DETERMINATION OF COLISTIN IN POULTRY FEED BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT. Colistin, or also known as polymyxin E is considered as the last-resort treatment for multiantibiotic-resistant gram-negative bacterial infections in humans. Usage of colistin in animal feed raises a global concern of antimicrobial resistance occurrence, hence the banning of colistin in animal feed through Malaysian Feed Act 2009 regulations since 2019. A confirmatory method for the determination of colistin in poultry feed was developed and validated. In this study, colistin A and colistin B were extracted from samples with the mixture of 10 % trichloroacetic acid-acetonitrile and purified with mixed-mode weak cation exchange cartridges. Analytes were separated from matrix components using ultra-high performance liquid chromatography, which were detected with electrospray ionization on a triple quadrupole mass spectrometer. Colistin A was determined by precursor ion of 390.9 m/z with major fragmentation of 101.2 m/z and 384.9 m/z, while colistin B was detected at 386 m/z with major fragments of 101 m/z and 380 m/z. Calibration curves were linear from 0 to 1000 µg/kg for colistin A and B. The range of mean recoveries were between 99.9-113.8 % and 99.4-106.5 % with intra-day RSD of 3.0-4.1 % and 2.2-5.3 %, and inter-day RSD of 3.3-11.3 % and 5.1-8.9 % for colistin A and B respectively. The limit of detection (LOD) was 15.0 µg/kg, and the limit of quantification (LOQ) was capped at 50.0 µg/kg. The developed method was applied to 30 random poultry feed samples and one sample was found to contain 89.75 µg/kg colistin A and 127.38 µg/kg colistin B. The presence of colistin in poultry feed used locally indicates the importance of implementing regular monitoring programs on animal feed to detect the presence of banned substances.

Keywords: poultry feed, colistin, residue analysis, mass spectrometry

INTRODUCTION

Polymyxin E, a colistin, was discovered in 1949. Polymyxin E1 (colistin A) and polymyxin E2 (colistin B) are peptides (cyclic lipodecapeptides) that are used in veterinary medicine to treat food producing animals. Gram-negative bacteria such as *Acinetobacter* species, *Klebsiella* species, *Pseudomonas aeruginosa*, and *Enterobacter* species are treated with these peptides (Fu *et al.,* 2018). The chemical structures for the polymyxins E1 and E2 are as shown in Figure 1.

In the last few decades, colistin as well as other antimicrobial agents have been widely used in farms for their significant growth-promoting effect on livestock (Mazutti *et al*., 2016). As colistin is reported as the last resort antibiotic for human medicine, the resistance phenomenon raises issues on the usage of colistin in veterinary medicine (Gaugain *et al*., 2021). The European Commission as well as national health authorities worldwide have established maximum residues limit (MRLs) for colistin in food of animal origin. MRLs were set for animal tissue, eggs, and milk to regulate the use of colistin (FAO & WHO, 2018). Malaysia has also established maximum MRL for colistin in

chicken, cattle, pig, rabbit, and sheep (Malaysian Food Act, 1983) for muscle, liver, fat, kidney, milk, and eggs. Ministry of Agriculture Malaysia approved suggestions for stopping colistin use in animal feed and animal feed additives for treatment, prevention, and boosting purposes in food-producing animals starting 1 January 2019 to prevent the occurrence of resistant antibiotics especially to humans (Poultry World, 2018). It is therefore very important for the Department of Veterinary Services (DVS) to monitor the use of colistin in animal feed.

Several analytical methods have been reported for measurement of colistin in various biological matrices, including human plasma (Binhashim *et al.,* 2021) chicken muscle and egg (Kumar *et al.,* 2021), as well as animal feed (Fu *et al*. 2018; Tao *et al*., 2018). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is becoming more frequently used in these methods due to its higher sensitivity and selectivity (Leporati *et al.,* 2014). However, there seems to be very few methods that described on the determination of colistin in animal feed. This is caused by the challenging combination of the difficulties associated with the chemical properties of the drug and the complexity of a compound feed matrix containing a high diversity of ingredients such as fat, proteins, and minerals (Gaugain *et al*., 2021). The method used in this study was a modified method described by Fu *et al*. (2018), where samples were extracted using solid phase extraction method, then colistin A and B were determined using UPLC-MS/MS. This study's objective is to develop a reliable, accurate, as well as robust method to determine the presence of colistin A and colistin B in poultry feed by using LC-MS/MS for monitoring purposes in Malaysia.

