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MULTI-CLASS ANALYSIS OF ANTIMICROBIAL SUBSTANCES IN POULTRY FEED AT CROSS-CONTAMINATION LEVELS BY UHPLC-MS/MS – METHOD ESTABLISHMENT, VALIDATION, AND APPLICATION

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The Commission Delegated Regulation (EU) 2024/1229 establishes maximum limits (MLs) for the cross-contamination of antimicrobial substances in feed intended for food-producing animals. This study presents the development and in-house validation of two multi-class analytical methods for detecting antimicrobial substances in poultry feed at cross-contamination levels, using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Methods with and without solid-phase extraction (SPE) purification were developed for 26 antimicrobial substances across eight classes, covering a concentration range of 5–300 μg kg^{-1}. The methods were validated for parameters including selectivity, limit of quantification, within- and between-day precision, accuracy, decision limit, matrix effect, and short-term analyte stability in the corresponding extracts. Both methods met the required performance criteria for detecting antimicrobial substances at cross-contamination levels, except for reduced sensitivity to lincomycin in the SPE method. Between-day precision was below 18 % and 19 %, with recoveries ranging from 95 to 103 %, for the method without SPE, and from 88 % to 101 % for the method with SPE, respectively. The analytes remained stable after two days of storage in the dark under two temperature conditions. These methods were applied to 39 poultry feed samples, revealing that approximately 41 % contained one or more antimicrobial substances, some of which exceeded the cross-contamination MLs. This study underscores the importance of simple, rapid, sensitive, and reliable analytical methods for controlling the presence of antimicrobials at cross-contamination levels. Such methods enable stringent control to prevent uncontrolled antimicrobial use in poultry production systems, thereby mitigating the development of antimicrobial resistance.

Keywords: antimicrobial substances; poultry feed; multi-class UHPLC-MS/MS method; matrix effect; stability study

МУЛТИКЛАСНА АНАЛИЗА НА АНТИМИКРОБНИ СУПСТАНЦИИ ВО ХРАНАТА ЗА ЖИВИНА НА НИВО НА ВКРСТЕНА КОНТАМИНАЦИЈА СО УПОТРЕБА НА UHPLC-MS/MS – ВОСПОСТАВУВАЊЕ, ВАЛИДАЦИЈА И ПРИМЕНА НА МЕТОДОТ

Максималните граници за вкрстена контаминација на антимикробните супстанци во храната за живина која се одгледува за човекова исхрана се пропишани во Регулативата на Европската Комисија (ЕУ) 2024/1229. Во ова истражување се претставени развојот и валидацијата на два аналитички метода за испитување на супстанции од повеќе класи на антимикробни соединенија во храната за живина на ниво на вкрстена контаминација, со примена на ултра-високоперформансна течна хроматографија спрегната со масена спектрометрија (UHPLC-MS/MS).

За испитуавње на 26 антимикробни супстанции од осум класи соединенија, со опсег на концентрации од 5 до 300 μg kg^{-1}, развиени се методи со и без користење на пречистување со екстракција од цврста фаза (SPE). Методите се валидирани за параметрите селективност, граница на квантификација, прецизност во тек на еден и помеѓу повеќе денови, точност, граница на одлучување, ефект на матрица и студија за стабилноста на аналитите во екстракти во тек на краток временски период. Карактеристиките на двата метода ги исполнуваат пропишаните критериуми за испитување на антимикробни супстанции на ниво на вкрстена контаминација, со исклучок на намалената чувствителност на линкомицин при примена на методот SPE. Определената прецизност во тек на повеќе денови е помала од 18 % односно 19 %, со аналитички приноси од 95 до 103 % за методот без SPE, и од 88 % до 101 % за методот со SPE. Испитаните аналити демонстрираа задоволителна стабилност по чување на темно на две различни температури во текот на два дена. Методите беа применети за анализа на 39 примероци храна за живина, при што е утврдено дека приближно 41 % од примероците содржат една или повеќе супстанции, од кои некои и со концентрации кои ги надминуваат дозволените максимални вредности за вкрстена контаминација. Оваа студија ја нагласува важноста за развој на едноставни, брзи, чувствителни и веродостојни аналитички методи за контрола на вкрстената контаминација на антимикробните супстанции во храната за животни. Воспоставените методи овозможуваат строга контрола во системите за производство на живина, со цел да се спречи неконтролираната употреба на антимикробни соединенија, со што се придонесува за намалување на појавата на антимикробна резистенција.

Клучни зборови: антимикробни супстанции; храна за живина;
мултикласен UHPLC-MS/MS метод; ефект на матрица; студија за стабилност

1. INTRODUCTION

Veterinary medicinal products (VMPs), particularly antimicrobial agents, were widely used in animal husbandry for both therapeutic and prophylactic purposes. One common route of administration in livestock production was through incorporation into animal feed, resulting in "medicated feed".[1] The uncontrolled use of antimicrobial agents for prophylactic purposes significantly contributed to the emergence of antimicrobial resistance (AMR).[2] In the Netherlands, research showed that antibiotic concentrations in 87 % of feed samples ranged from 0.1 to 154 mg kg^{-1}, which was expected to influence the development of AMR.[3] Furthermore, improper and excessive use of non-targeted feed in food-producing animals led to uncontrolled VMP residues in animal-derived foods. This posed a threat to consumers due to potential adverse effects and significantly contributed to the spread of AMR.[4]

European Union Regulation (EU) 2019/4 introduced the concept of "cross-contamination" in feed, referring to the transfer of trace amounts of VMPs from medicated to non-medicated feed during production.[5–7] As a result, this regulation restricted the use of VMPs as feed additives for metaphylactic purposes.[7] The European Food Safety Authority (EFSA) provided scientific risk assessments to establish maximum limits (MLs) for antimicrobial substances in non-medicated animal feeds.[2]

The recently issued EU Commission Delegated Regulation (EU) 2024/1229 established maximum levels for the cross-contamination of antimicrobial active substances in non-target feed and outlines methods for analyzing these substances in feed.[8] The regulation sets a harmonized cross-contamination level at 1 % of the active substances present in medicated feed. Exceptions applied to feed intended for food-producing animals during egg and milk production, as well to animals intended for slaughter, with respect to the withdrawal period of the substance.[8] For these feeds, cross-contamination levels were set at the limits of quantification (LOQs) defined in the regulation. For antibiotics and sulfonamides, cross-contamination levels ranged from 10 to 500 μg kg^{-1}.[8] Enforcing this stringent regulation required the use of sensitive and reliable analytical methods, preferably multi-class methods, to meet the target concentration levels in non-medicated feed. Feed typically consisted of cereals, seeds, fats of plant and/or animal origin, and various additives such as enzymes, acidifiers, minerals, vitamins, and mineral-vitamin premixes.[9] The presence of these substances made the matrix highly complex and variable, posing significant challenges for analysis. Consequently,

developing analytical methods to determine low concentrations of a wide range of antimicrobial substances in feed was both labor-intensive and time-consuming.

The availability of analytical techniques capable of simultaneously detecting antimicrobial substances from different chemical classes for both screening and confirmatory purposes has been crucial to ensuring reliable feed control. This has been vital for supporting the implementation and monitoring of measures aimed at combating AMR.[10] For decades, bioassays were used to test for the presence of antimicrobial substances in animal feed; however, these assays were limited to a small number of substances, often within a single class.[11,12] Recent trends in antimicrobial analysis in both medicated and non-medicated feed have predominantly relied on liquid chromatography techniques coupled with various detectors, including fluorescence,[13,14] diode-array,[1,15] tandem mass spectrometry,[4,9,16–18] and high-resolution mass spectrometry (HRMS).[12,19,20]

Analytical methods using liquid chromatography with fluorescence or diode-array detection for quantifying antimicrobials in feed have typically been limited to single-class substances such as fluoroquinolones,[12] sulfonamides,[9] and tetracyclines.[1,11,14,15] These methods were primarily developed for analyzing antimicrobials in medicated feeds. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been widely used to determine antimicrobial agents in both medicated[9,16–18,21,22] and non-medicated feeds.[9,10,17,23–25] Previously published LC-MS/MS methods for assessing cross-contamination levels in non-target feeds were designed for a limited range of antimicrobial substances and classes.[9,16–18,21,24] Additionally, some developed multi-class methods[23,24] have not demonstrated the capability to detect all substances at the MLs specified in the regulation.[8] Recently, only a few publications have addressed the newly established performance requirements for analytical methods intended for feed control at the required MLs.[10,25] Consequently, the limited availability of analytical methods capable of achieving the required sensitivity to detect antimicrobial substances at the MLs specified by the regulation[8] highlights the need for further research into method development, optimization, and validation.

