

Simultaneous Determination of Aminoglycoside Residues in Livestock and Fishery Products by Phenylboronic Acid Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry

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A rapid and simple method has been developed for simultaneous determinations of the concentrations of nine aminoglycosides (AGs) in livestock and fishery products using phenylboronic acid (PBA) solid-phase extraction (SPE) clean-up. Unlike the widely employed SPE approaches, based on cation-exchange, PBA SPE relies on the reversible formation of covalent bonds with the analytes. The advantage of using PBA lies in the fact that this compound strongly and stably retains analytes, and the pH of the loading solution can be easily adjusted using a high-concentration buffer. The clean-up conditions, such as the pH of the loading solution and the acetonitrile concentration in the elution and wash solvents, were optimized. The degree of recovery measured for nine AGs in six samples (bovine muscle, bovine liver, milk, chicken egg, fish and shrimp) were in the 73–98% range, and the values for the relative standard deviation were 9.3% or less.

Keywords Aminoglycoside, phenylboronic acid, solid-phase extraction, livestock and fishery product

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Introduction

Aminoglycosides (AGs) are antibiotics comprising two or more amino sugars joined to an aminocyclitol scaffold *via* glycosidic bonds. These compounds inhibit bacterial protein synthesis by binding to prokaryotic ribosomes,¹ and are widely used as veterinary drugs to treat bacterial diarrhea and mastitis in livestock.² However, the irrational use of veterinary drugs may increase the health risks for humans associated with the effects of residual amounts of drugs and the growth of drug-resistant bacteria. Therefore, it is important to monitor veterinary drug residues in livestock and fishery products.³

Analytical methods for determining the presence and concentration of AGs in livestock and fishery products have been reported.^{4,5} Many of them rely on acidic solvents for analyte extraction and on solid-phase extraction (SPE) for clean-up. Since AGs are strongly hydrophilic, they are not adsorbed to a satisfactory level onto conventional reverse-phase sorbents. Therefore, reverse-phase SPE cartridges with ion-pair reagents^{6,7} or cation-exchange cartridges^{8,9} have been used for clean-up. Given that the retention strength of sorbents varies depending on the structure of AGs and the pH of the loading solution, some AGs are characterized by low degrees of recovery, resulting from the fact that they are not adequately adsorbed onto SPE cartridges or effectively eluted from them, as a consequence of strong binding interactions. To address these issues, a method using two kinds of cation-exchange SPE cartridges, for instance a strong and a weak cation-exchange cartridge,¹⁰ and a method

relying on two loading solutions with different pH values¹¹ have been reported. Although the development of these methods has certainly afforded an improvement of the degree of recovery of AGs, their implementation has complicated the necessary analytical operations.

In this study, we aimed to develop a simple analytical approach for determining the presence and concentration of AG residues in livestock and fishery products. The substances to be analyzed were apramycin, gentamycin, kanamycin, neomycin, spectinomycin, streptomycin, and dihydrostreptomycin (Fig. 1), which are sold as veterinary drugs in Japan.¹² We examined the clean-up procedure performed using one loading solution and one SPE cartridge based on phenylboronic acid (PBA), which reversibly forms covalent bonds with *diol*-containing compounds.^{13,14} One of the advantages of using PBA is that this acid's covalent bonds, albeit relatively strong, are readily cleaved at low pH. Another advantage of PBA use is that, unlike ionic bonds, the covalent bonds that this compound forms with *diol*-containing target analytes are relatively unaffected by the salt concentration in solution. Therefore, the pH of the loading solution can be easily adjusted using highly concentrated buffer solutions. We have thus developed a rapid and simple analysis method of the nine AGs mentioned above in livestock and fishery products that is based on PBA SPE clean-up followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. To the best of our knowledge, this is the first report on the use of PBA SPE for determining the AGs concentration.