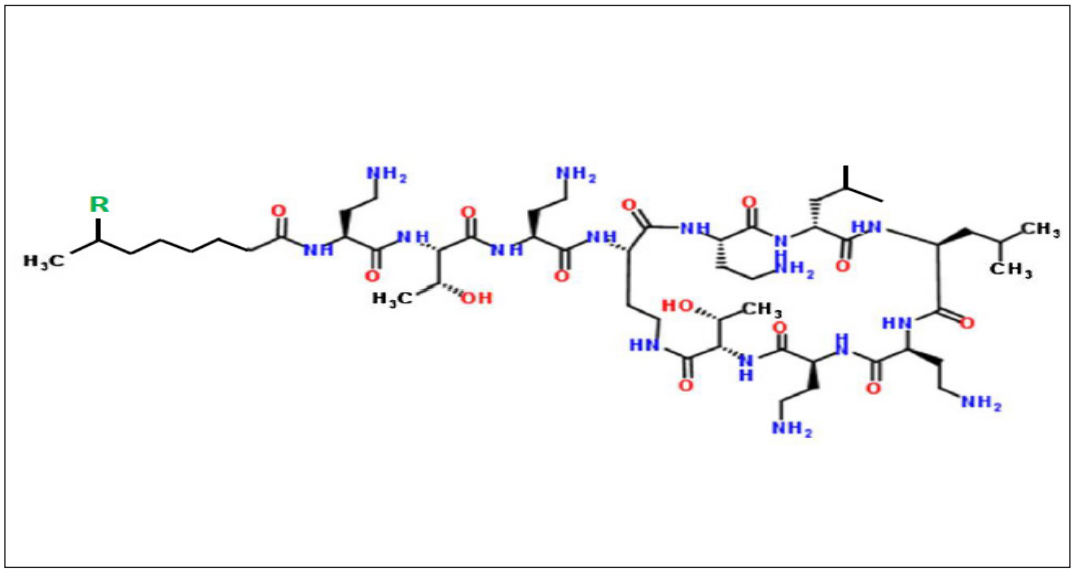

Figure 1. Chemical structure of the polymyxins E1 (colistin A) and E2 (colistin B) where R = CH3 for colistin A and R = H for colistin B (Choosakoonkriang et al., 2013).

MATERIALS AND METHOD

Chemicals and Reagents

Colistin A and B standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). LC-MS grade acetonitrile was obtained from Fisher Scientific (Waltham, Massachusetts, USA). Analytical grade acetonitrile, methanol, trichloroacetic acid (TCA), formic acid, and sodium hydroxide (NaOH) were also acquired from Fisher Scientific (Waltham, Massachusetts, USA). Ultrapure water was produced by Milli-Q Integral 5 water purification system (Merck-Millipore, Germany). Measurement of pH was conducted using Accumet® XL 150 (Fisher Scientific, Waltham, Massachusetts, USA). Oasis WCX (3 cc, 60 mg) solid phase extraction (SPE) cartridge, acrodisc syringe filter and UPLC C18 column were purchased from Waters (Milford, Massachusetts, USA).

Nitrogen (N_2) (99.999 %) (for desolvation and nebulizer gas) used for sample evaporation by Turbovap LV Classic (Biotage, Uppsala, Sweden) were generated through nitrogen generator, Simplex 12 from IMT Gasgen (Grasbrunn, Germany). Argon (99.999 %) (MS-MS collision gas) was obtained from Malaysian Oxygen (Kuala Lumpur, Malaysia).

Preparation of Standard Solutions

Stock standard solution of colistin A and colistin B were prepared by dissolving 10 mg of the compound in 10 mL of ultrapure water to a concentration of 1000 µg/mL and was kept at -20 ºC. Intermediates in concentration of 100 µg/mL were then prepared from the stock and stored at the same degree. Working standard solutions were prepared freshly before use.

