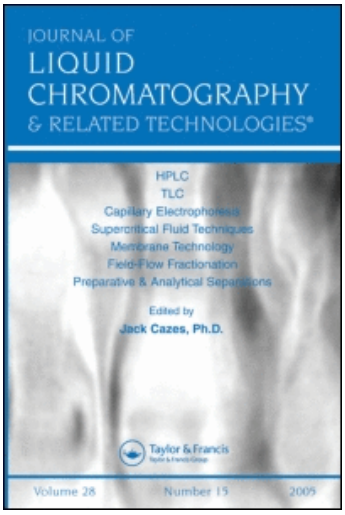

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Determination of Kanamycin A in Animal Feeds by Solid Phase Extraction and High Performance Liquid Chromatography with Pre-Column Derivatization and Fluorescence Detection

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Abstract: A selective method was developed for the determination of kanamycin A in medicated animal feeds by reversed-phase high performance liquid chromatography (HPLC) with precolumn derivatization. Samples were extracted with 0.1 mol/L hydrochloric acid solution and cleanup was achieved with MCX solid phase extraction (SPE). The purified extract was derivatized with *O*-phthaladehyde (OPA), separated on a reversed-phase C_{18} column and the fluorescence detection was performed at the excitation and emission wavelengths of 230 and 389 nm, respectively. The run time was approximately 14 min, at the flow rate of 0.8 mL/min. This method provides the average recoveries 98.4%–106.0% in feeds spiked in the range of 10–2000 g/ton, with the coefficients of variation 1.17%–9.78%. The limit of detection in feeds was 5 g/ton and the limit of quantification in feeds was 10 g/ton, which are well below the effective dose.

Keywords: Kanamycin A, HPLC, Animal feeds, Precolumn derivatization, SPE, Fluorescence

INTRODUCTION

Kanamycins are aminoglycoside antibiotics, which are synthesized by *Strep-comtomyces kanamyceticus* as a complex of three structurally similar analogues: kanamycin A, B, and C (Figure 1). In this mixture, kanamycin A

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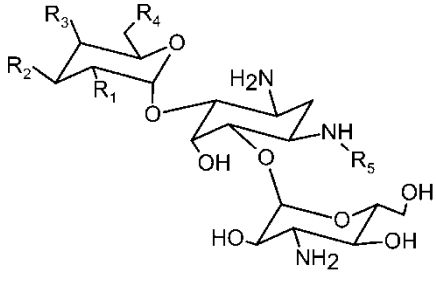
is the main component (>95%), kanamycin B and C are minor components (<5%). They are effective against a broad spectrum of gram-negative and gram-positive bacteria. Kanamycins are popularly applied to prevent diseases and promote growth performance of farm animals. However, the potential problems with ototoxicity and nephrotoxicity were the main appearance of drug residues.[1] Maximum residue limits of kanamycins in tissues have been set by many countries in the world. Therefore, aminogloside antibiotics are not regulated as a feed additive used in animal feeds in China, except apramycin and neomycin, which could only be used as therapy for pigs and poultry at levels between 80–100 mg/kg complete feed for 7 d, withdrawal period is at least 21 d.[2] However, the illicit use of kanamycins as a feed additive in farm animals can pose a food safety risk. Therefore, exploration of a reliable analytical method is critically necessary to ensure the detection of kanamycins in a high sensitivity from animal feeds, in order to indirectly protect humans from harmful antibiotics residue in the animal's tissues.

As mentioned above, kanamycin A is the main component in the kanamycin mixture, so it is usually chosen as the detected target compound. The official pharmaceutical British method used for the detection of kanamycin A (kanamycin acid sulfate raw material and formulations) in food and tissue is the microbiological method.[3] However, this method is time consuming and has a low sensitivity, which can hardly provide reliable results. There are also other analytical methods for determination of kanamycin A in animal tissue, human serum, and plasma, which include: nuclear magnetic resonance spectroscopy,[4] mass spectrometry,[5] and high performance liquid chromatography (HPLC).[6,7] Compared to nuclear resonance spectroscopy and mass spectrometry methods, HPLC is a simple procedure for detecting aminoglycosides,[8] relatively easy to develop, and



	R_1	R_2	R_3	R_4	R_5
Kanamycin A	OH	OH	OH	NH_2	H
Kanamycin B	NH_2	OH	OH	NH_2	H
Kanamycin C	NH_2	OH	OH	OH	H

Figure 1. Structure of kanamycin components.

is well suited for the analysis of small molecular compounds. Simple chromatographic methods are not applicable due to the lack of volatility, chromophores, and strong hydrophility of aminoglycosides.[5] Therefore, two types of derivatization can be used for HPLC separation, precolumn derivatization and post column derivatization. o-phthaladehyde (OPA),[9–12] 1-fluoro-2,4-dinitrobenzene (FDNB),[13] and 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl)[14,15] have been used as derivative reagents for this purpose. However, no research of a precolumn HPLC method applicable to feeds has been published thus far.