This study aimed to establish and validate two sample preparation approaches – with and without solid-phase extraction purification – for simple, rapid, and sensitive determination of 26 antimicrobial substances in poultry feed using ultra-high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS). The target concentration range reflected cross-contamination levels set by legislation, ranging from 10 to 150 μg kg^{-1}.[8] Both methods were validated in accordance with the EU regulatory framework.[26] The validated methods targeted eight classes of antimicrobial substances, encompassing one or more compounds: tetracyclines (4), quinolones (4), penicillins (4), macrolides (4), cephalosporins (2), lincosamide (1), pleuromutilin (1), and sulfonamides (6), many of which have regulated cross-contamination limits.[8] In presenting and discussing the validation data, we critically assessed the strengths and limitations of the methods employed in this study, referencing existing literature. The applicability of the validated methods was confirmed by testing real feed samples used in poultry rearing systems.

2. EXPERIMENTAL SECTION

2.1. *Reagents and materials*

Methanol, acetonitrile, and water of LC-MS/MS quality were used for preparation of analytical standards, mobile phases, and sample extraction (Fisher Scientific, Hampton, NH, USA). Analytical-grade dimethyl sulfoxide (DMSO) was used for preparing analytical standards and samples (Merck, Darmstadt, Germany). Formic acid (purity ≥ 98 %), supplied by Merck (Darmstadt, Germany), was used as both a mobile phase component and an extraction solvent modifier.

For solid-phase extraction (SPE), Bond Elut Plexa cartridges containing 200 mg of sorbent and a 6 ml volume were used (Agilent Technologies, Santa Clara, CA, USA). The autosampler vials (1.5 ml) were conical and LC-MS-certified (Agilent Technologies, Santa Clara, CA, USA).

McIlvine buffer was prepared by dissolving 11.8 g of citric acid, 13 g of $Na_2HPO_4 \cdot 2H_2O$ (both from Merck, Darmstadt, Germany), and 33.62 g of ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) (VWR Chemicals, Solon, Ohio, USA) in a 1000 ml volumetric flask filled with deionized water obtained using the Simplicity® system (Merck, Darmstadt, Germany).

2.2. *Analytical standards*

Analytical standards for lincomycin hydrochloride (LIN), sulfadoxine (SDX), sulfamonomethoxine (SMM), sulfamethoxazole (SMX), sulfisoxazole (SSX), enrofloxacin (ENR), ciprofloxacin (CIP), marbofloxacin (MAR), amoxicillin trihydrate (AMX), ampicillin trihydrate (AMP), nafcillin (NAF), ceftiofur (CEF), cephapirin sodium (CEP), and tilmicosin (TIL) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetracycline hydrochloride (TTC), chlortetracycline hydrochloride (CTC), doxycycline (DOX), flumequine (FLU), tulathromycin (TUL), and tildipirosin (TLD) were sourced from HPC Standards (Cunnensdorf, Germany). Penicillin V potassium salt (PEN), oxytetracycline hydrochloride (OTC), tylosin tartrate (TYL), sulfamethazine (SMT), and sulfadimethoxine (SDM) were acquired from CPA Chem (Bogomilovo, Bulgaria), and tiamuline (TIA) was purchased from Supelco (Bellefonte, PA, USA).

Individual stock solutions were prepared at a concentration of 1000 mg l^{-1} by dissolving approximately 10 mg of dry substances (corrected for purity and salt content) in methanol (TTC, OTC, DOX, CTC, AMX, AMP, CLX, PEN, LIN, TIL, TIA, and TYL), acetonitrile (SDX, SMM, SMX, SSX, SMT, and SDM), and DMSO (CEF, TUL, and TIL). An intermediate mix of all analytes at a concentration of 10 mg l^{-1} was prepared in methanol from the stock solution. Working solutions of 1 mg l^{-1} and 100 µg l^{-1} in acetonitrile were obtained through further serial dilution.

Individual tuning solutions of all analytes at a concentration of 10 mg l^{-1} were prepared in a 50:50 (v/v) mixture of acetonitrile and LC-MS/MS-grade water, with the addition of 0.1 % (v/v) formic acid. These solutions were used to optimize the mass spectrometer parameters.

2.3. *Instrumentation*

Instrumental analysis was performed using an ultra-high-performance liquid chromatograph coupled with an electrospray ionization quadrupole mass spectrometer (UHPLC-ESI-MS/MS). This system consisted of an Acquity UPLC H-class liquid chromatograph and XEVO TQ-S micro tandem mass spectrometry detector equipped with electrospray ionization (ESI) (Waters Corporation, Milford, MA, USA). Instrumental control and data processing were performed using MassLynx 4.2 software (Waters Corporation, Milford, MA, USA). An Acquity UPLC®BEH C18 (2.1 × 100 mm × 1.7 µm; Waters Corporation, Milford, MA, USA) was used as the analytical column.

2.3.1. *Instrumental conditions for UHPLC-ESI-MS/MS*

Ultra-high-performance liquid chromatography (UHPLC) was used to separate analytes. Mobile phase A consisted of water modified with 0.1 % (v/v) formic acid, while mobile phase B was a 50:50 (v/v) mixture of methanol and acetonitrile containing 0.1 % (v/v) formic acid. The column temperature was set to 45 °C, the total analysis time was 10 min, and injection volume was 10 µl. The optimized linear solvent gradient for analyte separation was as follows: 0 – 1 min 95 % A, 1 – 4.5 min 5 % A; 4.5 – 5.0 min 5 % A; 5.0 – 10 min 95 % A.

The tandem quadrupole mass spectrometer operated in positive ion mode (ESI+) under the following conditions: capillary voltage, 3.00 kV; cone, 25 V; desolvation temperature, 450 °C; source temperature, 150 °C; cone gas flow rate, 20 l hr^{-1}; desolvation gas flow rate, 900 l hr^{-1}. For each analyte in multiple reaction monitoring (MRM), the cone voltage and collision energy were optimized for two transitions. The transition with the higher signal was selected as the quantification ion, while the other was used as the confirmation ion. The intensity of the confirmation ion was calculated relative to that of the quantification ion.

2.4. *Sample preparation*

2.4.1. *Method 1*

Four grams of homogenized sample were placed into a 50 ml tube and extracted using a 15 ml of a methanol, acetonitrile, and McIlvaine buffer (37.5:37.5:25, v/v). The samples were shaken manually for 30 s and then placed in an ultrasonic bath for 15 min. After sonication, the samples were centrifuged at 3000 rpm for 10 min. Three milliliters of the supernatant were diluted to a final volume of 10 ml with 7 ml of a 0.1 % formic acid/methanol solution (50:50, %v/v). The diluted extract was filtered through a 0.22 µm membrane filter before being injected into the analytical instrument.

