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アプリケーションノート

Development and Validation of a Confirmatory Method for the Determination of Aminoglycosides in Foods Using LC-MS/MS With a Zwitterionic HILIC Column

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Abstract

Aminoglycosides (AMGs) are broad-spectrum antibiotics that have bactericidal activity against aerobic bacterial infection and are commonly used as veterinary drugs on food-producing animals and in human medicine. Thus, it is important to monitor residues in food to control AMG use. Many countries have established maximum residue limits (MRL) for aminoglycosides approved for use on animals. AMGs are often analyzed in honey, eggs, milk, tissues, and biofluids of food-producing animals for control and monitoring purposes. AMGs are highly polar compounds and show little to no retention in reversed phase columns. Although ion-pairing reagents have been utilized successfully to chromatograph AMGs on C_{18} columns, when used with liquid chromatography tandem quadrupole mass spectrometry (LC-MS/MS), this approach can suffer from ion suppression and contamination of the LC and MS/MS systems. The introduction of hydrophilic interaction chromatography (HILIC) provided a more MS-compatible option for the analysis of polar compounds. Here we show the results from the successful evaluation of the Atlantis™ Premier BEH™ Z-HILIC Column, which has a sulfobetaine zwitterionic chemistry, for the determination of AMGs. Samples of milk, eggs, and honey were extracted using a solution that contained 10 mM ammonium acetate, 0.4 mM ethylenediamine tetraacetic acid (EDTA), 0.5% NaCl, and 2% trichloroacetic acid (TCA) and subjected to clean-up by Solid-Phase Extraction (SPE) on Oasis™ HLB cartridges prior to LC-MS/MS. The performance of the method was successfully verified

according to Commission Implementing Regulation (EU) 2021/808. The method is suitable for reliable confirmation of residues to check compliance with MRLs globally and in cases where use of the substances is not allowed.

Benefits

- Chromatographic retention and separation were provided by the Atlantis Premier BEH Z-HILIC Column, using an MS friendly mobile phase, without resorting to using an ion-pair reagent
- The method is suitable for reliable determination of residues to check compliance with MRLs and in cases where use of the substances is not allowed
- The method was successfully validated in three sample types, according to Commission Implementing Regulation (EU) 2021/808

Introduction

Aminoglycosides (AMGs) are broad-spectrum antibiotics that have bactericidal activity against aerobic bacterial infection. They are used as veterinary drugs, feed additives and as growth promoters on food-producing animals. Due to their low cost, there is concern about the overuse of these drugs in commercial animal production. The presence of AMGs in food may represent a risk to consumer health, due to possible allergenicity and/or tissue toxicity, and exposure to them may lead to an increase in antimicrobial resistance. Misuse of veterinary medicines can lead to unacceptance residues in the tissues of the animals. Maximum Residue Limits (MRLs) have been set for some AMGs in a range of tissues, eggs, and milk of food producing species.[1] Some AMGs are not approved for use in animals from which milk or eggs are produced for human consumption and so no MRLs exist for those AMGs in milk or eggs. In addition, there are no MRLs for AMGs in honey, as use for apiculture is not authorized.

AMGs are weak bases, water soluble, and are too polar to be included in the scope of multiresidue approaches and rely on separate class-specific methods.[2,3] They are also not amenable to reversed phase chromatography without resorting to using ion-paring agents (eg HBFA), which leads to ion suppression and contamination of the LC-MS/MS system. Alternatively, AMGs can be determined using hydrophilic interaction liquid chromatography (HILIC), but limited separation selectivity for these compounds is observed using amide or aminopropyl HILIC stationary phases. In this study, a method based upon the Atlantis™ Premier BEH™ Z-HILIC Column, developed previously, was implemented using UniSpray™, and the performance further evaluated for the determination of eight AMGs in milk, eggs, and honey.[4,5]

Experimental

Sample Description

Samples of milk, eggs, and honey, purchased from local retailers, were prepared, and analyzed by the laboratory of the State Office for Agriculture, Food Safety and Fisheries Mecklenburg-West Pomerania, Germany. Samples were homogenized, extracted using a solution that contained 10 mM ammonium acetate, 0.4 mM ethylenediamine tetraacetic acid (EDTA), 0.5% NaCl, and 2% trichloroacetic acid (TCA) and subjected to SPE clean up using Oasis HLB, prior to LC-MS/MS. Full details are provided below in Figure 1.

