of the supernatant through an Agilent ValueLab nylon filter (p/n 5191-4286).
7. Transfer the filtered samples to HPLC vials for analysis.

Data acquisition and data analysis

All samples were acquired using Agilent MassHunter data acquisition software (version 12.0). Chromatograms were viewed using Agilent MassHunter Qualitative Analysis software (version 12.0). Quantitation of each batch was carried out using MassHunter Quantitative Analysis software (version 12.1). QC parameters, including linearity, reproducibility, recovery, specificity, and sensitivity in terms of limit of quantification (LOQ), were evaluated to ensure acceptable method performance.

Results and discussion

Figure 2 shows a zoomed view of representative extracted ion chromatograms (EICs) for veterinary drugs in a pre-spiked sample at 100 ng/mL.

Table 4. Spiking levels for the preparation of matrix-based calibration and quality control samples for apramycin, kanamycin, and spectinomycin.

Conc. of Working Standard Solution (ng/mL)	Volume Spiked (μL)	Sample Volume (mL)	Concentration Spiked (ng/mL)
1,000	25	1.0	25
1,000	50	1.0	50
1,000	100	1.0	100
1,000	150	1.0	150
1,000	200	1.0	200
1,000	250	1.0	250
1,000	300	1.0	300

Table 5. Spiking levels for the preparation of matrix-based calibration and quality control samples for all other analytes.

Conc. of Working Standard Solution (ng/mL)	Volume Spiked (μL)	Sample Volume (mL)	Concentration Spiked (ng/mL)
100	25	1.0	2.5
100	50	1.0	5.0
1,000	10	1.0	10.0
1,000	15	1.0	15.0
1,000	25	1.0	25.0
1,000	50	1.0	50.0
1,000	100	1.0	100.0

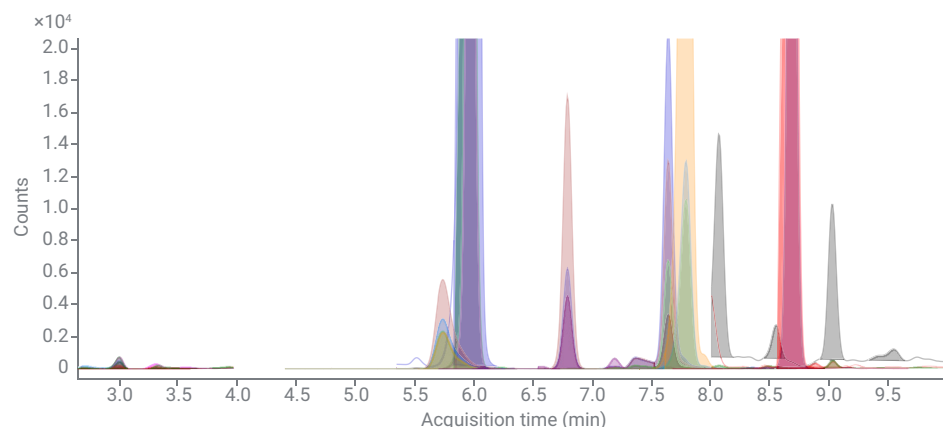


Figure 2. Extracted ion chromatograms (EICs) of veterinary drugs at 100 ng/mL.

Including EDTA in the sample extraction significantly improves extraction recoveries by chelating divalent metal ions, particularly calcium, which are known to form complexes with aminoglycoside antibiotics. This chelation reduces analyte–metal interactions, resulting in more efficient

extraction and improved method robustness. The effect is especially notable for compounds with a strong affinity for metal ions.

Calibration curves were established to ensure quantitative accuracy. For six analytes, linearity was achieved across concentrations ranging from

2.5 to 100 ng/mL (Figures 3 and 4). However, kanamycin, spectinomycin, and apramycin required higher ranges, with calibration curves spanning 25 to 300 ng/mL and constructed using linear regression and 1/x weighting (Figures 5 and 6). Accuracies for calibration points were within $\pm 20\%$.