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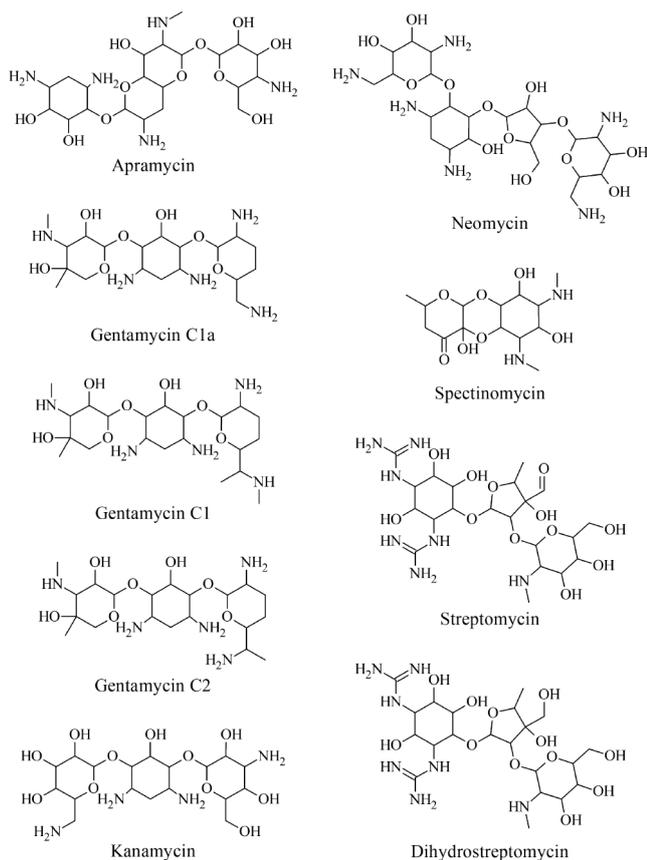


Fig. 1 Chemical structures of the nine aminoglycosides evaluated in this study.

Experimental

Reagents and chemicals

Kanamycin, gentamycin (C1, C1a, and C2 mixture), streptomycin, dihydrostreptomycin, spectinomycin, and neomycin were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Apramycin was purchased from Kanto Chemical Company (Tokyo, Japan). Individual stock standard solutions (200 mg L^{-1}) were prepared in acetonitrile (ACN)/water (2:3, v/v). Working standard solutions A (10 mg L^{-1} each) and B (2 mg L^{-1} each) were prepared from stock standard solutions in ACN/water (2:3, v/v). Both of the stock standard solutions and the working A and B solutions were stored in polypropylene tubes in the dark at 4°C .

Solvents used as the mobile phase for liquid chromatography were of LC/MS grade, whereas the others were of analytical grade. ACN, formic acid (FA), *n*-hexane (hexane), trichloroacetic acid (TCA), ammonia solution (28%, w/w), and sodium hydroxide (NaOH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ethylenediamine-*N,N,N',N'*-tetraacetic acid disodium salt dihydrate (EDTA) was purchased from Dojindo Laboratories (Kumamoto, Japan). Ammonium formate (AF) was purchased from Sigma Aldrich (Buchs, Switzerland). Ultrapure water was produced by a Purelab flex-UV system (Organo, Tokyo, Japan).

Solutions of the other reagents were prepared as follows: (a) 5% (w/v) TCA. TCA, 25 g, was dissolved in 500 mL of ultrapure water; (b) 10% (w/v) EDTA. EDTA, 10 g, was dissolved in 100 mL of ultrapure water; (c) 1 mol L^{-1} NaOH. NaOH, 4 g, was dissolved in 100 mL of ultrapure water; (d)

0.4 mol L^{-1} AF (pH 8.5). AF, 5.04 g, was dissolved in ultrapure water, and the pH of the resulting solution was adjusted to 8.5 with an ammonia solution; the total volume of the solution was finally adjusted to 200 mL with ultrapure water; (e) 0.1 mol L^{-1} AF (pH 8.5). 25 mL of 0.4 mol L^{-1} AF (pH 8.5) and 75 mL of ultrapure water were mixed together, and the pH of the resulting solution was adjusted to 8.5 with an ammonia solution; (f) 0.01 mol L^{-1} AF (pH 8.5). 20 mL of 0.1 mol L^{-1} AF (pH 8.5) and 180 mL of ultrapure water were mixed together, and the pH of the resulting solution was adjusted to 8.5 with an ammonia solution.

The cartridges used for SPE were Bond Elut PBA (100 mg, 1 mL) from Agilent Technologies (California, USA).