2.4.2. *Method 2*

Two grams of the feed sample were weighed into a 50 ml plastic centrifuge tube, and 10 ml of an extraction solution consisting of acetonitrile and water (85:15, v/v) with 0.2 % formic acid was added. The mixture was vortexed for 1.5 min and centrifuged at 4000 rpm for 5 min at room temperature. The supernatant was filtered through a 0.45 µm membrane filter and further purified using solid-phase extraction (SPE). The SPE column was conditioned with 3 ml methanol followed by 3 ml of water. One milliliter of the extract was applied to the column and allowed to pass through to waste. A clean collection tube was placed beneath the column, and an additional 2 ml of the extract was passed through it.

The eluate was collected, and 1 ml was transferred to a concentration tube. Then, 50 µl of DMSO was added, and the eluate was concentrated to 50 µl under a gentle nitrogen stream at 40 °C. The residue was reconstituted in a mobile phase mixture of A and B (95:5, v/v), and vortexed. A 300 µl aliquot was mixed with 550 µl of 10 mM ammonium formate, and 10 µl of the final solution was injected into the UHPLC-MS/MS system.

2.5. *Method validation*

Methods 1 and 2 were rigorously validated to confirm their suitability for detecting antimicrobial substances in animal feed at cross-contamination levels. Validation was performed using blank poultry feed confirmed to be free of the analytes of interest. The validation process assessed several parameters, including selectivity, linearity, limit of quantification (LOQ), precision, trueness, measurement uncertainty, decision limit, matrix effect (ME), and stability. These parameters were calculated, and figures were generated using Excel 2017 (Microsoft Corporation, Redmond, WA, USA). The performances of the validated methods were then evaluated against the criteria established in Commission Delegated Regulation 808/2021.[26]

2.5.1. *Specificity, linearity and limit of quantification*

Specificity was assessed by analyzing two types of poultry feed intended for broilers and laying hens. Ion chromatograms were examined for interfering peaks that shared the same ion transitions and retention times as the analytes of interest.

Linearity was evaluated over the range of 5 – 300 µg kg^{-1} using seven matrix-matched calibration standards in triplicate. These standards were created by spiking blank feed samples with concentrations of 5, 10, 20, 50, 100, 200, and 300 µg kg^{-1} prior to the extraction procedure. The acceptance criterion for linearity was a coefficient of determination (R^2) ≥ 0.98.[26]

Limits of quantification were defined as the concentration levels at which the quantification ion in the matrix solution exhibited a signal-to-noise ratio of at least 10, in accordance with the target values set by legislation.[8] This approach was used determine LOQs for both methods to ensure they met required sensitivity for further validation. Signal-to-noise ratios for analyte peaks in the matrix were calculated using MassLynx 4.2 software.

2.5.2. *Precision and accuracy*

To evaluate the precision and accuracy of the proposed methods, spiking experiments were conducted on previously tested blank poultry feed confirmed to be free of the analytes of interest. Method precision was assessed by repeatability (within-day precision), based on six replicate extractions under repeatability conditions, and reproducibility (between-day precision), determined from six spike replicates performed on three different days. Both within-day and between-day precision were evaluated at concentration levels corresponding to the determined LOQs and two higher concentration levels. Specifically, for TIA, precision and accuracy were assessed at 10, 50, and 100 µg kg^{-1}; for FLU, LIN (Method 1), SDX, SDM, SMM, SMT, SSX, and SMX at 25, 50, and 100 µg kg^{-1}; for PEN, NAF, TTC, OTC, CTC, DOX, CEP, CEF, ENR, CIP, MAR, LIN (Method 2), TYL, TIL, and TUL at 50, 100, and 150 µg kg^{-1}; and for AMX and AMP at 100, 150, and 200 µg kg^{-1}. Accuracy was evaluated by calculating the recovery values (expressed as percentages) at the same concentration levels used for precision, based on data obtained from six replicates across three different days.

2.5.3. *Measurement uncertainty and decision limit*

The combined measurement uncertainty of both methods was assessed metrologically following the EURACHEM/CITAC GUIDE.[27] Using a

bottom-up approach, measurement uncertainty components arising from the between-day reproducibility at the LOQ level, balance accuracy, volumetric equipment uncertainty (as stated in ISO 17025 calibration certificates), and analytical standard assay uncertainty (as specified in ISO 17034 certificates). The combined measurement uncertainty was estimated by summing all the aforementioned components.

The decision limit (*CCα*), expressed in μg kg^{-1}, was calculated from the combined measurement uncertainty using Equation 1:[26]

$$CC_\alpha = ML + \mathrm{k} \times MU \quad (1)$$

where *ML* is the maximum limit in μg kg^{-1} (in this context, the methods LOQs), k is the one-sided coverage factor for 95 % confidence, equal to 1.64, and *MU* is the combined measurement uncertainty.

2.5.4. *Relative matrix Effect*

The ME was assessed by adding a standard to the prepared extract after extraction, which was then analyzed alongside a pure analyte solution at the same concentration level.[28] For both methods, ME was evaluated by spiking 20 blank feed extracts with a standard mixture to achieve a concentration of 100 μg l^{-1}. The peak areas were used to calculate ME, as described in Equation 2.[28] The peak area of the standard solution was calculated as the average of ten injections.

$$\mathrm{ME}(\%) = \frac{\text{Peak area of the matrix standard}}{\text{Peak area of the solution standard}} \times 100 \quad (2)$$

2.5.5. *Stability*

This study included short-term stability experiments for both methods to simulate real-world conditions in a testing laboratory. Five replicates of spiked blank feed were prepared at low concentrations (LOQs) and two higher concentrations, and the extracts were divided into three aliquots. One aliquot was tested immediately after preparation, the second after 2 days of storage at 4 – 6 °C in the dark, and the third after 2 days of storage at less than –20 °C in the dark. The analyte concentrations in each stored aliquot were compared to those in the freshly prepared extracts by calculating the percentage of the remaining analyte concentration.[26]

2.5.6. *Sample collection and analysis*

The applicability of the validated UHPLC-ESI-MS/MS method was evaluated by analyzing poultry feed samples. Between April and July 2024, 39 feed samples were collected from poultry farm facilities and distributors. The samples were homogenized, properly packed in sealed containers, and stored at temperature below –20 °C until analysis. All samples were analyzed using both sample preparation procedures and quantified by preparing matrix-matched calibration curves alongside each sample batches.

3. RESULTS AND DISCUSSION

3.1. *Mass spectrometer and UHPLC optimization*

The mass spectrometer parameters were fine-tuned by injecting individual standard solutions prepared in acetonitrile/water with formic acid. All analytes were ionized in positive mode, forming $[M+H^+]$ adducts. Cone voltages and collision energies for the daughter product ions were optimized for each analyte. Table 1 presents the final results of the optimization study. Additional analyte identification was performed based on retention times, following the criteria outlined in Commission Delegated Regulation 2021/808.[26] During the study, retention times varied by no more than ±0.06 min compared to those listed in Table 1. The mass spectrometer acquisition method was optimized using a retention time window, ensuring that at least 17 data points defined the ion peak.

UHPLC conditions were optimized to achieve rapid separation and quantification of the analytes, completing the analysis within 10 min. The use of small stationary phase particles (< 2 μm) resulted in sharper and narrower peaks, thereby enhancing the separation of closely related compounds. Retention time were significantly reduced compared to conventional HPLC or when using analytical columns with particles larger than 2 μm,[9,19,23,29] without compromising selectivity. This, combined with tandem mass spectrometry, enhanced the specificity of the applied method.

