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Application Note

Determination of 8 Aminoglycosides in Pork Meat acc. to GB/T 21323-2007

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Abstract

Following the GB/T 21323-2007 method, an LC-MS/MS (ESI positive mode) method was developed to determine 8 aminoglycoside residues of spectinomycin, hygromycin B, dihydrostreptomycin, amikacin, kanamycin, tobramycin, gentamicin and neomycin in pork meat. The cleanup of the pork meat sample was done by SPE using a Discovery® DSC-18 tube. An Ascentis® Express C18 UHPLC column was used to separate and quantify the aminoglycoside residues. The R^2 values for the external calibration in the range of 1 to 5000 ng/mL were between 0.9927 and 0.9998 and the % recovery was 81.4 to 101.4%.The LOD and LOQ of the aminoglycosides in pork meat ranged from 0.2 to 0.8 µg/kg and 0.5 to 2.3 µg/kg respectively. The described method met the acceptance criteria of the official Chinese Method.

Introduction

Aminoglycosides are natural or semisynthetic antibiotics derived from actinomycetes.[1] Most of aminoglycosides are widely used against bacteria and parasites in the production of pork, chicken, beef, milk and eggs around the world, which could arouse side reactions and antimicrobial resistance to consumers (Schenck, 1998).[2] Thus, governments around the world, international regulatory bodies, including the European Union, the US Food and Drug Administration and the Codex Alimentarius are all intensifying their efforts to control veterinary antibiotics usage and issuing increasingly stringent regulations on maximum residue limits (MRLs).[3,4] In order to protect public health, China has released national standard methods GB/T 21323-2007 for determination of the residues of 8 aminoglycosides (**Figure 1**) in food.[5] Following this GB method, an application was developed for determination of 8 aminoglycosides in pork meat. In this study the pork samples were cleaned-up by SPE using a Discovery® DSC-18 SPE tube and the resulting sample was run on an Ascentis® Express C18 UHPLC column to quantify the 8 aminoglycosides by LC-MS/MS.