Sample preparation

Bovine muscle, bovine liver, chicken egg, fish (rainbow trout), and shrimp (whiteleg shrimp) samples were homogenized with a food processor and then stored at -30°C until analysis. Milk was stored at 4°C and analyzed within the expiration date.

A 2.5 g aliquot of each sample was weighed in a 50-mL polypropylene centrifuge tube, and 13 mL of 5% (w/v) TCA and 0.3 mL of 10% (w/v) EDTA were added to the tube. The resulting mixture was homogenized for 1 min using an ULTRA-TURRAX T25 basic (IKA, Germany), and then 8 mL of hexane was added to the tube. The tube was shaken for 5 min and centrifuged at 3300 rpm for 5 min at 4°C . The hexane layer was discarded, and the supernatant solution left in the tube was transferred to a 25-mL volumetric flask. To the residue left in the tube were then added 9 mL of 5% (w/v) TCA, 0.2 mL of 10% (w/v) EDTA and 5 mL of hexane. The tube was then shaken for 5 min and centrifuged at 3300 rpm for 5 min at 4°C . The hexane layer was discarded, and the supernatant left in the tube was transferred to the same 25-mL volumetric flask mentioned above; the volume of the solution inside the flask was then adjusted to 25 mL with 5% (w/v) TCA. An aliquot of 2 mL of the extracts was transferred to a new polypropylene tube, and its pH was adjusted to 8–10 using 1 mol L^{-1} NaOH; to this solution was then added 1.25 mL of 0.4 mol L^{-1} AF (pH 8.5) to obtain Extract-A.

A PBA SPE cartridge was conditioned with 3 mL of the elution solvent, which consisted of ACN/water/FA (30:70:1, v/v/v), and 3 mL of 0.1 mol L^{-1} AF (pH 8.5). Extract-A was loaded onto the cartridge, and the cartridge was washed with 2 mL of 0.01 mol L^{-1} AF (pH 8.5) and 0.01 mol L^{-1} AF (pH 8.5)/ACN (1:1, v/v). AGs were eluted with 2 mL of the elution solvent into a new polypropylene tube. Approximately 1 mL of this solution was transferred to a 1.5-mL centrifuge tube, which was centrifuged at 15000 rpm for 10 min at 4°C . The supernatant in the 1.5 mL-centrifuge tube was then transferred to a polypropylene vial for LC-MS/MS analysis.

LC-MS/MS conditions

The LC-MS/MS equipment comprised an Agilent Triple Quadrupole LC/MS 6495 system with Agilent 1260 liquid chromatography (Agilent Technologies). Chromatographic separation was performed using a CAPCELL PAK ST (2.0 \times 100 mm) column (Shiseido, Tokyo, Japan) at 40°C . A binary mobile phase was used at a flow rate of 0.4 mL min^{-1} , and a total run time of 20 min. Mobile phase component A was a 0.4% FA aqueous solution, and component B was a 0.4% FA ACN solution. The gradient elution program was as follows: from 0 to 2 min, 10% A, 90% B; at 5 min, 80% A, 20% B; at 11 min, 80% A, 20% B; at 11.5 min, 10% A, 90% B, and the equilibration time was 8.5 min. The sample injection volume was $5 \mu\text{L}$.

Table 1 Liquid chromatography–tandem mass spectrometry acquisition parameters for the nine aminoglycosides evaluated in the present study

Compound	Retention time/min	Molecular weight	Precursor ion (<i>m/z</i>)	Quantitation		Qualitation	
				Product ion (<i>m/z</i>)	Collision energy/eV	Product ion (<i>m/z</i>)	Collision energy/eV
Apramycin	7.5	539.6	540	217	29	378	17
Gentamycin C1	7.6	477.6	479	322	13	157	17
Gentamycin C1a	7.6	449.5	450	322	13	160	25
Gentamycin C2	7.6	463.6	464	322	9	160	21
Kanamycin	7.4	484.5	485	163	25	324	14
Neomycin	7.6	614.6	615	161	35	293	30
Spectinomycin	6.9	332.3	333	98	29	140	21
Streptomycin	7.2	581.6	582	263	35	246	35
Dihydrostreptomycin	7.2	583.6	584	263	30	246	35

Electrospray ionization in the positive mode was used for ion detection and quantitation. Acquisitions were performed in multiple reaction monitoring. The transitions are shown in Table 1. The other optimized parameters were as follows: gas temperature, 200°C; flowrate, 20 mL min⁻¹; nebulizer pressure, 50 psi; sheath gas temperature, 400°C; sheath gas flow rate, 12 L min⁻¹; capillary voltage, 3000 V; ion funnel HPRF, 150 V; ion funnel LPRF, 60 V.