Although the method does not cover all antimicrobial substances listed in the regulation,[8] it could be expanded to include additional compounds such as trimethoprim, amphenicols, amprolium, tylvalosin, valnemulin, and oxolinic acid. Due to the need for ion-pairing chromatography

for the separation and quantification of aminoglycosides,[10] their simultaneous analysis with other antimicrobial agents was not feasible. Additionally, the distinct chemical properties of polymyxins (polypeptide substances) required different chromatographic conditions, typically involving varied pH values and mobile phase compositions.[10] Therefore, for analyzing these two antimicrobial classes, it was advisable to use separate analytical methods.[10,30]

3.2. *Sample preparation*

Two sample preparation methods were used as the starting point for this study. The first method, developed for the determining antimicrobials at cross-contamination levels, did not include sample purification.[31] The second method, originally designed for analyzing antimicrobial substances in tissue samples and employing SPE for extract purification, was modified for feed matrix.[32] Moreover, a recent study applied this sample preparation technique to analyze antibiotics in feed.[25]

In the first method (Method 1), extraction was performed using a mixture of organic solvents and McIlvaine buffer containing Na_2EDTA, followed by sonication. This combination of extraction solvents had previously used in developing sample preparation procedures for LC-MS/MS to determine antibiotic substances in feed without further SPE clean-up.[31,33] In earlier studies, sample preparation methods for LC-MS/MS or LC-HRMS determination of antibiotic substances in feed used only EDTA,[23] a mixture of acetonitrile, methanol, and water (65:25:10, v/v) with 1 % formic acid,[19] or a mixture of acetonitrile and water (90:10, v/v) with 0.1 % acetic acid.[29]

In Method 2, extraction was performed using a mixture of acetonitrile and water acidified with formic acid. The Bond Elut Plexa sorbent used for purification consisted of a styrene-divinylbenzene copolymer, which facilitated efficient extraction of a wide range of polar and non-polar compounds.[34] These cartridges effectively removed over 95 % of common matrix interferences, including phospholipids, salts, and proteins, thereby reducing matrix effects and enhancing the analytical performance.[34] Similar extraction solvents were used in studies by Gaugain et al.[30] and Varenina et al.,[25] where styrene-divinylbenzene copolymer cartridges were also employed for purification. The use of SPE with these cartridges was further discussed in a recent Joint Research Center (JRC) recommendation document.[10]

3.3. *Validation study*

The validation study for both methods was conducted in accordance with the European Commission's requirements[8] for selectivity and LOQ, as well as the criteria for precision (both within-day and between-day), accuracy, and decision limits, as specified for testing pharmacologically active substances in animal-derived foods.[26] Furthermore, in line with regulatory guidelines,[26] experiments were performed to assess the ME on analyte signals and the stability of the analytes in the final extracts. At this stage of the validation, ruggedness was not evaluated, as no variations in the method conditions were applied. Notably, the validation parameters included in this study were more comprehensive than those reported in previously published methods for the analysis of antimicrobials in feed.[10,23,25,29,31,33]

3.3.1. *Specificity*

In the specificity assessment, two feed samples, comprising broiler and laying hen feed types, were examined for endogenous peaks with a signal-to-noise ratio exceeding three at the retention times of the target compounds. Both methods yielded clean background for all analytes across the analyzed matrices. This method also met the criteria for compound identification, achieving four identification points through the detection of a precursor ion and two product ions at retention times that varied by no more than ± 2.0 %.[26] The ion ratio for each analyte in the samples matched the ion ratio for the standards (Table 1) within each run, with differences between the calculated ratios remaining below the maximum permitted tolerance of 30 %.[26]

Chromatograms of the quantification ions, along with those of the blank poultry feed sample used in the validation study, are presented in Supplementary Figures 1S and 2S.

T a b l e 1

Retention times, optimized multiple reaction monitoring conditions, and ion intensities for analytes included in the method scope

Analyte	Abbrev.	t_R (min)	ESI[a]	CV[b] (V)	DT[c] (ms)	Quantification ion	CE[d] (eV)	Intensity (%)	Confirmation ion	CE (eV)	Intensity (%)
Penicillins											
Penicillin V	PEN	3.91	+	25	30	351.4>229.0	25	100	351.4>257.1	20	56
Amoxycillin	AMX	3.81	+	20	30	365.4>208.3	10	100	365.4>113.9	18	34
Ampicillin	AMP	2.79	+	46	40	350.2>105.9	15	100	350.2>160.0	10	29
Nafcillin	NAF	4.31	+	25	40	415.1>199.1	14	100	415.1>300.05	14	98
Tetracyclines											
Tetracycline	TTC	2.88	+	25	30	445.1>410.2	20	100	445.1>427.2	12	50
Oxytetracycline	OTC	2.91	+	25	30	461.1>426.1	18	100	461.1>443.1	10	30
Chlortetracycline	CTC	3.27	+	25	30	479.1>443.9	19	100	479.1>461.7	15	76
Doxycycline	DOX	3.41	+	25	30	445.0>428.0	17	100	445.0>154.0	30	11
Cephalosporins											
Ceftiofur	CEF	3.48	+	70	40	524.0>241.0	15	100	524.0>95.0	40	46
Cephapirin	CEP	2.42	+	25	40	424.1>292.1	14	100	424.1>292.1	20	65
Quinolones											
Enrofloxacin	ENR	2.85	+	25	30	360.2>316.2	18	100	360.2>245.2	27	21
Ciprofloxacin	CIP	2.77	+	25	30	332.2>288.2	17	100	322.2>314.15	17	60
Marbofloxacin	MAR	2.65	+	35	30	363.1>72.0	20	100	363.1>320.0	15	24
Flumequine	FLU	3.92	+	35	30	262.1>244.0	15	100	262.1>202.0	35	36
Lincosamides											
Lincomycin	LIN	2.54	+	25	30	407.2>126.1	27	100	407.2>359.3	17	43
Macrolides											
Tylosin	TYL	3.89	+	30	30	916.5>174.1	40	100	916.5>101.0	45	85
Tilmicosin	TIL	3.47	+	60	30	869.6>174.2	42	100	869.6>695.5	37	49
Tulathromycin	TUL	2.81	+	25	30	806.6>230	26	100	806.6>577.5	26	15
Tildipirosin	TLD	2.47	+	25	30	734.6>98.1	47	100	734.6>174.1	47	12
Sulfonamides											
Sulfadoxine	SDX	3.47	+	35	30	311.0>156.0	15	100	311.0>192.0	32	78
Sulfadimethoxine	SDM	3.47	+	25	30	311.0>155.9	19	100	311.0>92.0	35	99
Sulfamonomethoxine	SMM	3.06	+	35	30	281.0>92.0	35	100	281.0>156.0	22	39
Sulfamethazine	SMT	2.90	+	30	30	279.0>186.0	15	100	279.0>92.0	25	61
Sulfisoxazole	SSX	3.23	+	35	30	268.0>92.0	28	100	268.0>156.0	13	89
Sulfamethoxazole	SMX	3.13	+	30	30	254.1>92.0	25	100	254.1>156.0	15	52
Pleuromutilins											
Tiamuline	TIA	3.50	+	25	30	494.6>192.2	22	100	494.4>119.1	38	44

Abbrev. – Abbreviation; [a] ESI – electro-spray ionization; [b] CV – Cone voltage; [c] DT – Dwell time; [d] CE – Collision energy

3.3.2. *Linearity*

The linearity of both methods was assessed by matrix-matched calibration within the concentration range of 5 – 300 μg kg^{-1} for FLU, LIN, SDX, SDM, SMM, SMT, SSX, SMX, and TIA with seven calibration points. The correlation coefficients (R^2) obtained were greater than 0.98, as outlined in the regulation.[26] For the analytes PEN, AMX, AMP, NAF, TTC, OTC, CTC, DOX, CEF, CEP, ENR, CIP, MAR, TYL, TIL, TUL, and TLD, linearity was determined using six calibration points in the range of 10 – 300 μg kg^{-1}, which also met regulatory requirements.[26]

Matrix-matched calibration addressed matrix interference (suppression or enhancement) by pre-

paring calibration standards within the same sample matrix.[19] This approach provided a more accurate representation of analyte behavior in real samples during preparation, as absolute recovery values were highly dependent on the actual matrix characteristics. Matrix-matched calibration was only feasible when a real blank feed was available, as was the case in this study. In the absence of blank feed material, the standard addition method was more suitable for analyte quantification.[19]