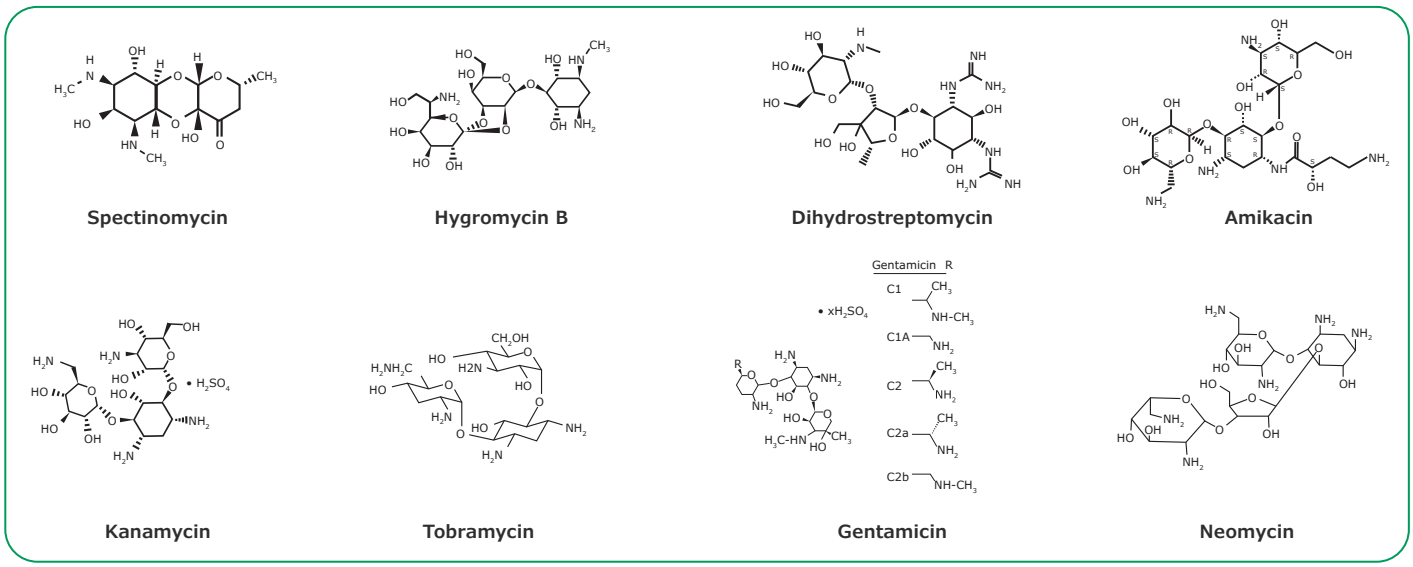

Figure 1. Structures of aminoglycosides determined.

Experimental

Standards and samples were prepared to following procedures:

Standard Preparation

1. Weigh 4.28 g perfluorobutanoic acid and transfer to a 1 L volumetric flask. Dilute to volume with water for 20 mM perfluorobutanoic acid aqueous solution.
2. Weigh 10 mg each of the 8 aminoglycoside reference material into individual 10 mL volumetric flasks (1 mg/mL).
3. Add 7 mL methanol to each flask and sonicate for 5 mins. Top-up to mark with methanol and mix well.
4. Transfer 100 µL of each standard into a 10 mL volumetric flask and top up with 20 mM perfluorobutanoic acid (10 µg/mL).
5. Dilute the 10 µg/mL standard to 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL with 20 mM perfluorobutanoic acid. Mix well before use.

Sample Preparation

1. Phosphate buffer solution: Weigh 1.36 g of potassium dihydrogen phosphate, 0.15 g of Na_2EDTA and 20 g of trichloroacetic acid in a 1 L volumetric flask. Add 980 mL of water, adjust the pH to 4.0 using 1 M hydrochloric acid and make up to 1000 mL with water. This is phosphate buffer pH 4.0 containing 0.4 mM EDTA and 2% trichloroacetic acid.
2. Homogenize pork samples with a high-speed tissue homogenizer.
3. Weigh 5 g of homogenized pork sample into a 50 mL centrifuge tube and add 10 mL of phosphate buffer solution.
4. Shake tubes for 10 min followed by centrifugation at 4500 rpm for 10 min.
5. Transfer the supernatant to another 50 mL centrifuge tube.
6. Repeat extraction with 10 mL phosphate buffer and pool the supernatant into one tube. Adjust pH to 3.5 ± 0.2 with 1.0 M HCl solution.
7. Add 2.0 mL of 100 mM perfluorobutanoic acid aqueous solution and mix well for SPE clean-up.
8. Perform SPE clean up according to protocol in **Table 1**.

Table 1. SPE Conditions

SPE Conditions	
Sample/matrix:	Pork sample extract
SPE tube/cartridge:	Discovery® DSC-18, 500 mg/3 mL (**52603-U**)
Conditioning:	3 mL methanol followed by 3 mL 20 mM perfluorobutanoic acid
Sample Load:	Transfer total collected extract volume and set a flow rate of ~1 drop/s
Washing:	3 mL of 20 mM perfluorobutanoic acid followed by 2 x 3 mL water. Drain for 5 min.
Elution:	5 mL of acetonitrile : 20 mM perfluorobutanoic acid (80:20 %v/v)
Eluate post-treatment:	Evaporate to dryness under nitrogen at 40 °C and reconstitute to 1 mL 20 mM perfluorobutanoic acid.

LC-MS/MS Analysis

Standards and samples were analyzed by LC-MS using the method described in **Table 2**.