Sample			Erythrom...	Erythromycin Results			Qu...
Name	Type	Level	Exp. Conc.	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Mat...			7.642	0.5469		
MB 2.5 ppb	Cal	1	2.5000	7.642	2.8274	113.1	32.7
MB 5.0 ppb	Cal	2	5.0000	7.642	5.1800	103.6	36.2
MB 10.0 ppb	Cal	3	10.0000	7.633	8.9394	89.4	33.5
MB 15.0 ppb	Cal	4	15.0000	7.642	12.6420	84.3	35.2
MB 25.0 ppb	Cal	5	25.0000	7.642	27.1040	108.4	32.3
MB 50.0 ppb	Cal	6	50.0000	7.642	50.4061	100.8	31.7
MB 100.0 ppb	Cal	7	100.0000	7.642	100.4012	100.4	31.8

Sample			LINCOM...	LINCOMYCIN Results			Qu...
Name	Type	Level	Exp. Conc.	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Mat...			5.823	0.3866		0.1
MB 2.5 ppb	Cal	1	2.5000	5.973	2.5673	102.7	4.4
MB 5.0 ppb	Cal	2	5.0000	5.965	4.8294	96.6	4.5
MB 10.0 ppb	Cal	3	10.0000	5.965	9.4872	94.9	4.5
MB 15.0 ppb	Cal	4	15.0000	5.965	15.7075	104.7	4.7
MB 25.0 ppb	Cal	5	25.0000	5.965	23.3341	93.3	4.9
MB 50.0 ppb	Cal	6	50.0000	5.973	49.6012	99.2	5.0
MB 100.0 ppb	Cal	7	100.0000	5.973	108.5915	108.6	5.1

Sample			NEOMYC...	NEOMYCIN Results			Qu...
Name	Type	Level	Exp. Conc.	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Mat...			5.726	0.2441		51.9
MB 2.5 ppb	Cal	1	2.5000	5.743	2.8645	114.6	42.5
MB 5.0 ppb	Cal	2	5.0000	5.735	5.1560	103.1	42.6
MB 10.0 ppb	Cal	3	10.0000	5.760	10.0754	100.8	42.7
MB 15.0 ppb	Cal	4	15.0000	5.793	14.6885	97.9	42.8
MB 25.0 ppb	Cal	5	25.0000	5.743	20.6175	82.5	39.8
MB 50.0 ppb	Cal	6	50.0000	5.735	47.0542	94.1	42.1
MB 100.0 ppb	Cal	7	100.0000	5.735	107.0439	107.0	40.9

Sample			SPIRAM...	SPIRAMYCIN Results			Qu...
Name	Type	Level	Exp. Conc.	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Mat...			6.823	0.4500		815...
MB 2.5 ppb	Cal	1	2.5000	6.789	2.7897	111.6	34.0
MB 5.0 ppb	Cal	2	5.0000	6.789	5.3049	106.1	36.1
MB 10.0 ppb	Cal	3	10.0000	6.789	8.9835	89.8	36.0
MB 15.0 ppb	Cal	4	15.0000	6.789	12.2158	81.4	35.0
MB 25.0 ppb	Cal	5	25.0000	6.789	26.6563	106.6	31.6
MB 50.0 ppb	Cal	6	50.0000	6.789	52.8668	105.7	28.5
MB 100.0 ppb	Cal	7	100.0000	6.789	98.6831	98.7	26.9

Sample			Tylosin M...	Tylosin Results			Qu...
Name	Type	Level	Exp. Conc.	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Mat...			7.743	0.7787		216...
MB 2.5 ppb	Cal	1	2.5000	7.793	2.9381	117.5	24.3
MB 5.0 ppb	Cal	2	5.0000	7.785	5.1089	102.2	22.8
MB 10.0 ppb	Cal	3	10.0000	7.785	9.1006	91.0	21.7
MB 15.0 ppb	Cal	4	15.0000	7.785	13.3284	88.9	20.1
MB 25.0 ppb	Cal	5	25.0000	7.785	24.3297	97.3	19.9
MB 50.0 ppb	Cal	6	50.0000	7.793	50.4208	100.8	19.2
MB 100.0 ppb	Cal	7	100.0000	7.793	102.2734	102.3	19.4

Sample			VIRGINIA...	VIRGINIAMYCIN M1 Results			Qu...
Name	Type	Level	Exp. Conc.	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Mat...			8.717	0.7385		100...
MB 2.5 ppb	Cal	1	2.5000	8.683	2.6425	105.7	60.0
MB 5.0 ppb	Cal	2	5.0000	8.683	4.7996	96.0	57.1
MB 10.0 ppb	Cal	3	10.0000	8.683	9.2745	92.7	58.0
MB 15.0 ppb	Cal	4	15.0000	8.683	12.8838	85.9	62.5
MB 25.0 ppb	Cal	5	25.0000	8.683	23.9825	95.9	56.2
MB 50.0 ppb	Cal	6	50.0000	8.683	55.6366	111.3	51.1
MB 100.0 ppb	Cal	7	100.0000	8.683	112.4670	112.5	51.2

Figure 3. Snapshots of quantitation tables for erythromycin, lincomycin, neomycin, spiramycin, tylosin, and virginiamycin M1 across the concentration range of 2.5 to 100 ng/mL in a milk matrix.