T a b l e 2

Limit of quantification, signal-to-noise ratios, combined measurement uncertainties, and decision limit (CCα) for substances analyzed using Methods 1 and 2

Analytes	Method 1				Method 2			
	LOQ [a] (μg kg^{-1})	S/N [b]	MU [c] (μg kg^{-1})	CCα [d] (μg kg^{-1})	LOQ [a] (μg kg^{-1})	S/N [b]	MU [c] (μg kg^{-1})	CCα [d] (μg kg^{-1})
Penicillins								
Penicillin V	50	32	5.90	59.68	50	15	8.79	64.41
Amoxycillin	100	33	17.66	128.96	100	17	18.72	130.71
Ampicillin	100	408	11.21	118.39	100	13	17.23	128.25
Nafcillin	50	41	8.59	64.09	50	21	7.80	62.79
Tetracyclines								
Tetracycline	50	104	7.16	61.74	50	19	7.89	62.93
Oxytetracycline	50	75	6.55	60.74	50	24	8.72	64.30
Chlortetracycline	50	69	7.18	61.78	50	13	7.54	62.36
Doxycycline	50	138	7.71	62.65	50	17	8.36	63.72
Cephalosporins								
Ceftiofur	50	138	4.73	57.76	50	12	9.48	65.55
Cephapirin	50	112	6.72	61.03	50	15	8.75	64.35
Quinolones								
Enrofloxacin	50	284	7.64	62.53	50	25	9.41	65.43
Ciprofloxacin	50	136	6.55	60.74	50	12	8.26	63.55
Marbofloxacin	50	379	7.33	62.02	50	21	8.96	64.70
Flumequine	25	284	3.14	30.15	25	136	4.70	32.71
Lincosamides								
Lincomycin	25	122	4.70	32.71	50	12	9.46	65.52
Macrolides								
Tylosin	50	69	4.79	57.85	50	22	8.50	63.95
Tilmicosin	50	454	6.65	60.90	50	91	8.58	64.08
Tulathromycin	50	81	7.64	62.53	50	30	9.46	65.51
Tildipirosin	50	1107	6.73	61.03	50	88	8.85	64.52
Sulfonamides								
Sulfadoxine	25	1205	3.62	30.93	25	30	4.07	31.67
Sulfadimethoxine	25	82	2.72	29.46	25	24	4.13	31.77
Sulfamonomethoxine	25	38	4.58	32.52	25	13	4.31	32.06
Sulfamethazine	25	73	3.74	31.13	25	20	4.40	32.21
Sulfisoxazole	25	272	4.33	32.10	25	12	3.95	31.47
Sulfamethoxazole	25	246	2.98	29.88	25	24	4.63	32.60
Pleuromutilins								
Tiamuline	10	3454	1.22	12.01	10	21	1.56	12.55

[a] LOQ – limit of quantification; [b] S/N – signal-to-noise ratio; [c] MU – combined measurement uncertainty; [d] CCα – decision limit

3.3.3. *Limit of quantification*

The LOQ is a critical parameter in the development of methods to analyzing cross-contamination MLs in non-targeted feed intended for food-producing animals.[8] This is because the LOQs required by regulatory standards essentially represent the maximum permissible levels of cross-contamination in non-target feeds.

In this study, LOQs were established based on the signal-to-noise ratio criterion of at least 10:1 (Table 2). Almost all LOQs were determined at concentrations equal to or below the levels specified by the regulation.[8] The LOQs for both methods ranged from 10 to 100 μg kg^{-1} (Table 2). Based on the presented results, LOQs achieved for PEN, FLU, SDX, SDM, SMM, SMT, SSX, SMX, and TIA met the regulatory requirements.[8] Additionally, for AMX, TTC, OTC, CTC, DOX, and TYL, the determined LOQs were lower than the corresponding values specified by the regulation.

The method was also validated for analytes not regulated by the legislation,[8] such as AMP, NAF, CEF, CEP, ENR, CIP, MAR, TIL, TUL, and TLD, with LOQs ranging from 50 – 100 μg kg^{-1} (Table 2).

Method 1 demonstrated higher signal-to-noise ratios, indicating its potential for achieving lower LOQs for the tested substances (Table 2). The reduced signal-to-noise ratios observed in Method 2, along with the higher LOQ for LIN, which exceeded the regulatory threshold of 25 μg kg^{-1}, were likely attributable to the SPE purification step. While SPE improves sample purity and reduces matrix interference, it may also lead to analyte dilution and potential loss of sensitivity if not optimally configured.[33] Therefore, to enhance the sensitivity of Method 2 for LIN, further optimization of the SPE protocol is necessary to achieve an effective balance between purification and retention of analyte concentrations sufficient for accurate and sensitive detection at the required MLs.[8]

The LOQs obtained in this study for both methods were generally consistent with and comparable to previously published data for poultry feeds. For example, a method without SPE purification by Borrás et al.,[33] reported LOQs for various antimicrobial classes: 8.5 – 21.4 μg kg^{-1} for macrolides, 12.3 – 228 μg kg^{-1} for fluoroquinolones, 8.7 – 58.5 μg kg^{-1} for sulfonamides, 70.3 – 78.0 μg kg^{-1} for tetracyclines, and 0.6 μg kg^{-1} for tiamuline. Additionally, a method developed for 37 antimicrobial substances without SPE purification reported LOQs in the range of 10 – 30 μg kg^{-1}.[31] Valese et al. reported LOQs ranging from 25 to 50 μg kg^{-1} for sulfonamides, tetracyclines, and fluoroquinolones.[29] In contrast, Patyra et al.[23] and Avolio et al.[24] reported higher LOQs, ranging from 133 to 217 μg kg^{-1} and 25 to 1000 μg kg^{-1}, respectively.

For Method 2, recent findings[10] reported LOQs ranging from 3 μg kg^{-1} for tiamulin (TIA) to 60 μg kg^{-1} for amoxicillin (AMX). A more recent study by Varenina et al.[25] reported LOQs ranging from 10 to 50 μg kg^{-1} for most analytes, except sulfisoxazole, which had an LOQ of 500 μg kg^{-1}.

3.3.4. *Precision and accuracy*

Due to the unavailability of certified reference materials for all analytes under investigation, the accuracy and precision of the method were assessed in terms of recovery, repeatability (within-day precision), reproducibility (between-day reproducibility), and combined measurement uncertainty. This evaluation was performed using blank feed samples spiked with known analyte quantities.

Precision and accuracy were assessed using three validation series for each method, conducted on three separate occasions. Each series consisted of six replicates of blank samples spiked at three different validation levels. The results are presented in Table 3. Repeatability values were below 15 % for both methods, whereas the reproducibility values ranged from 10 % to 20 %. These precision values met regulatory requirements, as all values were below two-thirds of 20 % for repeatability and below 20 % for reproducibility.[26]

Accuracy was determined based on relative recovery, with values for Method 1 ranging from 95 % to 103 %, and for Method 2, from 88 % to 101 % (Table 3). The high recovery values observed were attributed to the use of matrix-matched calibration, which compensates compensated for the complex MEs present in real feed samples. The recovery values obtained were within the acceptable range specified by methods validation guidelines,[26] which set a range of 80 – 120 %.