Preparation and Purification of Samples

Sample extraction was performed based on Fu *et al*. (2018) with modification in extraction steps and matrix extracted standard preparation. Feed or premix sample (2.0 ± 0.02 g) was weighed into a 50 mL polypropylene centrifuge tube. Then, 10 mL of 10 % TCA:acetonitrile (40:60, v/v) was added to the tube and vortex to mix. Instead of vortexing the samples for 2 minutes as in the original method, the sample was shaken using rotary shaker for 10 minutes and subsequently centrifuged at 4000 rpm for 10 minutes at 2 ^{0}C. The supernatant was collected into a 50 mL centrifuge tube. Sediments were re-extracted with 10 mL, 10 % TCA:acetonitrile (40:60, v/v), shaken and centrifuged and the supernatant combined. The pH of combined supernatant was adjusted to 9.0 ± 0.3 using 1 M NaOH. The pH 9.0 combined supernatant was then centrifuged at 4000 rpm for 10 minutes at 2 ^{0}C. Five mL of supernatant was taken for SPE clean-up.

The sample was transferred into Oasis WCX SPE cartridge preconditioned with 3 mL methanol and 3 mL water. The sample was then rinsed with 3 mL methanol, followed by 3 mL water and lastly 3 mL of 5 % formic acid in water. Finally, the targeted analyte in the sample was eluted with 5 mL of 20 % formic acid in methanol into a 15 mL glass tube. The eluent was evaporated at 50 ºC under nitrogen gas flow. Following UPLC-MS/MS analysis, sample was reconstituted with 1 mL of water, then vortexed for 1 minute. The sample was then transferred into microcentrifuge tube and centrifuged at 11000 rpm for 20 minutes at 2 ^{0}C. Before injection, sample was filtered through a 0.22 µm GHP membrane filter into LCMS vial.

For the preparation of standard solution to obtain the calibration curve, instead of matrix matched used in most reported methods, matrix extracted standard were prepared. Six portions of approximately 2.0 ± 0.02 g of blank feed sample were weighed into 50 mL polypropylene centrifuge tubes. Then, 2 mL of 10

% TCA:acetonitrile (40:60, v/v) was added to the tube and vortex to mix. Standards were spiked into each tube according to concentration points and the mix were vortexed and left to stand for 10 minutes. The balance of 10 % TCA:acetonitrile (40:60, v/v) was added to each standard to a total of 10 mL.The standards were then treated in similar way as the sample extraction procedure.

UPLC–MS/MS Determination

Prior to sample analysis, the instrument was conditioned, and the column acclimatized to mobile phase A and B (0.5 % formic acid in water and 0.5 % formic acid in acetonitrile respectively). Targetted analytes were analyzed using ultraperformance liquid chromatography system, a tandem quadrupole (triple quadrupole) mass spectrometry, Xevo TQ-S (Waters, Milford, Massachusetts, USA). Chromatography separations were conducted through Acquity BEH C18 column (2.1 × 100 mm) with 1.7 µm particle size at column oven temperature of 30 °C. Injection volume was set at 2.0 µL and flow rate of gradient program for mobile phase A and B was set at 0.400 mL/min. The gradient program is as shown in Table 1.

The triple quadropole mass spectrometer applied ESI (electropsray ionization) ion source in positive mode using tuned and optimized parameter of 35 V cone voltage, 2.5 kV capillary voltage, 150 °C source temperature, 500 °C desolvation temperature, 150 L/h cone gas (N_2) flow rate, 800 L/h desolvation gas (N_2) flow rate and 0.15 mL/min collision gas flow. Positive ions were acquired in the ESI multiple reaction monitoring (MRM) mode, allowing for quantification of analytes in the sample. Colistin A was detected at mass-to-charge ratio (m/z) of 390.9 > 384.9 (cone, 35 V and collision energy, 10 eV) and m/z 390.9 > 101.2 (cone, 35 V and collision energy, 14 eV), while colistin B was determined at m/z 386 > 380 (cone, 35 V and collision energy, 10 eV) and m/z 386 > 101 (cone, 35 V and collision energy, 14 eV). Dwell time was set at 0.035 seconds for both colistin A and B and automatically applied to ensure multiple data points were acquired for each chromatographic peak. Data processing and acquisition were carried out using MassLynx software v4.1 (Waters, Milford, Massachusetts, USA).

Method Validation

The reliability of this method was evaluated in terms of specificity, linearity, recovery, precision, LOD and LOQ (EC, 2002). To determine the specificity, blank samples (n = 20) which were obtained from ten different sources of poultry feed collected from several regions in the Peninsular of Malaysia were extracted and evaluated for interference near the colistin retention times. The linearity was assessed

Table 1. Gradient program for colistin analysis.