The aim of this work was to develop and validate a simple, precise, reliable, and sensitive method for separation, detection, and qualitative determination of kanamycin A in animal feeds. Special attention is paid to sample preparation as well as to improve HPLC determination sensitivity. The submission method is used to detect kanamycin A in poultry and pig complete feed, concentration feed, and premixed feed.

EXPERIMENTAL

Materials and Reagents

All chemicals and solvents used were analytical or HPLC grade unless otherwise stated. Milli-Q water, 18.2 MΩ · cm. was from the Millipore Corp. The standard of kanamycin A (kanamycin sulfate salt) was purchased from Sigma Chemicals Co. and the pure raw material was the gift from Qilu Pharmaceutical General Factory (Shandong, China). OPA (grade for fluorogenic detection) was bought from Merck (Darmstadt, Germany). Ammonium acetate, acetic acid, hydrochloric acid, formic acid, and ammonia solution were purchased from Beijing Chemical Company (Beijing, China).

The standard stock solution was composed of 1.0 mg/mL of pure standard product, which was dissolved with deionized water and stored at + 4°C, for three months. The working standard solution was diluted with borate buffer and prepared fresh daily. The borate acid buffer was 0.4 mol/L (24.73 g borate acid was dissolved in 1.0 L of distilled water and the pH was adjusted to 9.5 with 6.0 mol/L sodium hydroxide solution). The OPA derivative reagent was prepared by dissolving 0.134 g OPA into 5.0 mL of methanol, adding 100 μL of mercaptoethanol, then diluting to 25.0 mL with borate buffer (pH 9.5), protected from light and prepared fresh daily.

The extracted solution of 0.1 mol/L hydrochloric acid was prepared by diluting 8.4 mL of hydrochloric acid in 1 L of water. Five percent ammonia methanol solution was used as the initial eluting solution, concluding with 5 mL of ammonia solution and 95 mL of methanol. Solid phase extraction columns Oasis® MCX 3 cc (60 mg), purchased from Waters (China), were used for cleanup.

Apparatus and Chromatographic Conditions

The HPLC system used for method development consisted of Waters 600 controller, Waters™ 600 pump, Waters 717 plus autosampler, and Waters 2475 multi λ fluorescence detector (Waters, USA). The system was controlled by data station Empower 1154. An HPLC analytical column (RP_{18} 4.6 mm × 250 mm) (Waters XTerra™, USA) and guard column (4.6 mm × 20 mm) packed with 5 μm particle size (Waters Symmetry®, USA) were used. The mobile phase consisted of an ammonium acetate solution (0.77 g ammonium acetate was added to 800 mL water then added to 40 mL acetic acid with water to equal 1 L) and acetonitrile (50:50, v/v). The flow rate was 0.8 mL/min and the injected volume was 100 μL at room temperature. The analyte was monitored by fluorescence with excitation wavelength at 230 nm and emission wavelength at 389 nm. The cleanup system included a vacuum manifold processing station of Agilent Technologies and a vacuum pump (Agilent Technologies, USA). The centrifuge used was TGL-20M (Changsha, China).

Sample Preparation and Extraction

Typical commercial free kanamycin feed used in this study included complete feed, concentrated feed, and premixed feed for pigs and poultry. All feed samples were made at the pilot workshop of Ministry of Agriculture Feed Industry Center (MAFIC) in (Beijing, China). These feeds were used to provide the presence of interfering peaks, carry out accurate and precise experiments, and were also, further used to examine the applicability of the method in medicated feeds.