In comparison, methods developed for analyzing antimicrobials at cross-contamination levels without SPE purification reported recovery values between 90.4 % and 103 %,[31] 76 % and 120 %,[23] and 65 % and 106 %.[35] For Method 2, similar studies employing SPE sample preparation achieved recovery values ranging from 78 to 125 % and from 75 % to 121 %.[10,25]

T a b l e 3

Validation results for within-day precision, between-day precision, and recovery, at various spiking levels for Methods 1 and 2

Analyte	Spiking level µg kg^{-1}	Method 1			Method 2		
		Within-day RSD (%) (N = 6)	Between-day RSD (%) (N = 18) [b]	Recovery (%) (N = 18) [c]	Within-day RSD (%) (N = 6)	Between-day RSD (%) (N = 18) [b]	Recovery (%) (N = 18) [c]
Penicillin V	50[a]	7.34	11.76	96.13	10.45	17.55	91.93
	100	7.48	10.09	98.52	8.76	15.86	93.32
	150	7.70	11.18	98.14	4.27	12.45	93.61
Amoxycillin	100 [a]	8.14	17.62	95.91	11.87	18.70	88.66
	150	9.03	12.10	97.43	7.18	12.40	90.34
	200	10.14	14.03	95.36	6.35	9.50	94.42
Ampicillin	100 [a]	6.20	11.17	101.28	11.21	17.25	92.06
	150	5.16	7.62	100.79	8.46	12.19	93.85
	200	5.39	8.48	97.99	6.35	7.99	95.99
Nafcillin	50 [a]	8.89	17.16	102.30	9.71	15.57	95.06
	100	5.05	6.78	97.49	7.84	12.56	96.02
	150	6.81	9.19	98.94	5.83	9.01	96.18
Tetracycline	50 [a]	11.93	14.27	97.51	7.24	15.74	99.87
	100	7.92	11.97	99.46	6.22	15.60	95.35
	150	6.56	8.92	100.14	5.60	8.40	96.76
Oxytetracycline	50 [a]	9.41	14.31	99.65	10.80	17.41	89.90
	100	6.74	9.30	97.16	6.96	11.52	92.65
	150	3.21	5.07	98.64	6.25	8.57	96.24
Chlortetracycline	50 [a]	7.85	14.33	99.79	9.08	15.40	93.33
	100	7.14	10.41	98.64	7.44	13.03	94.04
	150	5.73	8.61	98.81	4.83	9.22	96.25
Doxycycline	50 [a]	9.25	15.39	99.77	13.05	18.39	92.27
	100	8.47	10.98	99.03	8.64	13.03	91.56
	150	7.06	11.13	97.60	5.44	7.37	95.81
Ceftiofur	50 [a]	8.15	9.41	102.90	11.60	18.94	92.79
	100	5.22	8.08	99.54	8.94	11.91	98.61
	150	3.90	6.82	100.03	5.37	8.59	95.66
Cephapirin	50 [a]	9.49	13.41	99.19	10.15	17.47	93.55
	100	4.91	7.50	99.88	8.87	14.25	94.14
	150	5.95	9.53	98.92	5.77	9.25	94.58
Enrofloxacin	50 [a]	6.85	15.25	98.98	12.48	18.79	92.46
	100	4.80	8.92	97.81	9.90	14.16	95.67
	150	2.96	8.25	100.24	7.26	10.88	96.17
Ciprofloxacin	50 [a]	9.10	13.06	96.84	8.34	16.50	91.84
	100	6.97	12.31	99.28	8.15	13.48	94.85
	150	5.15	7.09	95.72	4.69	7.19	95.47
Marbofloxacin	50 [a]	11.03	14.63	101.32	10.92	17.90	96.70
	100	8.42	10.90	99.61	8.89	11.97	95.83
	150	5.28	9.78	98.03	4.66	9.54	97.04
Flumequine	25 [a]	9.04	12.52	97.59	10.31	18.79	94.26
	50	7.77	13.82	99.34	11.92	18.57	92.67
	100	6.61	10.65	96.25	10.97	15.72	94.79
Lincomycin	25 [a]	9.13	14.91	99.86	/	/	/
	50 [a]	11.09	18.77	95.83	11.70	18.90	92.84
	100	8.63	12.74	97,46	7.28	12.93	92.05
	150	/	/	/	5.20	8.29	98.20
Tylosin	50 [a]	5.77	9.53	99.15	10.07	16.98	97.05
	100	5.61	10.23	95.22	8.96	13.84	92.09
	150	7.70	11.19	99.30	4.89	8.22	95.15
Tilmicosin	50 [a]	5.73	13.26	101.24	9.29	17.14	92.79
	100	6.07	8.08	101.10	11.79	17.91	92.69
	150	3.89	6.76	100.89	5.15	8.12	96.81
Tulathromycin	50 [a]	7.19	15.18	97.43	11.55	18.83	93.40

	100	4.29	8.18	99.63	8.31	15.46	95.00
	150	6.30	9.32	99.19	5.28	7.84	95.93
	50 [a]	9.73	13.35	99.02	10.27	17.67	91.42
Tildipirosin	100	5.05	8.54	100.59	8.81	16.42	93.43
	150	6.13	9.22	101.79	5.02	10.59	96.42
	25 [a]	11.42	14.43	97.17	10.39	16.25	96.11
Sulfadoxine	50	5.77	10.74	101.13	7.81	16.95	94.73
	100	7.15	8.66	100.76	7.63	13.36	97.49
	25 [a]	7.17	10.83	97.57	9.31	16.49	97.50
Sulfadimethoxine	50	6.10	8.51	101.66	8.36	17.38	96.32
	100	6.75	8.36	99.52	6.75	10.8	100.38
	25 [a]	11.62	18.31	98.32	12.01	17.20	94.72
Sulfamonomethoxine	50	8.96	13.20	100.54	11.65	18.20	92.62
	100	6.43	8.77	99.08	8.23	15.11	95.58
	25 [a]	7.51	14.92	97.30	10.34	17.56	96.73
Sulfamethazine	50	7.48	11.95	99.85	9.47	15.18	96.29
	100	5.18	6.47	98.19	6.82	12.00	98.97
	25 [a]	9.21	17.28	97.05	9.26	15.76	96.32
Sulfisoxazole	50	6.29	11.75	100.25	11.44	18.27	93.98
	100	7.22	9.34	98.24	6.71	9.68	98.54
	25 [a]	7.41	11.87	98.19	10.93	18.50	100.47
Sulfamethoxazole	50	8.10	12.79	98.44	11.16	18.04	96.82
	100	5.03	10.42	99.11	9.82	15.18	97.17
	10 [a]	12.19	14.94	95.19	9.90	15.53	100.19
Tiamuline	50	8.02	12.19	97.59	7.35	10.70	97.68
	100	9.55	11.67	101.11	7.80	12.06	95.28

[a] Limit of quantification; for lincomycin: Method 1, LOQ = 25 μg kg^{-1}; Method 2, LOQ = 50 μg kg^{-1}; [b]N = 3 × 6 = 18; six replicates over three days; [c]Average from six replicates over three days.

3.3.5. *Determination of decision limit (CCα)*

The decision limit (CCα) is defined as the concentration at or above which a sample was deemed non-compliant, with an associated error probability of α.[26] Various methodologies were available for calculating CCα values, all relying on between-day reproducibility experiments.[26] In this study, the CCα calculation was based on the estimated combined measurement uncertainty at the maximum permitted levels,[26] specifically the LOQs determined for each analyte for both methods, using Equation 1. The calculated results are presented in Table 2.

To estimate the combined measurement uncertainty (MU), a bottom-up approach was used, incorporating uncertainty components arising from reproducibility and sources of type B uncertainties.[27] This approach required the individual quantification of each uncertainty source, which, when applied to a large number of analytes, was both time-consuming and challenging to implement. Nevertheless, the detailed calculations provided by this method ensured that no sources of uncertainty were overlooked.[29] A comprehensive uncertainty study showed that relying solely on empirical data from in-house validation could lead to an underestimation of MU values for certain analytes, while neglecting other sources that significantly contributed to the overall uncertainty.[29]

The calculated combined measurement uncertainties (MU) for both methods were then used to determine the CCα values for the analytes included in the study, using Equation 1.[26] When calculating the CCα values for authorized substances, it was crucial to achieve values as close as possible to the levels of interest.[26] Because the between-day reproducibility for all analytes, when tested using both methods, was below the maximum permitted reproducibility limit[26] of 20 % RSD (Table 3), the obtained CCα values demonstrated satisfactory proximity to the maximum contamination levels from the regulation.[8]

3.3.6. *Relative matrix effect*

Electrospray ionization (ESI) is widely considered as preferred ionization source for LC-MS analysis, owing to its broad compound coverage capabilities. However, ESI is known to significantly affect ionization efficiency in the presence of a matrix, potentially affecting detection reliability.[29] Therefore, it was crucial to assess the ME of analytes during ionization. The relative MEs determined using both methods are shown in Figure 1.