Time (Min)	% Mobile Phase A	% Mobile Phase B
0	95.0	5.0
0.50	95.0	5.0
3.00	50.0	50.0
4.00	5.0	95.0
4.10	5.0	95.0
5.50	95.0	5.0

by plotting seven levels of matrix extracted calibration curves with concentration of 0, 50, 100, 300, 500, 700 and 1000 µg/kg.

The accuracy was expressed in terms of recovery, which was assessed by spiking blank samples at three concentration levels (50, 100, and 300 µg/kg). Each three-fortification levels were analysed in six replicates. Calculation of the recoveries was conducted by comparing the measured concentrations with the spiked concentrations of the samples. The precision was represented by the intra-day (six replicates) and inter-day (five replicates, 3 occasions) relative standard deviations (RSDs). The limit of detection (LOD) and limit of quantification (LOQ) were defined as lowest concentrations with a signal-to-noise (S/N) ratio of 3 and 10, respectively. Further analysis was done to validate the LOQ level with satisfactory recovery within 80-120% and RSD of less than 20 %.

Analysis of Real Samples

A total of 30 feed samples were randomly selected from animal feed samples received in the laboratory from various states and were analysed using the developed method. For quality control (QC) purposes, two blank samples and three spiked samples (at 500 µg/kg) were prepared for each batch of sample analysis. QC recoveries in the range of 80-120% were considered as acceptable. To cater for matrix effect, matrix extracted standards which were extracted together with the samples were used to plot matrix calibration curves for quantification.

RESULTS AND DISCUSSION

UPLC-MS/MS Optimisation and Anaytical Performance

Colistin was found to exhibit a significant peak tailing with the stationary phase of the C18 column. The peak tailing was caused by the terminal amine groups in colistin chemical structure (Fu *et al.,* 2018). As with other compound analysis by UPLC-MS/MS, the addition of formic acid (0.5 % in this study) as ion pairing agent has provided the best peak shape and ionization efficiency. Furthermore, the use of acetonitrile as mobile phase B yielded better sensitivity and peak shape than usage of other solvent such as methanol. The separation was further optimized in order to counter the problem of 'carry over' after the injection of the standard solution. The cleaning and re-equilibration step was done using mobile phase A and achieved for both colistin A and colistin B in 5.5 min. The mass parameters were optimized by manual tuning which involved infusing a standard solution mixture of both colistin A and colistin B at the concentration of 500 ng/mL. A full scan in ESI positive ionization mode was performed to select the most abundant precursor ion and corresponding cone voltage for both targeted analytes.

The gradient programme was optimised so that all polar interference was eluted before the target analyte. No positive signal and interfering peaks were observed at the expected retention time for the blank samples, signifying the specificity of the method. The run time was increased to 6 minutes to ensure that all matrix interferences associated with target analytes were completely eluted and will not affect subsequent injections of standards and samples. In this study, the acidic mobile phase provided optimal retention, symmetrical peak shape, and reproducible signal with retention time and in agreement with Kumar *et al.* (2021). Figure 2 shows chromatographic separation obtained for a spiked sample and the blank sample.

The mass parameters of colistin were manually tuned by infusing standard solutions of colistin A and colistin B, respectively, at the

concentration of 500 µg/kg. The most dominant precursor ion cone voltage was obtained by running a full scan in ESI positive ionisation mode with appropriate collision energy. A triply charged ion with a mass of 390.9 was seen as dominant for colistin A and chosen as the precursor ion. For colistin B, a triply charged ion at m/z 386 was chosen. Colistin A and B major fragments were ions with m/z 384.9, 101.2 and m/z 380, 101, respectively after collision energy was optimised as shown in Figure 2.

Sample Extraction

Feed sample is regarded as a difficult and complex matrix for analyte extraction and purification. Previous research (Gorissen *et al*., 2015; Fu *et al*., 2018; Tao *et al*., 2018) reported the use of acetonitrile as an efficient extraction solvent for colistin extraction from various matrices. In this study, acetonitrile was used in combination with trichloroacetic acid to precipitate protein and other substances during extraction, which may affect the analysis of target analytes (Jansson *et al*., 2008).