Quantitation

Matrix-matched calibration curves were obtained for analyte quantitation. For this purpose, a blank solution was prepared with a blank sample that underwent the same sample preparation procedure as described above. The mixed standard solution was then diluted with the blank solution, and matrix-matched standard solutions prepared in the 0.001–0.150 mg L⁻¹ concentration range were used to draw calibration curves.

Recovery tests

The degree of recovery of the analytes and the repeatability of analysis results were determined by performing five replicate analyses ($n = 5$) on each of the six samples (bovine muscle, bovine liver, milk, chicken egg, fish and shrimp) that had been spiked with the mixed standard solution at two concentrations (0.1 and 0.5 mg kg⁻¹). Since gentamicin was a mixture of components C1, C1a, and C2, the mixed standard solution was added to the samples so that the sum of the three components matched the mentioned spiking level. The repeatability of the analytical results was expressed in terms of the relative standard deviation (RSD).

Results and Discussion

Optimization of liquid chromatography conditions

In the HPLC analysis of AGs, the use of hydrophilic interaction liquid chromatography (HILIC) columns that do not require ion-pair reagents has recently been increasing.^{4,15} In preliminary tests, the performances of a silica column (Atlantis HILIC Silica, Waters, Milford, MA, USA), an amide column (InertSustain Amide, GL Sciences, Tokyo, Japan) and a special column for streptomycin analysis (CAPCELL PAK ST) were examined. Analyses of the standard solution were performed under three isocratic elution conditions to examine the retention and the peak shape. The isocratic conditions were as follows: an aqueous 0.1% FA solution as mobile phase A and a 0.1% FA ACN solution as mobile phase B; the mixing ratios (A:B) of these mobile phases were 9:1, 8:2 and 7:3. When the silica and

the amide columns were used, gentamycin and neomycin were detected only under conditions whereby A:B = 9:1, which is the most easily eluted condition among the three conditions. And they showed strong tailing of peaks and low peak area values. Therefore, we concluded that these compounds were strongly retained by the columns. On the other hand, when the CAPCELL PAK ST (ST) column was utilized, although peak tailing was observed with gentamycin and neomycin, all AGs were successfully eluted under the conditions whereby A:B = 8:2 and A:B = 9:1. In addition, the peak area values were the largest among the three columns. Based on these results, tests for the optimization of HPLC conditions were performed with an ST column.

Evidence indicated that gradient elution using aqueous 0.1% FA as mobile phase A and 0.1% FA in ACN as mobile phase B did not sufficiently improve the tailing of the gentamycin and neomycin peaks. In the analysis with HILIC, the peak shape could be improved by adding AF or increasing the concentration of FA.^{15,16} First, AF was added to 0.1% FA to turn mobile phase A into 0.1% FA in 10 mM AF. As a consequence of this change in the composition of mobile phase A, however, the tailing of the neomycin peak was not sufficiently reduced. Next, the FA concentration in mobile phases A and B was made to increase to 0.2 and 0.4%. As a result of these increases in FA concentration in the mobile phases, AG elution time decreased and the analyte peaks became sharper; in fact, no tailing of the neomycin peak was observed when mobile phases containing 0.4% FA were utilized. We therefore decided to use mobile phases containing 0.4% FA (in either water or ACN, for mobile phase A or B, respectively) for our sample analyses.