For Method 1, using Equation 2, the calculated MEs revealed substantial signal suppression

due to feed matrix. For most analytes (16 of 26), the observed ME suppression was approximately 80 %. For NAF, TTC, OTC, CTC, CEP, ENR, and TYL, the suppression was approximately 70 %. In contrast, LIN and TLD showed suppression of approximately 65 % and 40 %, respectively. Previous studies that used sample preparation without SPE purification have not extensively investigated the ME of the feed.[31,10,23,25,35] In a study by Valese et al.,[29] the signals for tetracyclines were suppressed by approximately 80 %, whereas those for sulfonamides ranged from 40 % to 50 %. In the same study, other substances showed suppression varying between 20 % and 40 %, whereas some substances, such as amoxicillin, exhibited a slight signal enhancement due to ME.[29] A study conducted on pig feed reported ME for antimicrobial substances ranging from –50 % to +50 %.[33]

In Method 2, the experiments conducted to evaluate the ME in the analysis yielded variable outcomes (Fig. 2). Among all analytes, 14 exhibited an ME suppression of less than 20 %. The signal suppression for TTC, OTC, DOX, CIP, MAR, LIN, SDX, SDM, SMT, and SMX ranged from 20 % to 60 %. In contrast, signal enhancement was observed for CEP, FLU, TYL, TIL, TLD, and TIA, with TIL showing a value of approximately 50 %.

Given that these matrix suppressions were significantly higher than the recommended 20 % threshold,[26] it was concluded that matrix-matched calibration curves should be used for quantification provided that the corresponding blank feed was available. The complete feed components created a highly complex and variable matrix[9], with significant differences between feeds intended for different livestock animals. In the absence of a suitable blank feed matrix, the use of standard addition effectively addressed MEs.[29]

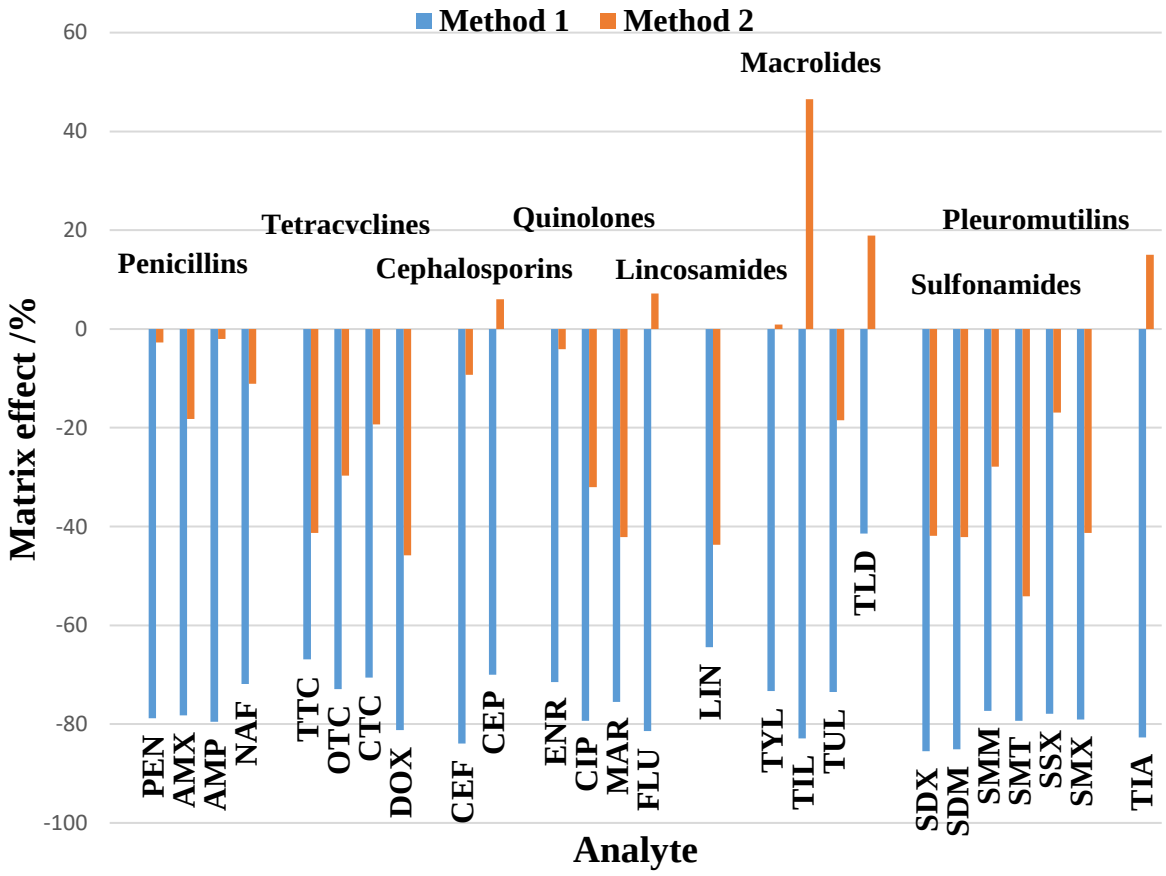


Fig. 1. Relative matrix effect of the analytes in Methods 1 and 2

3.3.7. *Stability of analytes in sample extracts*

The stability of the analytes in sample extracts during storage or analysis, was thoroughly evaluated because any instability could have compromised the reliability of the test results. If stability data for analytes were already available, as in previous studies, generating new data was unnec-

essary. However, referencing the existing stability data for analytes in a solution or matrix was permissible only when identical methods and conditions were applied.[26] Notably, stability data were rarely included in validation studies of feed analysis methods, with most studies primarily addressing the stability of standard solutions under various storage conditions.[29,30,35–38] This suggested that analyte stability in the final extracts during the storage stage was frequently overlooked in the method validation process.

In this study, we assessed the stability of analytes in prepared extract solutions intended for injection into UHPLC-MS/MS to determine the feasibility of short-term storage of samples before analysis. Stability experiments were conducted for both methods, and the results were presented in Supplementary Table 1S. According to the specified criteria outlined in the regulation,[26] the difference between the average of five stored replicates and the average of five fresh replicates was required to be lower than 15 % RSD. Figures 2 and 3 present the data for both methods regarding the remaining concentrations of the analytes in extracts from spiked feed at LOQs obtained from two aliquots after storage for two days at 2 – 6 °C and below –20 °C. The results indicated that in both temperature regimes, the examined methods exhibited satisfactory stability according to the specified criteria.[26]

The extracts obtained using Method 1 and 2, with analytes belonging to the tetracycline, quinolone, macrolide, lincosamide, sulfonamide, and pleuromutilin groups, exhibited high stability when compared to compounds belonging to the penicillin and cephalosporin group. Our findings were consistent with those of other studies,[29,30,35] which concluded that β-lactam substances could be a limiting factor for long-term storage of prepared extracts. For long-term stability studies, other factors influencing the analyte degradation process should have been considered, such as the solvents present in the extracts and the pH of the extracts.[37,38]