The 0.1% kanamycin A premix was prepared by diluting commercially pure raw material with calcium carbonate, then the feed samples with kanamycin A needed for this study were prepared by adding 0.1% kanamycin A premix of a certain amount in 1 kg of typical kanamycin A free commercial feeds, which were prepared as mentioned above. In order to make sure the uniformity of samples, kanamycin A premix was gradually diluted to 1 kg with the feeds. All samples were ground so that they passed through a 0.25 mm mesh screen before extraction.

Ground feed samples (complete feed, 5 g; concentrated feed, 3 g; premix, 1 g) were weighed into 50 mL polyethylene centrifuge tubes, and 40 mL of 0.1% hydrochloric acid extracted solution was added; the tubes were gently shaken for 25 min, then centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a filter paper into 100 mL volumetric flasks. The sediment was re-extracted with two additional 30 mL of the extracted solution. All the supernatant was combined and diluted to volume with the extracted solution. For the premix, the above solution should be further diluted 1:2 with the extracted solution, and then a part of the extract was added to 1.5 mL centrifuge tubes, centrifuged at 20,000 rpm for 10 min. The supernatant was prepared for further cleanup.

SPE Column Cleanup

Cleanup for all the samples was performed on MCX columns. The samples were applied to SPE cartridges that were conditioned previously with 3 mL of methanol and 2×3 mL of water, the sample extraction solution was loaded and washed twice. The flow rate through the SPE columns was at ca. 2 drops/s by applying positive pressure. When the eluting solvent passed through the column, the eluate was captured in a 10 mL tube and evaporated to near dryness by a gentle stream of nitrogen gas at room temperature; then 1 mL of borate acid buffer was added to the tube and a suitable solution was used to derivatize for HPLC analysis.

Derivatization and HPLC Determination

A sample extract of 400 μL was placed into a vial for HPLC analysis and 150 μL of OPA derivative reagent was added, using a vortex mixer. The sample and OPA derivative reagent were allowed a reaction time of 10 min prior to injection into the HPLC system. After the first injection, the next sample was being derivatized while the first sample was eluting from the HPLC system. The injection volume was 100 μL.

A standard calibration curve was generated by running a working standard solution and plotting the recorded peak area versus the corresponding concentration of analyte. The precision was expressed with relative standard derivation (RSD) by means of injecting OPA-kanamycin A working standard solutions, as well as feed samples with kanamycin A. The determination was repeated five to eight times. The recoveries were evaluated using six levels of concentration lying within the linear range. These assays were also repeated five times.

RESULTS AND DISCUSSION

Liquid Chromatography

Kanamycin A is highly polar and has no chromophore for absorption in the ultraviolet (UV) or visible region, thereby posing difficulties in its isolation from matrix components after base extraction. Therefore, pre- or post-column derivatization is necessary for UV or fluorescence detection. Compared with post-column, the pre-column procedure was very simple, fast, and it can occur at room temperature and does not require special equipment.[8] This procedure can reduce polarity for perfect separation, thereby, increasing retention and reducing tailing of kanamycin A through the C_{18} analytic column.

For choosing optimal derivatization, two common reagents, OPA[6,7,11,16] and FMOC-Cl[15] were compared. Derivatization with FMOC-Cl has very strong fluorescence[17] and the target peak is very near to the peak of

FMOC-Cl. Also the extra amount of FMOC-Cl in the sample solution must be eliminated by glycin, however, the extra OPA needn't be removed. So, OPA was used in further study. The reaction of this method was at pH 9.5, therefore, precolumn HPLC with OPA derivatization as the optimal method to detect kanamycin A in feeds was chosen in this study.

The time of derivatization and amount of derivative reagent were studied to optimize the derivatization conditions for the procedure of kanamycin A. The formation and decay of the OPA-kanamycin A derivative were measured by making repeated injections of 10 μg/mL standards and plotting peak area versus time. It was derivatized at 2, 3, 5, 6, 10, 15, and 20 min at room temperature, respectively, and the maximum peak area was reached at 10 min (Figure 2). The optimal amount of reagent was chosen by making repeated 10 μg/mL standard solutions, the derivative time was 10 min. At each derivatization, 400 μL standard solution with 50, 100, 150, 200, 250, and 300 μL of OPA derivative reagent were reacted. The data indicated that the optimal amount was 150 μL (Table 1). The analytical column used was reverse phase C_{18}, which easily separated kanamycin A after derivatization. Typically, OPA fluorescence derivatives were detected at 340 nm excitation and 450 nm emission wavelengths.[12] However, the fluorometric detection at 230 nm excitation and 389 nm emission wavelengths had higher sensitivities of OPA-kanamycin A derivative and lower interfering impurities. The composition of the mobile phase has a great effect on the quality of HPLC separation. Kanamycin A, which like all other aminoglycoside antibiotics, has polar amines, so the ion-pair mobile phase retention mechanism was found to be suitable for separation. Increasing the organic solvent portion resulted in decreasing the retention time, peak width, and asymmetry. Thus, the ratio of 50% acetonitrile was chosen as