Selection of the extraction solvent

Acidic solvents, like TCA and metaphosphoric acid, have been used for extracting AGs from biological samples.⁴ The addition of EDTA to such solvents improves the degree of AG recovery from metal-rich samples, such as those obtained from livers and kidneys.¹⁷ In this study, we used a solution containing TCA and EDTA to perform sample extraction, based on the fact that our target samples included the metal-rich liver and milk. Furthermore, given that when the extraction procedures were performed on lipid-rich muscle samples, the filter papers became clogged by lipids adhering to the paper during filtration, we tested a liquid–liquid extraction procedure aimed at removing hydrophobic contaminants. For this purpose, hexane, in a volume equal to half of the extraction solvent, was added to the tube containing the bovine muscle extracts, and the tube was shaken for 5 min and centrifuged at 3300 rpm for 5 min at 4°C. Subsequently, the hexane layer was discarded. By this

procedure, sufficient amounts of hydrophobic contaminants were removed from the mixture for clogging of the filter paper not to be observed. In fact, since fatty acid group contaminants become non-ionizing form and tend to be more soluble in the hexane layer under strong acidic condition, these contaminants could be efficiently removed.

Optimization of the PBA SPE clean-up procedure

Loading solution. Under alkaline conditions, PBA forms covalent bonds with diol-containing compounds.¹⁸ Notably, however, the pH value determines the charge state of the amino groups of AGs. Therefore, the pH of the loading solution will influence the formation of PBA-AG bonds. Thus, in order to evaluate the SPE performance under different pH conditions, the pH of 0.1 mol L⁻¹ AF solutions spiked with the nine AGs was adjusted to 7.0, 8.0, 9.0 and 10.0, and the resulting solutions were loaded onto conditioned PBA SPE cartridges (Fig. 2). When the loading solution at pH 7 was used, the measured degrees of recovery were in 7–56% range. At this pH, PBA was probably not activated. Overall, good degrees of recovery were obtained using loading solutions at pH 8 and 9, whereas at

pH 10 the degrees of recovery of apramycin and kanamycin decreased. We assumed that strong binding was responsible for the inadequate AG elution from the cartridge, since the AGs were hardly detected in either the loading solutions or the wash solvents passed through the cartridges. At pH 10, the AG structures changed as a consequence of the loss of charge from the amino groups, which might cause a change in the PBA binding mode. In fact, instead of PBA-diol bonds, more stable bonds, like the PBA- β -amino alcohol bond,¹⁸ might form. Based on the described results, we decided to adjust the pH of the loading solution to 8.5, chosen as an intermediate value between pH 8 and 9.

Elution solvent. PBA-diol bonds are cleaved by acidification, which results in the formation of the inactive neutral form of PBA and the release the bound compounds.¹⁸ Therefore, we decided to use an aqueous 1% FA as the elution solvent. For rapidity purposes, it is desirable to analyze the eluate directly by LC-MS/MS, without implementing the solvent exchange process. In this case, ideally the ACN concentration in the eluate should be the same as that in the mobile phase, which would help to avoid problems associated with the liquid

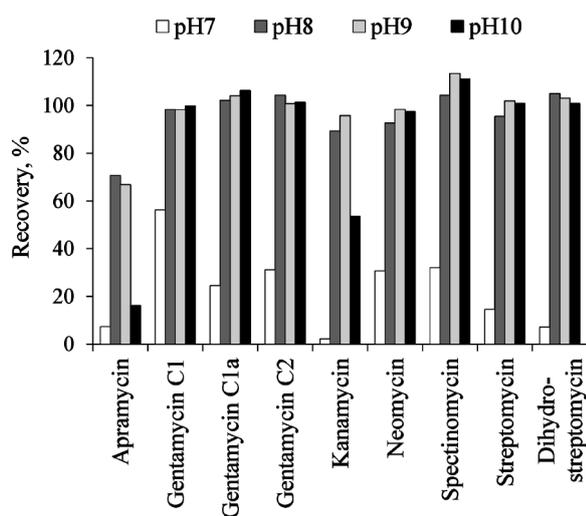


Fig. 2 Effect that the pH of the loading solution for phenylboronic acid solid-phase extraction clean-up had on the degrees of recovery of the nine aminoglycosides.