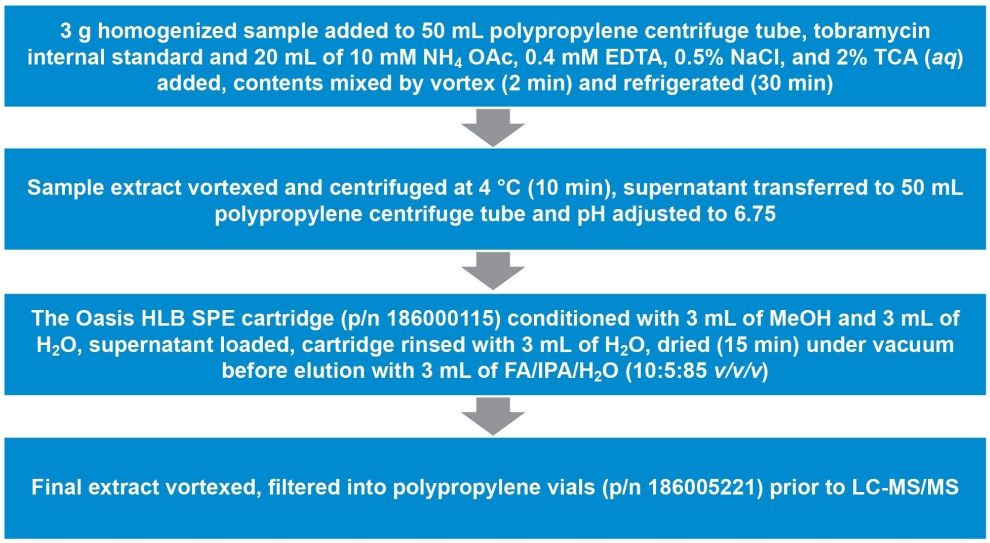

Figure 1. Overview of the details of sample extraction and clean up for the determination of aminoglycosides in milk, eggs, and honey.

Spikes were prepared in milk and eggs, previously shown to be blank, at 0.1, 0.5, 1, 1.5, and 2 times the level of interest (typically MRL where available and extrapolated if not). There are no MRLs for AMG residues in honey, so their presence in honey is not authorized. The level of interest for such unauthorized substances was set at the minimum method performance requirement (MMPR) provided by the EURL and spikes prepared at 1, 1.5, 2, and 3 times the MMPR (20 µg/kg).[6] Matrix-matched standards were prepared over the same range. Tobramycin was used as an internal standard (IS) throughout. The concentration of the levels of interest are shown in Table 1 below.

	Levels of Interest (MRLs or MMPRs; µg/kg)		
	Milk	Eggs	Honey
Apramycin	200*	200*	20
Dihydrostreptomycin	200	200*	20
Gentamycin	100	100*	20
Kanamycin	150	200*	20
Neomycin B	1500	500	20
Paromomycin	200*	200	20
Spectinomycin	200	200*	20
Streptomycin	200	100*	20

Table 1. Levels of interest (MRLs and MMPRs [honey] as µg/kg).