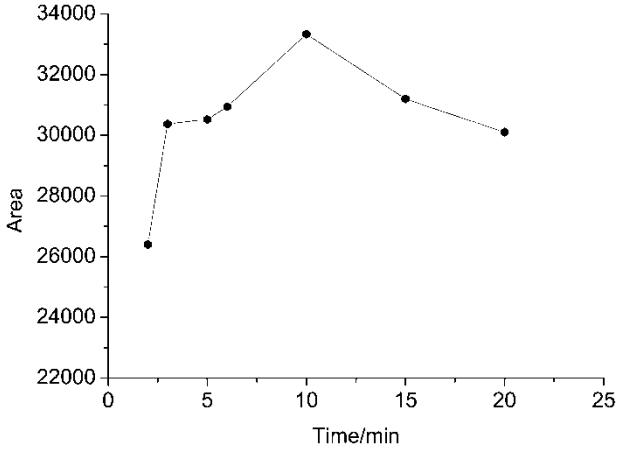


Figure 2. Formation and decay of OPA-kanamycin A derivative.

Table 1. The effect of amount on peak area of kanamycin A

Kanamycin A standard solution[a] (μL)	OPA derivatization agent[b] (μL)	Mean peak area (n = 5)
400	50	26785
400	100	28798
400	150	30817
400	200	28815
400	250	25618

Note: [a]The concentration of kanamycin A was 10 μg/mL.
[b]Solution was prepared by dissolving 0.134 g OPA into 5.0 mL of methanol, adding 100 μL of mercaptoehanol, then diluting to 25.0 mL with borate buffer (pH 9.5).

organic mobile phase and 50% of ammonium acetate solution. The peak symmetry was very good in this solvent system.

Choice of Extract Solvent and Clean-up

It is very important to make sure that kanamycin A was extracted completely from feed matrix. Kanamycin A is of high polarity because of numerous amines and hydrophilic groups. The organic solvents are not suitable as extraction solvents. Initial attempts were made to extract kanamycin A from feeds by borate buffer (pH 9.5) and 0.1 mol/L of sodium hydroxide aqueous solution, but this resulted in an incomplete recovery. Then, according to the characteristic that kanamycin A could be easily resolved in water and exists with a positive charge in acidic solution, the 0.1 mol/L hydrochloric acid solution was finally used as the extracting solution in this study and recovery over 98.4% was obtained. The procedures of the extraction and cleanup were very important for setting up a new method. Formic acid solution was used to extract aminoglycoside antibiotics completely and the recoveries achieved more than 90% in serum.[5] However, feed matrix, which is made of many different nutritional ingredients including soybean meal, corn, fat, mineral, vitamin, and other additives is very complicated. However, the recovery was lower than 0.1 mol/L hydrochloric acid solution using formic acid as the extraction solution. Sample extracts can not be analyzed directly because the analytical column is easy to be polluted by the presence of other components. The recovery and efficiency of cleanup by traditional liquid-liquid extraction methods are lower and/or in consistent, which is easily effected by feed matrix.[18] At the same time, kanamycin A will not move from the aqueous phase into organic immiscible solvents at any pH. Therefore, cleanup by liquid-liquid extraction could not be used. In recent years, solid phase extraction is used frequently for cleanup. The mixed mode

cation exchanger (Oasis®MCX) was chosen for kanamycin A cleanup in this study. MCX is a strong cation-exchanger; it is suitable for basic analytes in highly complex matrices, and for basic analytes that are too hydrophilic for Oasis HLB extraction. The SO_3^- groups of the MCX stationary phase are especially suitable for combining with hydrophilic amines; a high recovery was achieved, which exceeded 98.4%. The cleanup steps are shown in Table 2. Results indicate that the cleanup procedure is good for feed samples, and no interference peaks were observed near the target analyte.