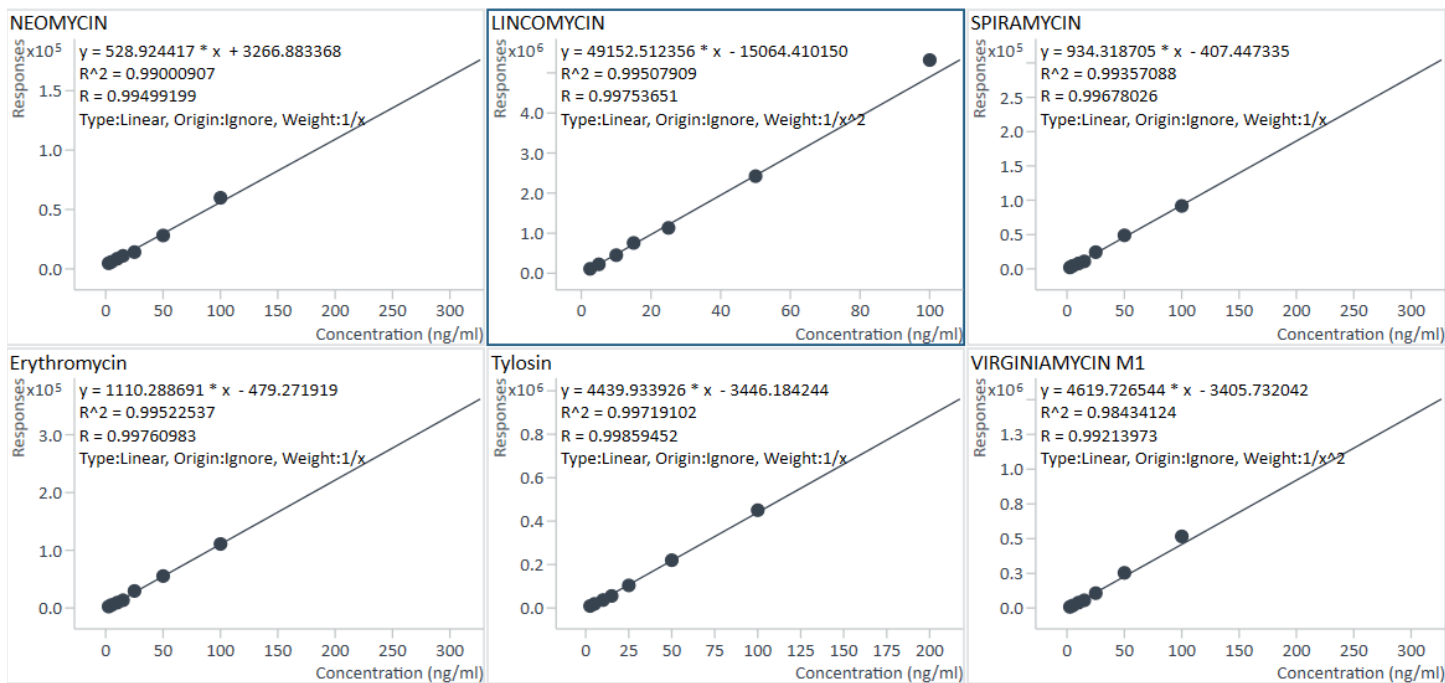


Figure 4. Calibration curves for erythromycin, lincomycin, neomycin, spiramycin, tylosin, and virginiamycin M1 over the concentration range of 2.5 to 100 ng/mL in a milk matrix.