*There are no MRLs for these analytes in this commodity, so the level of interest has been extrapolated from other MRLs for the spiking concentrations used in this validation plan

LC Conditions

LC system:	ACQUITY I-Class UPLC with FTN SM
Column(s):	Atlantis Premier BEH Z-HILIC (2.5 µm, 2.1 x 150 mm) p/n: 186009987
Column temperature:	50 °C
Sample temperature:	10 °C
Injection volume:	5 µL
Mobile phase A:	20 mM ammonium formate in water (pH 3.0)
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.60	10	90	Initial
1.0	0.60	75	25	6
5.0	0.60	85	15	6
8.0	0.60	85	15	6
8.1	0.60	10	90	6
10.0	0.60	10	90	6

MS Conditions

MS system:	Xevo TQ-XS
Ionization mode:	UniSpray (positive ion mode)
Impactor voltage:	3.0 kV
Source temperature:	150 °C
Desolvation temperature:	500 °C
Desolvation gas flow:	1000 L/hr
Cone gas flow:	150 L/hr

Compound	Retention time (min)	MRM	CV (V)	CE (eV)
Spectinomycin	1.47	351.2>207.2	30	22
		351.2>333.2	30	18
Dihydrostreptomycin	1.73	584.3>246.2	70	32
		584.3>263.1	70	35
Streptomycin	1.75	582.3>246.2	70	35
		582.3>263.2	70	40
Kanamycin	2.17	**485.3>163.1**	50	28
		485.3>324.2	50	15
Paromomycin	2.80	**616.3>163.0**	70	35
		616.3>293.2	70	20
Gentamycin C1	2.81	478.3>160.0	25	15
		478.3>322.2	25	22
Apramycin	2.87	**540.3>217.2**	50	30
		540.3>378.2	50	18
Gentamycin C2	2.89	464.3>160.0	25	15
		464.3>322.2	25	22
Gentamycin C1a	2.99	450.3>160.0	25	15
		450.3>322.2	25	25
Neomycin B	4.20	**615.3>161.0**	70	20
		615.3>163.0	70	20
Tobramycin (IS)	3.11	**468.3>163.0**	50	20

Table 1. MRM parameters for AMGs (quantitative transitions in bold).

Data Management

MS software: MassLynx v4.2

Informatics: TargetLynx™ XS

Method Validation

Validation was performed on milk, eggs, and honey based upon requirements of Commission Implementing Regulation (EU) 2021/808 using spiked representative samples.[7] Identification was assessed by examining retention times, ion ratios, and identification points. Trueness using measured recovery, repeatability (RSD_r), within-laboratory reproducibility (RSD_R) and decision limit (CCα) were derived from data from the analysis of replicate spiked samples, performed on three separate days. Unlike in the conventional validation approach, with the alternative validation approach a concentration range is validated instead of discrete concentration levels. It is therefore not necessary to fortify the samples used for validation at the exact concentration levels given in Annex I of Commission Implementing Regulation (EU) 2021/808. However, the required concentration

levels must be covered adequately by the selected validated concentration range. All calculations, for both MRL and unauthorized substances, were based on InterVAL 3.4.0.4, CD 2002/657 (alternative method).

Results and Discussion

The Atlantis Premier BEH Z-HILIC Column is packed with a zwitterionic sulfoalkylbetaine stationary phase attached to BEH particles. The zwitterionic sulfobetaine ligand has both positively and negatively charged groups, in a one-to-one ratio, making them net neutral. The sulfobetaine bonding provides a unique selectivity and creates a very hydrophilic surface. A dense/thick water rich layer on the surface further increases polar analyte retention. In contrast to high buffer concentrations in the mobile phase required for silica-based sulfoalkylbetaine stationary phases, a moderate buffer concentration (20 mM) provided the optimal separation of the analytes with the BEH sulfoalkylbetaine stationary phase. More details on the development of the HILIC method can be found in the earlier application note and recent publication.[4,5]

The Atlantis Premier BEH Z-HILIC Column provided sufficient retention for even the most polar analytes, meeting the requirement from Commission Implementing Regulation (EU) 2021/808: minimum acceptable retention time for the analyte(s) under examination shall be twice the retention time corresponding to the void volume of the column. All peaks eluted between 1.5 and 4.2 minutes with a total run time of 10 minutes. Chromatography was shown to be stable with no significant change in retention times across the batches analyzed (RSD 0.01–0.91%; n=57 in various matrices). This approach provided excellent retention and peak shape for all the AMGs without the need for an ion pair reagent or highly concentrated buffers in the mobile phase, which reduces mobile-phase related ion suppression and hence results in improved sensitivity.