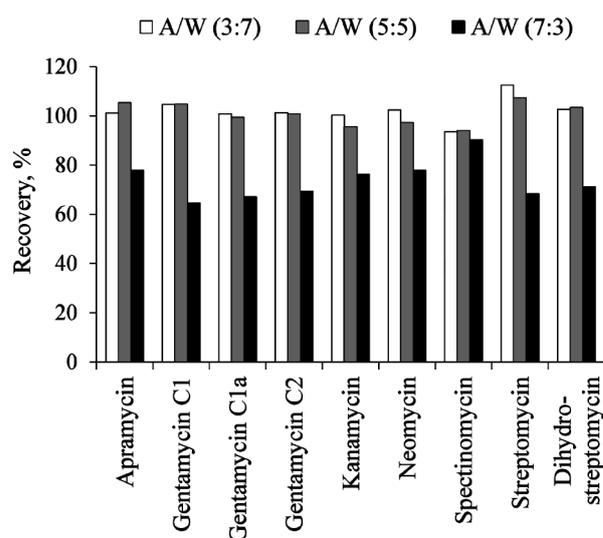


Fig. 3 Effect that the acetonitrile concentration in the elution solvent for phenylboronic acid solid-phase extraction clean-up had on the degrees of recovery of the nine aminoglycosides. A/W means acetonitrile ratio to water; the numbers in parentheses indicate the ratio.

Table 2 Values of the degrees of recovery and analysis repeatability ($n = 5$) for the nine aminoglycosides in the six samples of livestock and fishery products at two spiking levels

Analyte	LOQ/ mg kg ⁻¹	Recovery \pm relative standard deviation, %											
		Bovine muscle		Bovine liver		Milk		Chicken egg		Fish		Shrimp	
		0.5 mg kg ⁻¹	0.1 mg kg ⁻¹	0.5 mg kg ⁻¹	0.1 mg kg ⁻¹	0.5 mg kg ⁻¹	0.1 mg kg ⁻¹	0.5 mg kg ⁻¹	0.1 mg kg ⁻¹	0.5 mg kg ⁻¹	0.1 mg kg ⁻¹	0.5 mg kg ⁻¹	0.1 mg kg ⁻¹
Apramycin	0.01	86 \pm 2.6	79 \pm 2.2	77 \pm 6.3	78 \pm 4.5	75 \pm 3.7	88 \pm 3.2	74 \pm 3.8	81 \pm 2.6	79 \pm 3.0	87 \pm 5.1	84 \pm 4.9	80 \pm 2.0
Gentamycin C1	0.01	83 \pm 4.1	84 \pm 5.1	91 \pm 4.8	91 \pm 3.1	79 \pm 3.5	95 \pm 2.3	87 \pm 3.7	90 \pm 4.5	84 \pm 4.6	90 \pm 1.5	88 \pm 2.3	94 \pm 7.3
Gentamycin C1a	0.01	84 \pm 5.0	88 \pm 1.2	90 \pm 5.8	96 \pm 3.8	81 \pm 6.2	88 \pm 4.6	89 \pm 5.1	89 \pm 2.8	87 \pm 3.0	84 \pm 6.6	85 \pm 3.5	94 \pm 7.3
Gentamycin C2	0.01	86 \pm 4.4	84 \pm 9.2	91 \pm 4.1	97 \pm 3.6	80 \pm 3.2	90 \pm 8.6	93 \pm 4.3	93 \pm 5.0	89 \pm 6.7	88 \pm 3.9	83 \pm 4.7	97 \pm 7.2
Kanamycin	0.01	73 \pm 4.1	74 \pm 5.1	77 \pm 5.3	73 \pm 5.2	79 \pm 1.9	89 \pm 2.8	73 \pm 4.8	87 \pm 4.6	87 \pm 4.6	76 \pm 4.8	79 \pm 4.7	93 \pm 8.3
Neomycin	0.01	73 \pm 2.9	79 \pm 3.9	80 \pm 2.2	89 \pm 5.1	80 \pm 4.9	85 \pm 6.2	89 \pm 2.4	89 \pm 1.4	84 \pm 5.1	91 \pm 5.6	82 \pm 1.2	92 \pm 8.2
Spectinomycin	0.02	89 \pm 2.2	86 \pm 1.9	88 \pm 5.3	98 \pm 3.7	86 \pm 4.5	86 \pm 3.6	86 \pm 3.6	92 \pm 3.5	93 \pm 3.0	89 \pm 2.9	95 \pm 2.2	95 \pm 5.3
Streptomycin	0.02	74 \pm 4.6	83 \pm 3.8	77 \pm 4.5	88 \pm 7.7	73 \pm 4.6	86 \pm 7.2	87 \pm 9.0	82 \pm 5.3	79 \pm 9.3	91 \pm 5.7	86 \pm 3.1	89 \pm 2.5
Dihydrostreptomycin	0.01	85 \pm 6.5	90 \pm 2.4	89 \pm 5.0	90 \pm 4.5	75 \pm 5.9	92 \pm 4.8	91 \pm 5.9	87 \pm 4.7	89 \pm 4.6	89 \pm 3.9	87 \pm 2.3	97 \pm 3.1