Sample			KANAMY...	KANAMYCIN Results			Qu...
Name	Type	Level	Exp. Conc. Δ	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Sa...			2.882	7.2790		
MB 25.0 ppb	Cal	1	25.0000	2.606	29.5423	118.2	42.2
MB 50.0 ppb	Cal	2	50.0000	2.615	43.5628	87.1	46.8
MB 100.0 ppb	Cal	3	100.0000	2.615	92.7779	92.8	46.4
MB 150.0 ppb	Cal	4	150.0000	2.615	148.0012	98.7	47.4
MB 200.0 ppb	Cal	5	200.0000	2.615	195.4261	97.7	44.3
MB 250.0 ppb	Cal	6	250.0000	2.615	254.7526	101.9	47.8
MB 300.0 ppb	Cal	7	300.0000	2.615	310.9371	103.6	47.4

Sample			SPECTIN...	SPECTINOMYCIN Results			Qu...
Name	Type	Level	Exp. Conc. Δ	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Sa...			3.011	4.8125		34.9
MB 25.0 ppb	Cal	1	25.0000	3.011	26.2769	105.1	39.9
MB 50.0 ppb	Cal	2	50.0000	3.011	52.2233	104.4	42.0
MB 100.0 ppb	Cal	3	100.0000	3.011	94.0565	94.1	39.1
MB 150.0 ppb	Cal	4	150.0000	3.002	138.6447	92.4	42.6
MB 200.0 ppb	Cal	5	200.0000	3.002	193.3785	96.7	30.2
MB 250.0 ppb	Cal	6	250.0000	2.994	256.9566	102.8	37.9
MB 300.0 ppb	Cal	7	300.0000	3.002	313.4636	104.5	38.4

Sample			APRAMY...	APRAMYCIN Results			Qu...
Name	Type	Level	Exp. Conc. Δ	RT	Calc. Conc.	Accuracy	Ratio
Matrix Blank	Sa...			9.542	22.8147		3.8
MB 25.0 ppb	Cal	1	25.0000	9.024	22.5250	90.1	5.1
MB 50.0 ppb	Cal	2	50.0000	9.032	46.8630	93.7	5.5
MB 100.0 ppb	Cal	3	100.0000	9.024	102.5681	102.6	5.2
MB 150.0 ppb	Cal	4	150.0000	9.032	153.6570	102.4	4.2
MB 200.0 ppb	Cal	5	200.0000	9.032	200.3259	100.2	4.2
MB 250.0 ppb	Cal	6	250.0000	9.032	257.4022	103.0	5.3
MB 300.0 ppb	Cal	7	300.0000	9.032	291.6588	97.2	4.7

Figure 5. Snapshots of quantitation tables for kanamycin, spectinomycin, and apramycin across the concentration range of 25 to 300 ng/mL in a milk matrix.

Significant matrix effects were observed across several analytes, necessitating the use of a pre-extraction spike approach to generate pre-extraction spiked calibration points. This method provides reliable accuracy and demonstrates effective recovery of analytes from milk samples, ranging from 80 to 120% (Figure 7).

Consistency across multiple reaction monitoring (MRM) ion ratios further validates the robustness of this method. In addition, specific handling recommendations were noted for certain standards. Notably, apramycin and

neomycin should be dissolved in water and stored in plastic vials to prevent adsorption losses associated with glass surfaces. Overall, the study demonstrates a reliable workflow for aminoglycoside analysis in milk, combining optimized extraction with EDTA, tailored calibration ranges, and matrix-based recovery corrections. These measures collectively enhance accuracy, sensitivity, and reproducibility, demonstrating the suitability of the method for monitoring antibiotic residues in complex food matrices. The LOQ is 2.5 ppb for neomycin, erythromycin, lincomycin,

tylosin, spiramycin, and virginiamycin. For the other three analytes, kanamycin, spectinomycin, and apramycin, the LOQ is 25 ppb. The peak observed in the matrix blank shows a clear retention-time shift, and the MRM ratios fall above the +30% window compared to the standard, suggesting a matrix-derived interference rather than the analyte. This optimized method provides a strong foundation for monitoring aminoglycoside antibiotic residues in complex food matrices, supporting food safety and regulatory limits that vary from 10 to 200 ppb.