As matrix extracted standards were used for calibration curves, there was a need to ensure homogenization of samples with standards before extraction procedure. Hence for powder or dry samples like feed samples, the samples needed to be wetted first using 2 mL of extraction solutions, spiked with appropriate amounts of standards and left to stand for 10 minutes before commencing with the extraction steps. This ensures that the recoveries of standard are within an acceptable range of 80-120 %. This step was omitted in the study done by Fu *et al*. (2018).

A mixed-mode weak cation exchange cartridge, Oasis WCX (60 mg) was applied for sample clean-up and resulted in a satisfactory clean sample with good recovery for both colistin A and colistin B. In order to increase the absorption of targeted compounds to the cartridge, the pH value of loading solution was adjusted to pH 9. This is in accordance with study by Fu *et al*. (2018). Rinsing of SPE at 5 % formic acid resulted in high recovery rates and improved the clean-up effects, while elution with 20 % formic acid in methanol gave the highest yield of target analytes (Fu *et al.,* 2018). To improve sample purification, additional steps involving total sample evaporation, reconstitution, centrifugation, and filtration were performed as compared to the study by Fu *et al*. (2018).

Modification done on some steps in the method compared to method referred is to accommodate existing facilities without compromising the quality of analysis as well as to simplify and ease the extraction process, taking into account the high number of samples received in the National Veterinary Public Health Laboratory routinely. Based on validation conducted, the modification of certain steps yields a similar percentage of recovery as reported by Fu *et al*. (2018).

Matrix effect has been reported as one of the factors that can affect the recovery of analytes during LC-MS/MS analysis (Gorissen *et al*., 2015; Fu *et al*., 2018; Tao *et al*., 2018). In this study, the use of matrix extracted standards differs from any previous research where matrix matched standards were used instead, which involved spiking of pure standards after extraction of blank matrices. The advantage of using matrix extracted standards as calibration curve as applied in this study, will simultaneously eliminate errors that can occur during the extraction process and matrix effects that can hinder the detection of target analytes during instrumental analysis.

Method Validation

No peak was observed at targeted retention time for analyte (2.32 for colistin A and 2.20 for colistin B) during blank sample analysis (Figure 2(A) and

2(C)), showing the selectivity and specificity of the method. Clear peaks were observed at a spiked level (Figure 2(B) and 2(D)) of 500 µg/kg. Table 2 summarises the retention time the peak was observed for both colistin A and B as well as the precursor ions and its fragmentations as obtained during tuning and optimization.

Following injection of seven levels of matrix calibration curves with concentration of 0, 50, 100, 300, 500, 700, and 1000 µg/kg, good linearity was observed with coefficients of determination (R^2) at 0.9948 for colistin A and 0.9955 for colistin B. For the evaluation of method accuracy (reported as mean recovery

Table 2. Retention time, precursor, and fragments ion for colistin A and B.

Analyte	Retention time	Precursor Ion (m/z)	Fragmentations (m/z)
Colistin A	2.32	390.9	101.2, 384.9
Colistin B	2.20	386	101, 380