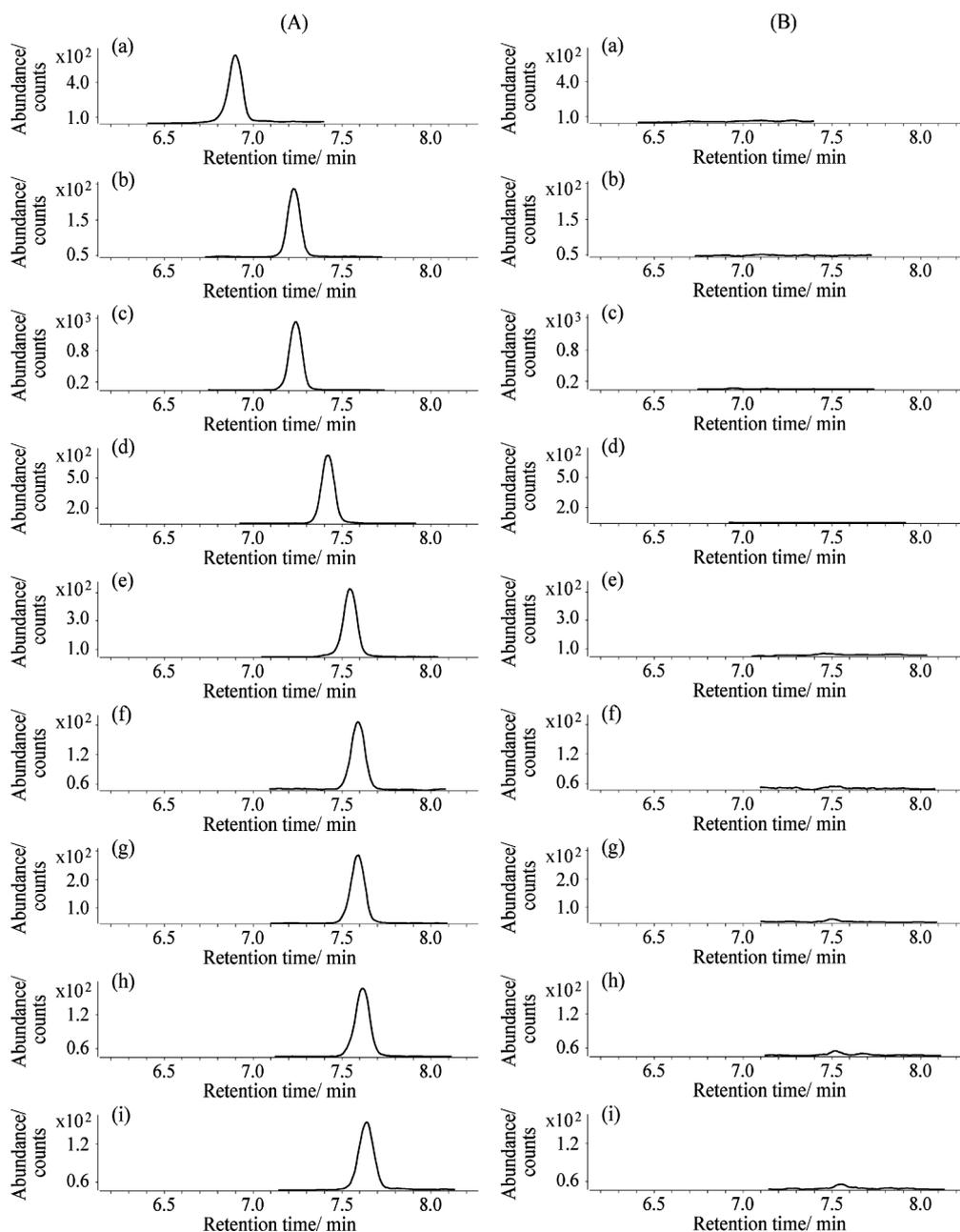


Fig. 4 Liquid chromatography-tandem mass spectrometry-multiple reaction monitoring chromatograms of the nine aminoglycosides spiked in a blank bovine muscle sample to a final 0.1 mg kg^{-1} concentration (A). The aminoglycosides are as follows: (a) spectinomycin, (b) streptomycin, (c) dihydrostreptomycin, (d) kanamycin, (e) apramycin, (f) gentamycin C1a, (g) gentamycin C2, (h) gentamycin C1, (i) neomycin. Chromatograms of a blank bovine muscle sample (B).

chromatography-based analysis, like the appearance of split and fronting peaks. On the other hand, given that the presence of ACN might affect the elution of AGs from the PBA SPE cartridge, AGs retained on the PBA SPE cartridge were eluted with 1% FA-containing ACN/water mixtures of various compositions (0:10, 3:7, 5:5 and 7:3, v/v); the effects of the ACN concentration on AG recovery were then examined. The results from these experiments are summarized in Fig. 3. In detail, the values for the relative degrees of recovery were good, between 93% and 113% (average 102%) in ACN/water = 3:7 (v/v) and ACN/water = 5:5 (v/v). However, the values for these parameters were as low as 65–90% (average 74%) when ACN/water = 7:3 (v/v) was used as the elution solvent. We speculated

that, at high ACN concentrations, the AG adsorption onto the PBA cartridge driven by intermolecular forces and ionic interactions increased in strength as a consequence of the decrease in the polarity of the elution solvent. We thus decided to use as the elution solvent ACN/water (3:7, v/v) containing 1% FA. *Wash solvent.* Hydrophilic contaminants are easily washed away with an aqueous solvent, and hydrophobic contaminants are easily washed away with an organic solvent. Therefore, we decided to carry out a two-stage washing procedure consisting of a first wash conducted with an aqueous solvent and a second one conducted with an organic solvent. Wash tests were conducted with 0.01 mol L^{-1} AF (pH 8.5; Wash-A), ACN or a mixed solution of both to investigate whether AGs were eluted