Table 2. LC-MS parameters used for the aminoglycoside determination

SPE Conditions			
Instrument:	Acquity UPLC I-class plus		
Column:	Ascentis® Express C18 UHPLC, 100 x 2.1 mm I.D., 2 µm (**50813-U**)		
Mobile phase:	[A] Water with 20 mM perfluorobutanoic acid [B] Acetonitrile/water (80/20, v/v) with 20 mM perfluorobutanoic acid		
Gradient:	Time (min)	A%	B%
	0	85	15
	1.5	85	15
	7.5	25	75
	8.9	25	75
	8.91	85	15
	10.0	85	15
Flow rate:	0.3 mL/min		
Pressure:	5441 to 6913 psi		
Column temp.:	25 °C		
Detector:	MS, MRM settings see **Table 3**		
Injection:	10 µL		
Samples:	Prepared as described		

Table 2. (cont.) LC-MS parameters used for the aminoglycoside determination

MS Conditions	
Instrument:	Xevo® TQ-S
Polarity:	Positive and negative
Spray voltage:	3.0 kV (ESI+)
Capillary temp:	500 °C
Desolvation (L/h):	1000
Cone (L/h):	150
Nebulizer (bar):	7

Table 3. MRM Transitions used for aminoglycoside determination

Compound name	Transition (m/z)	Cone (V)	Collision (V)	Dwell (s)
Spectinomycin	333.1 > 98.1	60	26	0.025
Hygromycin B	528.3 > 177.1	54	28	0.025
Dihydrostreptomycin	584.3 > 263.2	52	22	0.025
Amikacin	586.3 > 425.2	54	18	0.025
Kanamycin	485.4 > 163.2	66	24	0.025
Tobramycin	468.4 > 205.2	32	19	0.025
Gentamicin	478.3 > 157.3	34	22	0.025
Neomycin	615.4 > 293.1	62	24	0.025

Method & System Suitability

Acceptance Criteria as described in GB/T 21323-2007

- Critical ions must be present and exceed a signal-to-noise ratio >5.
- Linear correlation coefficient: R^2 > 0.99
- Aminoglycoside recovery with extraction and cleanup by SPE: 70 - 120%.
- For neomycin, hygromycin B, LOQ < 100 mg/kg; for other aminoglycosides, LOQ < 20 mg/kg.

Method & System Suitability

Chromatographic results for a standard, blank and spiked pork meat samples are displayed in the **Figures 2-4**. The retention times for the 200 ng/mL standard solution are shown in **Table 4**. The linearity and derived sensitivity for a pork meat sample can be seen in **Table 5**. All compounds showed R^2 values above 0.99. The repeatability for the injection of the 200 ng/mL standard ranged from1.72 to 3.29 %RSD (**Table 6**). All critical product ions are presented with signal-to-noise ratio (S/N) >5 during analysis. As example the calibration curve for spectinomycin is displayed in **Figure 5**. The method recovery for the 8 aminoglycosides spiked at 50 µg/kg into pork meat after SPE cleanup ranged from 81.4 to 101.4% (**Table 7**). The shown method met all acceptance criteria stated by the GB/T 21323-2007 method.