Excellent sensitivity was demonstrated from the analysis of the matrix-matched standards prepared from milk, egg, and honey extracts. Figure 2 shows chromatograms for the AMGs from the analysis of matrix-matched standards at 1x the level of interest (see Table 1), in milk. The chromatography, sensitivity and selectivity shown in the figure was representative of all three commodities, which indicates that the method is capable of being used for checking regulatory compliance, after suitable validation. The response for all the AMGs was linear over the range evaluated and graphs were created using 1/x weighting. The values for the coefficient of determination (R^2) were found to be >0.99.

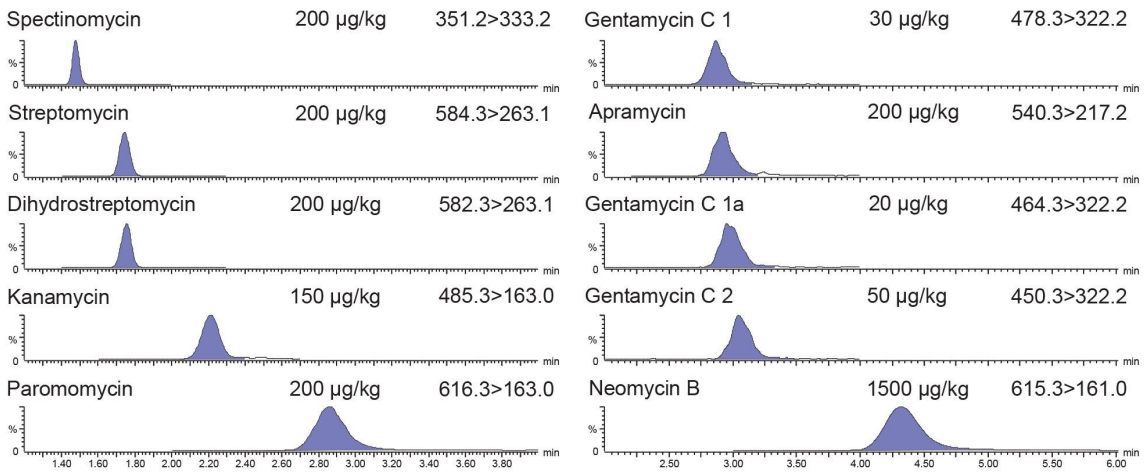

Figure 2. Chromatograms for a selection of aminoglycosides from the analysis of matrix-matched standards at 1x the level of interest in milk.

Commission Implementing Regulation (EU) 2021/808 states "Where, for a specific pharmacologically active substance validation of a concentration of 0.1 times the MRL is not reasonably achievable, the concentration of 0.1 times the MRL can be replaced by the lowest concentration between 0.1 times and 0.5 times the MRL, which is reasonably achievable." An initial assessment showed that the precision from the analysis of the spikes at 0.1x the level of interest was not acceptable, so these data were removed from further calculations and the spikes at 0.5x the level of interest became the lowest concentration.

The trueness, determined by measured recovery, was evaluated using the data from the analysis of the spiked samples. The mean values for measured recovery, for all the AMGs, in all three commodities, across all the spiking levels, were within the range the range 87 to 106%, which was within the tolerance set for minimum trueness (80–120% when expressed as measured recovery). The limits for precision for the repeated analysis of spiked materials, under within-laboratory reproducibility conditions, are specified in Commission Implementing Regulation (EU) 2021/808 and vary depending on the spiking concentration. The criteria for reproducibility (RSD_{wR} ⩽22% for >120–1000 µg/kg or ⩽25% for 10–120 µg/kg) and repeatability (RSD_r ⩽15 for >120–1000 µg/kg or ⩽17% for 10–120 µg/kg) were met for all but one case in milk and eggs, spiked at 0.5x to 2x the level of interest. For milk and eggs spiked at 0.5x to 2x the level of interest, all compounds passed the limit set for reproducibility and repeatability (10 to 19% RSD_{wR} and 1.6 to 16% RSD_r, respectively). In honey, which was spiked at lower concentrations (20 to 60 µg/kg), all compounds passed the limit set for reproducibility (11 to 22% RSD_{wR}). Repeatability was within the range 3.5 to 19% RSD_r and only one compound failed to meet the acceptance criterion for repeatability (⩽17% RSD_r) when spiked at 20 µg/kg.