Animal feeds usually contain some free amino acids, such as lysine, methionine, arginine, threonine, and tryptophan, which are used as additives to the feeds for improving animal production. All of these kinds of amino acids could react with the OPA derivative reagent and could be possibly eluted. So, it is very important to make sure that free amino acids in the feeds have no interferences with the peak of kanamycin A. Mixed amino acid standards solutions for amino acid analysis in feeds were used to check for interferences. The results showed that there were no peaks near kanamycin A; all peaks appeared before 6 min. The interference from other aminoglycoside antibiotics, including apramycin, neomycin, amikacin, tobramycin, streptomycin, and gentamycin was assured. The results showed that there were no effects on kanamycin A determination.

Linearity and Accuracy

Regression analysis of the data obtained from 0–10 μg/mL (0, 0.1, 0.5, 1.5, 2.5, 5.0, 6.0, 8.0, and 10.0 μg/mL) working solution and injecting 5–8

Table 2. The steps and purposes of solid phase extraction (SPE) in feed clean-up

Steps	Operating	Purposes	Eluant to
Condition/ equilibration	3 mL methanol and 2 × 3 mL water	Prepares sorbent for use	Waste
Load	3 mL of sample extraction solution	Basic analytes are retained by reverse-phase mechanism	Waste
Wash 1	3 mL of 0.1 mol/L hydrochloric acid solution	Removes cations absorbed	Waste
Wash 2	3 mL of methanol	Removes other reverse-phase interference retained	Waste
Elute	6 mL of ammonia methanol solution (5 + 95, v/v)	Elutes kanamycin A	10 mL tube

Table 3. Nominal value, fitted value ($y = 2842.2x - 958.04$) and RSD% calculated for each concentration tested

Standard solution concentration (μg/mL)	n	Mean peak area	RSD (%)
0.1	5	399.8	3.68
0.5	5	948.8	4.52
1.5	5	1912.4	3.56
2.5	5	4850.4	1.10
5.0	6	12386.9	2.02
6.0	5	15398.2	2.34
8.0	5	21774.3	0.44
10.0	8	28581.1	1.72

replicates was performed (Table 3). The results showed detector response to be linear ($R^2 = 0.9940$) and the intercept was close to zero. The samples were included with several levels of kanamycin A to yield 10, 20, 40, 80, 200, 300, and 2,000 μg/g. This method for included samples showed good linearity ($R^2 > 0.9985$). The results suggest that the linearity of the method would be quantitative by the external standard method.

The recoveries were higher than 98.4% by analyzing five replicated samples from each kind of pig and poultry feed. Each set was previously spiked with kanamycin A at different levels, based on the doses used in animal production (Table 4). The high recovery ranged from 98.4%–106.0% ($n = 5$). Raw materials, veterinary formulation, and culture media

Table 4. Recovery of kanamycin A from included feeds

Feeds	Included level (g/ton)	Recovery ($n = 5$), (%)			
		Maximum	Minimum	Mean	RSD (%)
Poultry	10	118.5	94.6	106.0	9.78
complete feed	40	106.7	100.4	103.4	2.51
	80	107.2	93.7	100.9	4.91
	200	104.4	97.2	100.1	2.78
Poultry premix	2000	104.1	98.3	101.5	2.60
Poultry	40	104.7	99.9	101.8	2.04
concentration feed	80	102.3	98.9	100.4	1.27
	200	101.2	97.4	99.3	1.53
	300	101.1	95.3	98.4	2.31
	10	116.4	99.6	101.5	6.99
Pig complete feed	20	104.1	99.6	101.0	1.81
	80	101.6	99.0	100.4	1.17
	200	108.1	96.4	101.9	4.19

Table 5. Accuracy of poultry complete feed recovery

Recovery of poultry complete feed			
Spiking level (g/ton)	40	80	200
Day 1-kanamycin A %			
Replicate 1	100.4	107.2	100.1
Replicate 2	103.2	93.7	100.8
Replicate 3	105.4	100.0	97.2
Average	103.0	100.3	99.4
RSD	2.44	6.74	1.94
Day 2-kanamycin A %			
Replicate 1	101.6	100.2	104.4
Replicate 2	106.7	103.2	98.2
Replicate 3	109.2	99.0	100.1
Average	105.8	100.8	100.9
RSD	3.91	2.12	3.13
Day 3-kanamycin A %			
Replicate 1	110.7	106.2	102.4
Replicate 2	104.7	102.4	100.6
Replicate 3	101.1	100.9	98.1
Average	105.5	103.2	100.4
RSD	4.60	2.65	2.16
Overall %			
Average	104.8	101.4	100.2
RSD	5.57	4.24	2.26

had high recovery results.[19] Method precision was evaluated for each day of analysis in which $n > 2$. The results showed that the low sample concentration, 40 g/ton, had a larger RSD than any other levels observed. This occurred during the entire three days (Table 5).