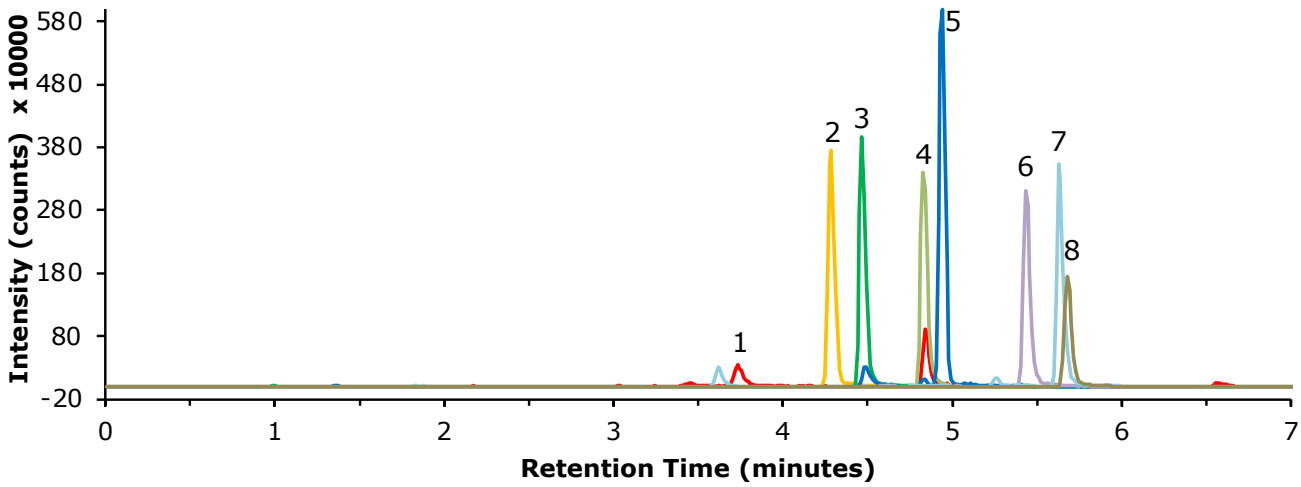

Figure 2. Injection of an aminoglycoside standard, 200 ng/mL (MRM product ion traces).

Table 4. Retention times of the aminoglycosides

Peak ID	Compound	Retention Time (min)
1	Spectinomycin	3.69
2	Hygromycin B	4.27
3	Dihydrostreptomycin	4.45
4	Amikacin	4.80
5	Kanamycin	4.90
6	Tobramycin	5.42
7	Gentamicin	5.60
8	Neomycin	5.65

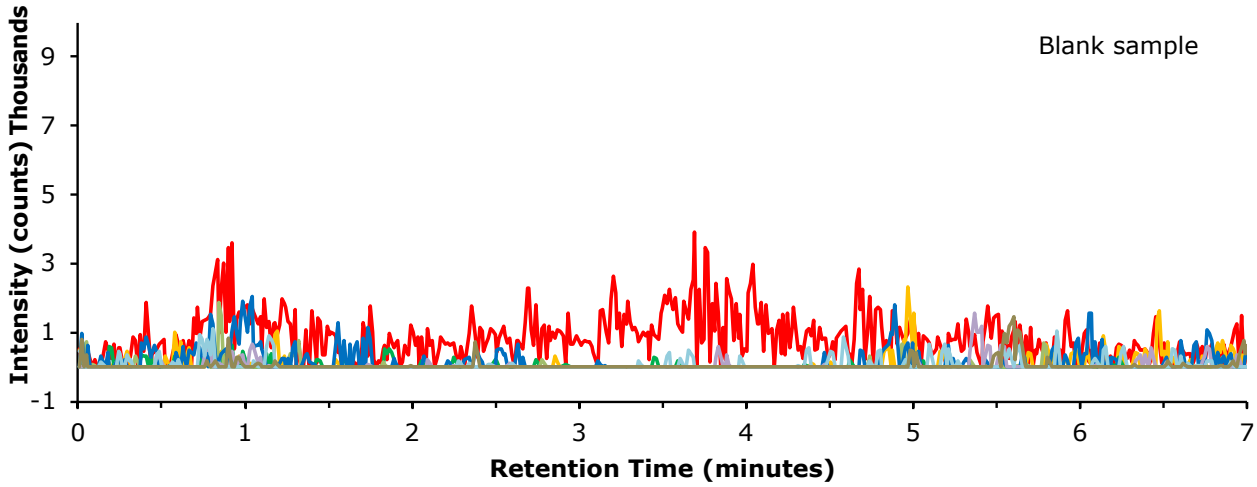

Figure 3. Injection of a blank pork meat sample.