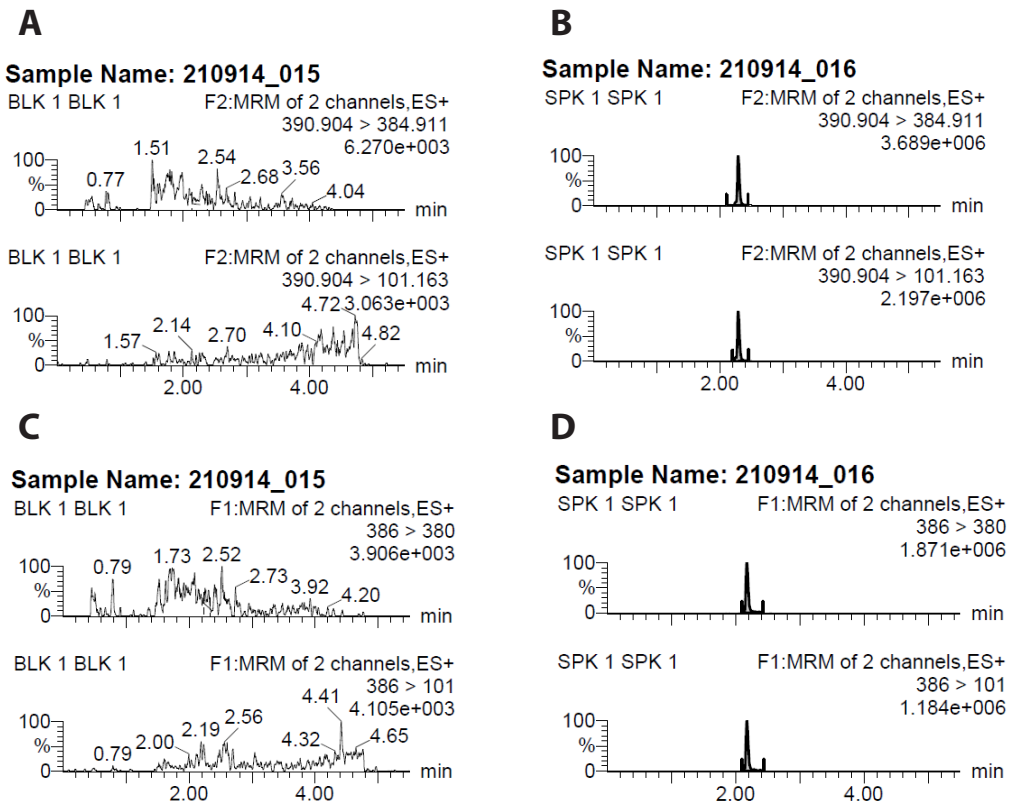

Figure 2. UPLC-MS/MS chromatograms of blank and spiked feed sample. (A) Blank sample (colistin A). (B) Spiked sample (colistin A, 500 µg/kg). (C) Blank sample (colistin B). (D) Spiked sample (colistin B, 500 µg/kg).

in percent) and precision (reported as RSD percentage), spiked samples were prepared for blank matrices at three levels, 50, 100, and 300 µg/kg. As shown in Table 3, the mean recoveries were between 99.9-113.8 % for colistin A and 99.4-106.5 % for colistin B, with intra-day RSDs of 3.0-4.1 % and 2.2-5.3 %, and inter-day RSDs of 3.3-12.2 % and 5.5-8.9 % for colistin A and colistin B respectively. The range was within the ±15 % limit set by the EC, 2002 demonstrating the good accuracy and repeatability of the method. The LOQ for colistin A and colistin B was determined at 50 µg/kg which offered acceptable recoveries within 80-120% with <20 % RSD for ten replicates, while LOD was obtained at 15 µg/kg, which was sufficient for detection of colistin for monitoring purpose.

Table 3. Validation parameter for colistin A and colistin B in poultry feed.

Analyte	LOD (µg/kg)	LOQ (µg/kg)	R^2 (Matrix calibration)	Spiked level (µg/kg)	Mean Recovery (%)	Precision (RSD, %)	
						Intra-day (n = 6)	Inter-day (n = 15)
Colistin A	15	50	0.9948	50	113.8	3.0	3.3
				100	99.9	3.2	12.2
				300	102.1	4.1	7.3
Colistin B	15	50	0.9955	50	106.5	5.3	5.5
				100	100.2	2.2	8.9
				300	99.4	4.2	5.9

Analysis of Samples

Analysis of 30 real poultry feed samples randomly chosen from samples received in the National Veterinary Public Health Laboratory using the developed method shows that one poultry sample of duck feed was found to contain 89.75 µg/kg colistin A and 127.38 µg/kg colistin B. Matrix extracted calibration curves were used and recovery corrections were performed to compensate for the matrix effect and prevent the accuracy and reliability of the results to be jeopardised by ion suppression or enhancement caused by co-eluting matrix components. Since colistin was banned, there should not be any amount of the drugs in the animal feed samples. Hence, the method developed is comparable with methods in previous research, suitable and sufficient to detect the presence of colistin in poultry feed.

CONCLUSION

This study describes a reliable and selective method for determination of colistin A and colistin B residues in poultry feed using UPLC-MS/MS. The developed method was validated and verified to be specific, accurate, and suitable for routine monitoring and regulating the use of colistin in feed. The use of colistin as a feed additive is prohibited in the production of food producing animals, hence it is crucial to enforce compliance through monitoring of the animal feed.

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