The chromatogram of the standards and samples are illustrated in Figure 3. The retention time of kanamycin A is about 14 min. The limit of detection in animal feeds was 5 g/ton and the limit of quantification was 10 g/ton.

CONCLUSIONS

A specific, accurate, and fast method for detection of kanamycin A in animal feeds using OPA with precolumn derivatization was developed. This procedure exhibited good reproducibility, sensitivity, and precision, resulting in a reliable method for detection of kanamycin A in feeds.

An important part of this analytical method was the sample preparation. Special attention was paid to the cleanup steps using the SPE MCX

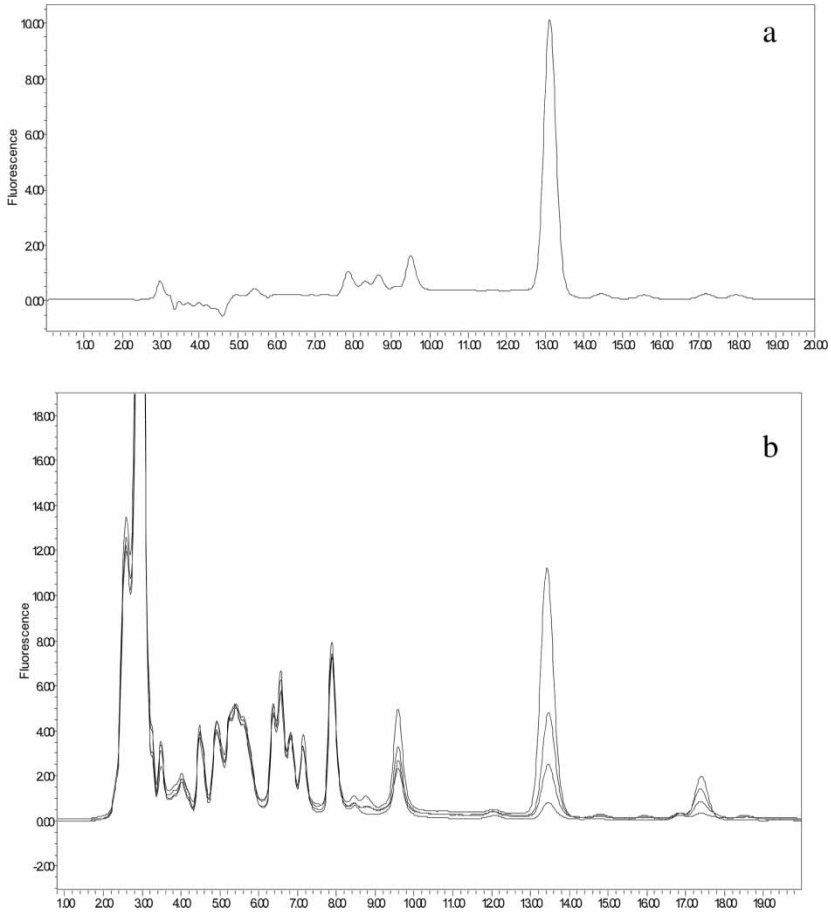


Figure 3. Typical HPLC examples of kanamycin A and poultry complete feeds. (a) 10 μg/mL kanamycin A HPLC working standard; (b) poultry complete feeds at level of 10 μg/g, 40 μg/g, 80 μg/g, and 200 μg/g.

cartridge, with elimination of interfering compounds, without loss of target analyte, as well as quantitative elution. The extraction, cleanup, and derivatization method can be applied to actual samples. The derivatization method demonstrated satisfactory results for its application in the studied matrices. Though the feed is a complex matrix, it achieved high recovery using Oasis®MCX SPE column cleanup and XTerra™ RP_{18} column separation. The accuracy and linearity of the method were adequate for monitoring feed quality. This method is suitable for determination of kanamycin A in feeds at low levels, with a simple, sensitive, and fast procedure.

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