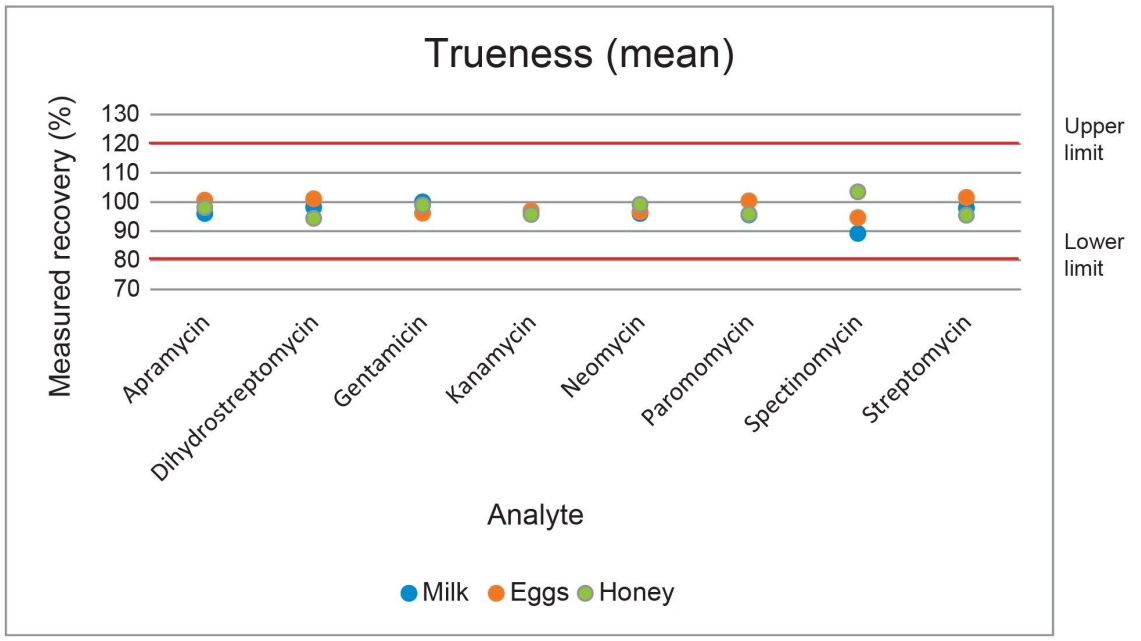

Figure 3. Summary of the mean values for trueness, as measured recovery, from the analysis of milk, eggs, and honey.