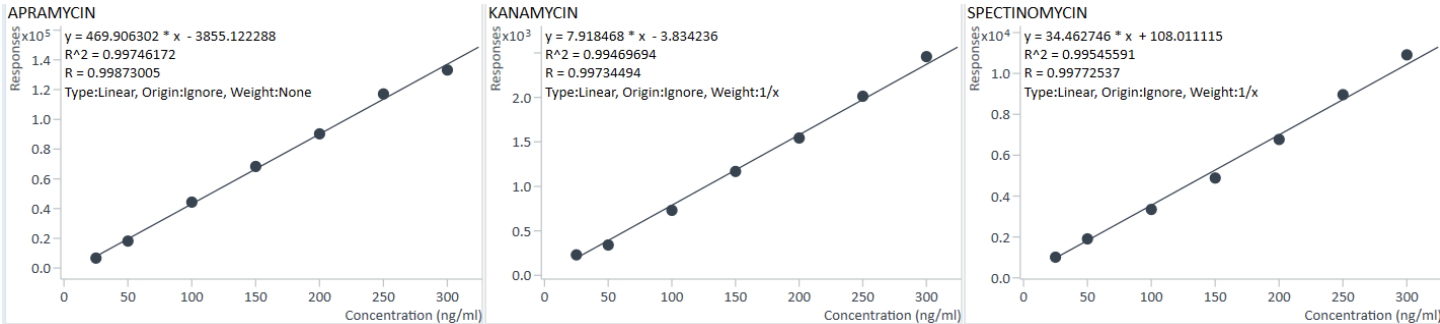


Figure 6. Calibration curves for kanamycin, spectinomycin, and apramycin over the concentration range of 25 to 300 ng/mL in a milk matrix.

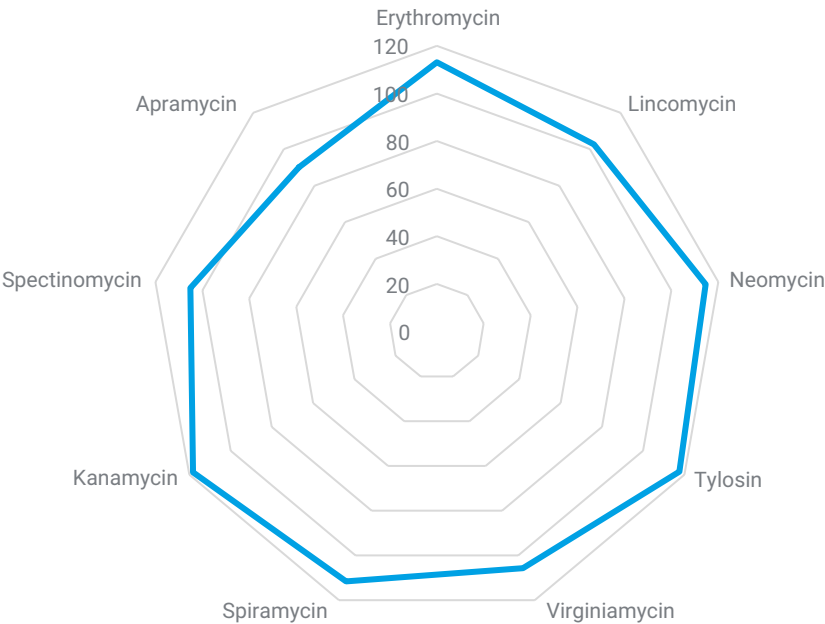


Figure 7. Radar plot showing the percent recovery of various aminoglycosides at their LOQ in a milk matrix.

Conclusion

A highly sensitive and robust multiple reaction monitoring (MRM) method was developed for the quantification of nine aminoglycoside antibiotics using an Agilent 6475 triple quadrupole LC/MS system. A simple and efficient sample preparation protocol was employed to extract these analytes from the milk matrix. Pre-extracted spiked calibration points for all analytes demonstrated excellent linearity across the specified concentration ranges using linear regression with 1/x weighting. The method achieved strong retention for analytes and effective matrix separation without the use of ion-pairing reagents, thereby ensuring optimal MS compatibility. Furthermore, the method exhibited consistent performance across multiple batches. Combined with simplified sample preparation and high-throughput capability, this practical method provides a reliable, reproducible, and MS-friendly solution for the routine monitoring of aminoglycoside residues in milk.

Reference

1. Zhai, A. Aminoglycosides in Milk using Agilent Bond Elute Plexa SPE, Agilent Poroshell 120, and LC/Tandem MS, *Agilent Technologies application note*, publication number 5991-1758 EN, **2013**.