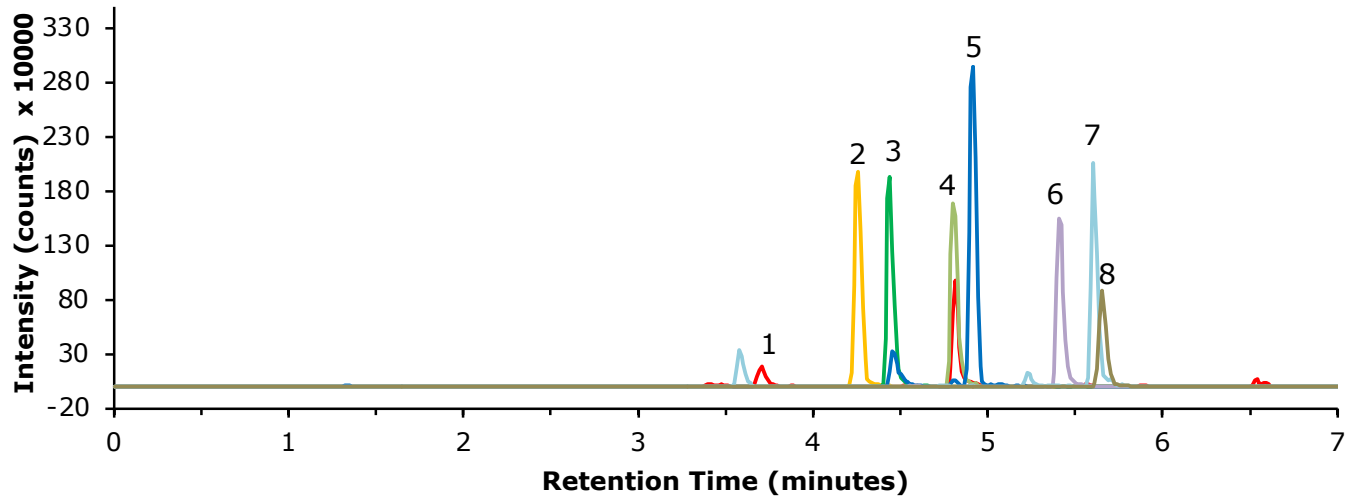

Figure 4. Analysis of aminoglycosides in pork meat spiked at 100 µg/kg after SPE cleanup.

Table 5. Calibration linearity data and derived sensitivity of 8 aminoglycosides for pork meat sample

Compound	Calibration (ng/mL)	No. of Calibrators	R^2	LOD (µg/kg)	LOQ (µg/kg)*
Spectinomycin	1 - 5000	12	0.9927	0.8	2.3
Hygromycin B			0.9963	0.2	0.5
Dihydrostreptomycin			0.9984	0.3	0.9
Amikacin			0.9976	0.2	0.6
Kanamycin			0.9998	0.3	0.9
Tobramycin			0.9970	0.2	0.6
Gentamicin			0.9991	0.4	1.3
Neomycin				0.3	1.0

*Note: Acceptance criteria for sensitivity by the GB method are for neomycin and hygromycin B an LOQ <100 mg/kg, for the other aminoglycosides an LOQ of <20 mg/kg