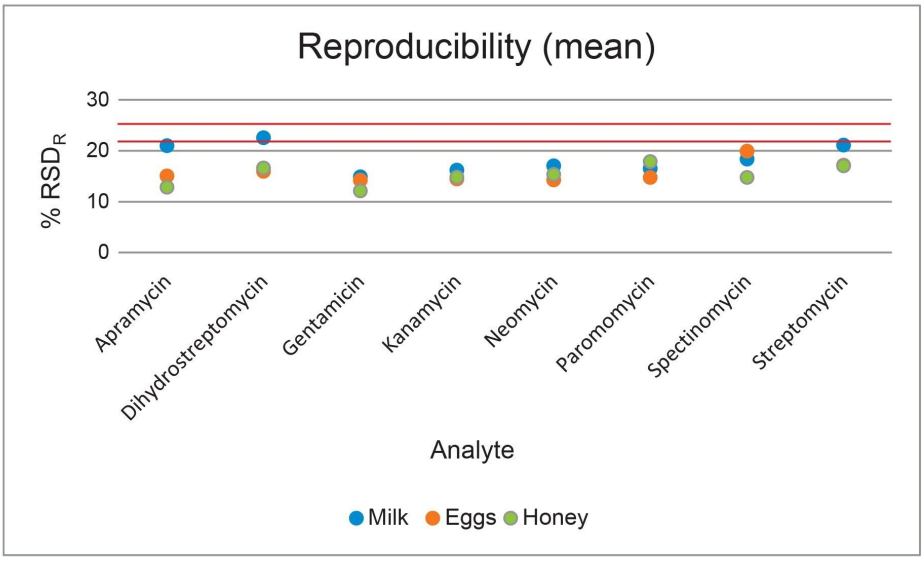

Figure 4. Summary of the mean values for reproducibility from the analysis of milk, eggs, and honey.

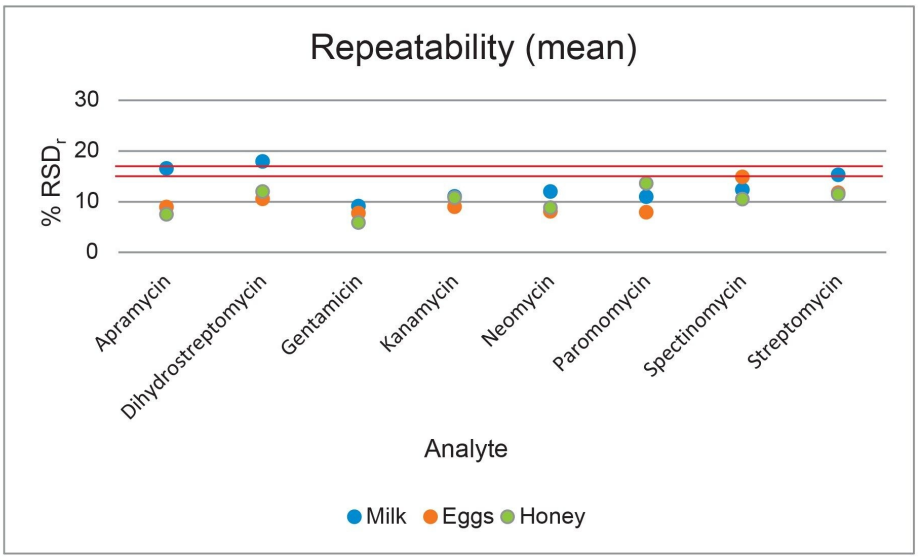

Figure 5. Summary of the mean values for repeatability from the analysis of milk, eggs, and honey.

The Decision Limit (CCα) for confirmation for authorized substances must be higher than but as close to the MRL as analytically achievable, whereas for the prohibited and unauthorized AMGs it must be as low as analytically achievable. Tables 2, 3, and 4 provide a summary of the critical validation parameters calculated including values for CCα.

Analyte	MRL (µg/kg)	Spiking level (µg/kg)	Measured recovery (%)	% RSD_r	% RSD_{wR}	CCα
Apramycin	none	100	96	14	17	53
		200	96	8.1	13	
		300	96	5.8	12	
		400	96	4.6	12	
Dihydrostreptomycin	200	100	97	16	19	254
		200	96	8.9	14	
		300	96	6.4	13	
		400	96	5.1	13	
Gentamicin	100	50	99	8.6	13	123
		100	98	5.3	12	
		150	97	4.0	12	
		200	97	3.2	11	
Kanamycin	150	75	95	10	14	185
		150	94	6.2	12	
		225	93	4.6	11	
		300	93	3.7	11	
Neomycin	1500	750	96	11	15	1854
		1500	95	6.5	12	
		2250	95	4.8	12	
		3000	95	3.9	11	
Paromomycin	none	100	96	10	15	40
		200	95	6.3	13	
		300	95	4.8	12	
		400	95	3.9	12	
Spectinomycin	200	100	89	12	17	259
		200	88	7.7	15	
		300	87	5.9	14	
		400	87	4.9	13	
Streptomycin	200	100	97	13	16	254
		200	96	7.0	14	
		300	96	4.9	13	
		400	95	3.8	13	

Table 2. Summary of the critical validation parameters calculated from the analysis of milk.