from the SPE cartridges as a consequence of the wash process. Evidence indicated that AGs were not eluted by any of the mentioned wash solvents. Hydrophobic contaminants can be removed to a satisfactory degree using a wash solvent with a higher ACN concentration than the elution solvent. Since the ACN/water ratio of the chosen elution solvent was 3:7, the ratio for the solvent to be used in the second wash was decided to be 5:5. Consequently, we decided to wash the SPE cartridge sequentially with Wash-A and ACN/Wash-A = 5:5 (v/v).

Recovery tests

We performed recovery tests for each bovine muscle, bovine liver, milk, chicken egg, fish and shrimp samples at two concentrations (0.1 and 0.5 mg kg⁻¹). The results from these tests and the typical chromatograms of a bovine muscle sample spiked with the nine AGs are given in Table 2 and Fig. 4. The values for the degree of recovery were in the 73 – 98% range, and values for the RSD, a parameter measuring the repeatability of the analyses, were 9.3% or less, with no observed influence of the concentration of the spiked analyte or of the sample type. The values of the determination coefficient of the calibration curves were 0.99 or more for all of the samples and the analytes. The lower limits of quantitation (signal-to-noise ratio ≥ 10) were 0.02 mg kg⁻¹ for spectinomycin and streptomycin, and 0.01 mg kg⁻¹ for the other analytes.

Conclusions

In this study, we have developed a rapid and simple method for the simultaneous determination of the concentrations of nine AGs in livestock and fishery products using PBA SPE clean-up. In the extraction process, an efficient procedure for the removal of hydrophobic contaminants was developed that relies on liquid-liquid extraction with hexane. Optimization of the PBA SPE clean-up was performed by investigating how the pH of the loading solution and the ACN concentration in the elution and wash solvents influenced the degree of recovery of the nine AGs. Recovery tests were performed on the six samples (bovine muscle, bovine liver, milk, chicken egg, fish and shrimp). Good degrees of recovery and analysis repeatabilities were obtained for all of the samples. The analytical method based on PBA proved to be useful for simultaneous determinations of the concentrations of nine AGs.

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