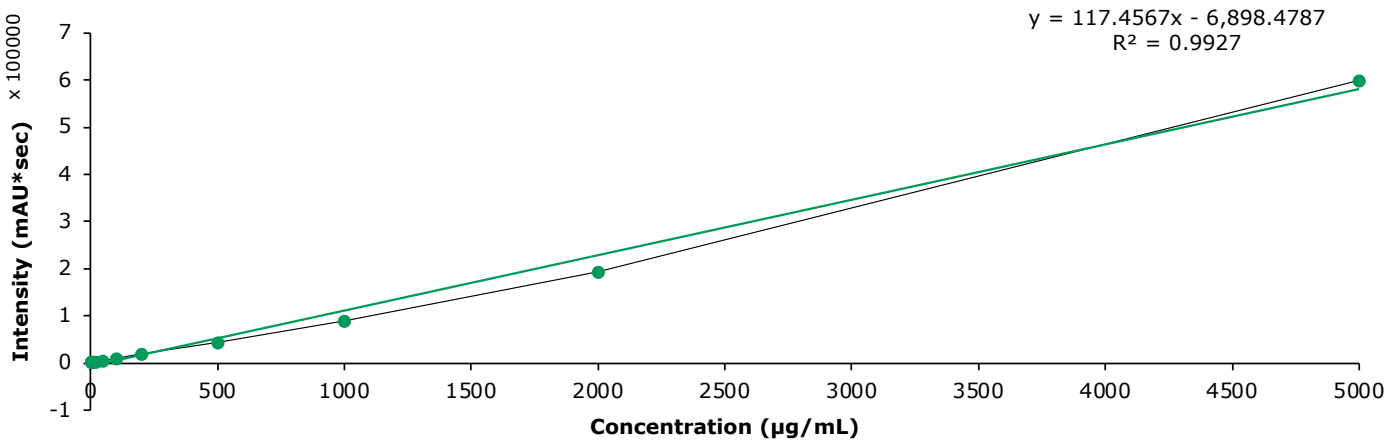

Figure 5. Calibration curve for spectinomycin.

Table 6. Repeatability of 8 aminoglycoside standard solution at 200 ng/mL (n=5)

Compound	Mean Area	Std Dev	RSD (%)
Spectinomycin	18469	318	1.72
Hygromycin B	177800	4392	2.47
Dihydrostreptomycin	171061	3927	2.30
Amikacin	148945	3851	2.59
Kanamycin	257395	6095	2.37
Tobramycin	143749	4728	3.29
Gentamycin	152902	4405	2.88
Neomycin	87728	2127	2.42

Table 7. Recovery (%) after SPE cleanup of 8 aminoglycosides spiked at 50 µg/kg into pork meat

Compound	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)	Mean	SD	RSD (%)
Spectinomycin	96.5	90.0	86.8	91.1	4.9	5.4
Hygromycin	87.9	88.5	87.9	88.1	0.3	0.4
Dihydrostreptomycin	81.0	86.2	84.2	83.8	2.6	3.1
Amikacin	81.8	84.2	78.2	81.4	3.0	3.7
Kanamycin	91.4	94.2	88.9	91.5	2.7	2.9
Tobramycin	102.2	102.7	99.4	101.4	1.8	1.8
Gentamicin	84.9	84.4	78.2	82.5	3.7	4.5
Neomycin	83.2	85.6	79.3	82.7	3.2	3.8

Conclusion

In this determination of 8 aminoglycoside residues in pork meat following the GB/T 21323 -2007 method, the SPE cleanup was performed with a Discovery® DSC-18 SPE tube and for the following LC-MS/MS analysis an Ascentis® Express C18 UHPLC column with 2 µm particles was used. The developed method showed good reproducibility, sensitivity, and linearity over a wide concentration range and met the acceptance criteria as described in GB/T 21323-2007. The R2 for the 8 aminoglycoside residues from 1 to 5000 ng/mL ranged from 0.9927 to 0.9998. The calibration curve derived LODs and LOQs ranged from 0.2 to 0.8 µg/kg and 0.5 to 2.3 µg/kg respectively. The % recovery of the aminoglycosides for a 50 µg/kg spiked sample after SPE cleanup ranged from 81.4 to 101.4%.

References

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5. Determination of aminoglycoside drug residues in animal tissues - HPLC-MS/MS method. GB/T 21323 -2007

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Standards & Reference Substances	
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Hygrovetine B, Vetec™	**V900372**
Streptomycin solution, 1 mg/mL in 1 mM EDTA, analytical standard	**PHR2687**
Kanamycin sulfate from Streptomyces kanamyceticus, Animal Component-free	**PHR1487**
Gentamicin sulfate, meets USP testing specifications, powder	**PHR1077**
Neomycin trisulfate salt hydrate, powder	**33492**
Dihydrostreptomycin, Pharmaceutical Secondary Standard; Certified Reference Material	**PHR1517**
Amikacin, Pharmaceutical Secondary Standard; Certified Reference Material	**PHR1654**
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Acetonitrile, gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
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