Analyte	MRL (µg/kg)	Spiking level (µg/kg)	Measured recovery (%)	% RSD_r	% RSD_{wR}	CCα
Apramycin	None	100	100	7.0	12	41
		200	100	3.7	11	
		300	99	2.6	11	
		400	99	2.0	11	
Dihydrostreptomycin	None	100	100	10	14	42
		200	99	5.8	12	
		300	99	4.3	11	
		400	99	3.4	11	
Gentamicin	None	50	96	6.1	12	18
		100	97	3.3	11	
		150	97	2.3	11	
		200	97	1.8	11	
Kanamycin	None	100	97	8.0	13	37
		200	97	4.8	11	
		300	97	3.6	11	
		400	97	2.9	10	
Neomycin	500	250	97	6.2	12	601
		500	97	3.3	11	
		750	97	2.3	10	
		1000	97	1.8	10	
Paromomycin	200	100	101	5.9	12	242
		200	101	3.1	11	
		300	102	2.1	11	
		400	102	1.6	11	
Spectinomycin	None	100	95	14	18	44
		200	95	9.3	15	
		300	95	7.3	14	
		400	96	6.1	14	
Streptomycin	None	50	101	10	14	22
		100	100	6.1	12	
		150	100	4.5	12	
		200	100	3.6	12	

Table 3. Summary of the critical validation parameters calculated from the analysis of eggs.

Analyte	MMPR (µg/kg)	Spiking level (µg/kg)	Measured recovery (%)	% RSD_r	% RSD_{wR}	CCα
Apramycin	20	20	99	11	15	5.5
		30	98	8.0	13	
		40	98	6.5	12	
		60	98	4.9	12	
Dihydrostreptomycin	20	20	94	17	20	8.2
		30	94	13	17	
		40	94	10	15	
		60	94	7.6	14	
Gentamicin	20	20	99	8.9	14	6.0
		30	99	6.3	12	
		40	99	5.0	12	
		60	99	3.5	11	
Kanamycin	20	20	97	15	18	6.8
		30	96	12	15	
		40	95	9.5	14	
		60	95	7.3	12	
Neomycin	20	20	99	13	17	6.4
		30	99	9.4	16	
		40	100	7.6	15	
		60	100	5.6	14	
Paromomycin	20	20	96	19	22	8.5
		30	96	15	19	
		40	96	12	17	
		60	96	8.8	14	
Spectinomycin	20	20	101	17	20	11
		30	103	11	15	
		40	105	8.4	13	
		60	106	5.6	12	
Streptomycin	20	20	95	17	20	7.8
		30	95	12	17	
		40	96	9.8	16	
		60	96	7.2	15	

Table 4. Summary of the critical validation parameters calculated from the analysis of honey.

In addition, the data from the analysis of the replicate spikes was assessed with respect to meeting required identification criteria. The two transitions for each analyte, enough to meet the required identification points for MRL and unauthorized substances (four and five points, respectively), gave peaks with ion ratios and retention times within the recommended tolerances, when compared with the standards.

Conclusion

This application note has described a method for the determination of AMG antibiotic residues in a variety of food products. After extraction and clean-up, AMG residues were determined by LC-MS/MS, using the Atlantis Premier BEH Z-HILIC Column. The performance of the method was successfully verified according to Commission Implementing Regulation (EU) 2021/808 in three types of animal products. The criteria for trueness, repeatability, and within-laboratory reproducibility were met with a few exceptions. Values for decision limit (CCα) demonstrated that the method is suitable for confirmation of residues to check compliance with EU MRLs and in cases where use of the substances is not allowed but also to check whether MRLs specified by other competent authorities are not being exceeded.

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