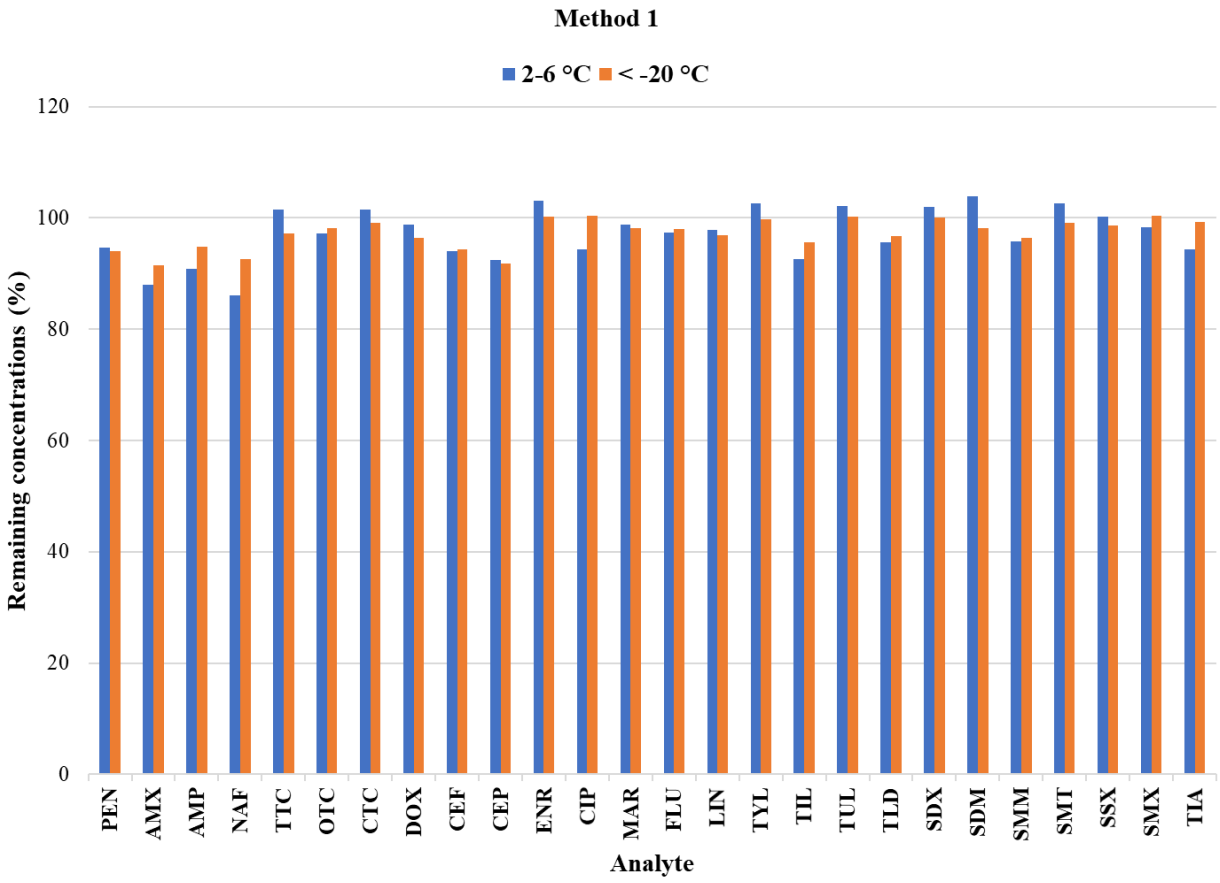


Fig. 2. The results of the short-term stability study of spiked extracts from blank feed obtained with Method 1 (without SPE) at the following LOQs: TIA (10 μg kg^{-1}); FLU, LIN, SDX, SDM, SMM, SMT, SSX, and SMX (25 μg kg^{-1}); PEN, TTC, OTC, CTC, DOX, CEF, CEP, ENR, CIP, MAR, TYL, TIL, TUL, and TLD (50 μg kg^{-1}); AMX and AMP (100 μg kg^{-1}).

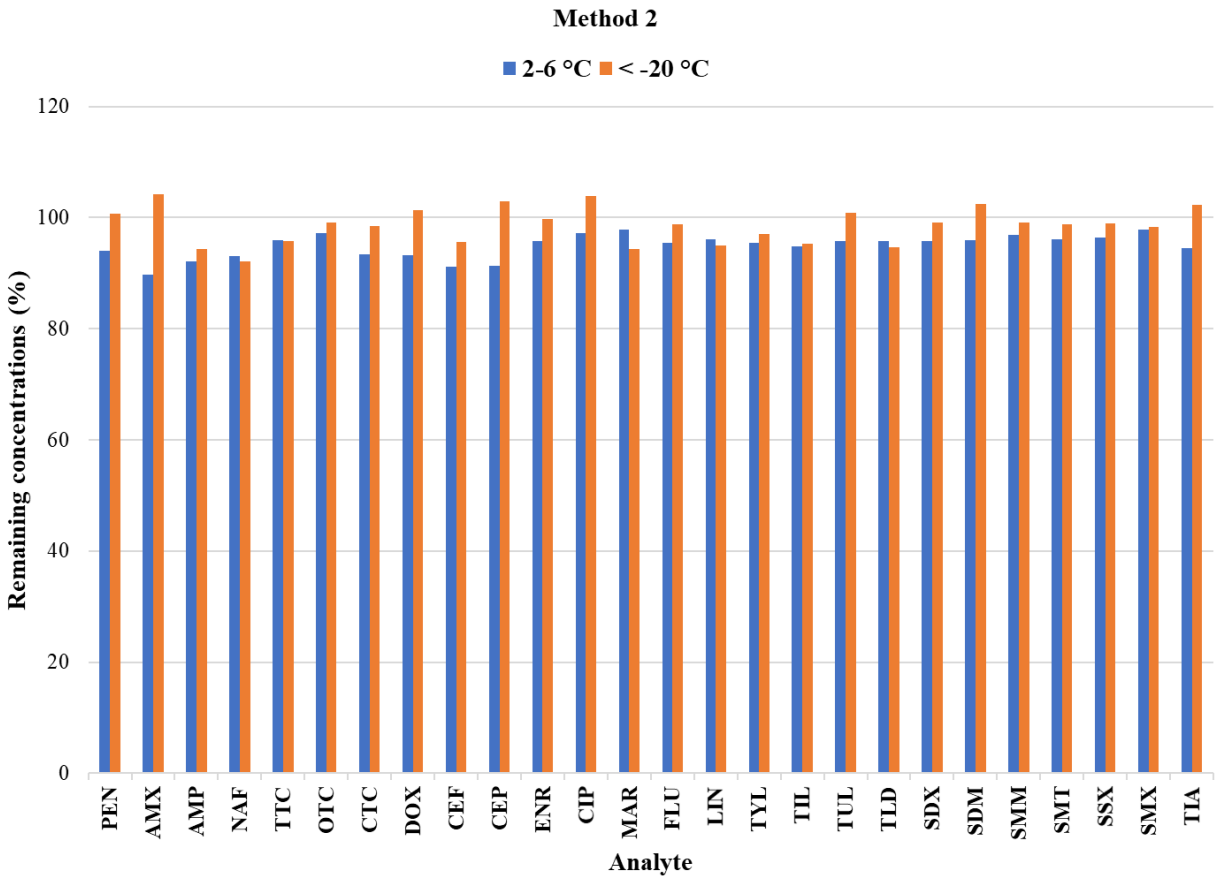


Fig. 3. The results of the short-term stability study of spiked extracts from blank feed obtained with Method 2 (with SPE) at the following LOQs: TIA (10 μg kg^{-1}); FLU, SDX, SDM, SMM, SMT, SSX, and SMX (25 μg kg^{-1}); PEN, TTC, OTC, CTC, DOX, CEF, CEP, ENR, CIP, MAR, LIN, TYL, TIL, TUL, and TLD (50 μg kg^{-1}); AMX and AMP (100 μg kg^{-1}).

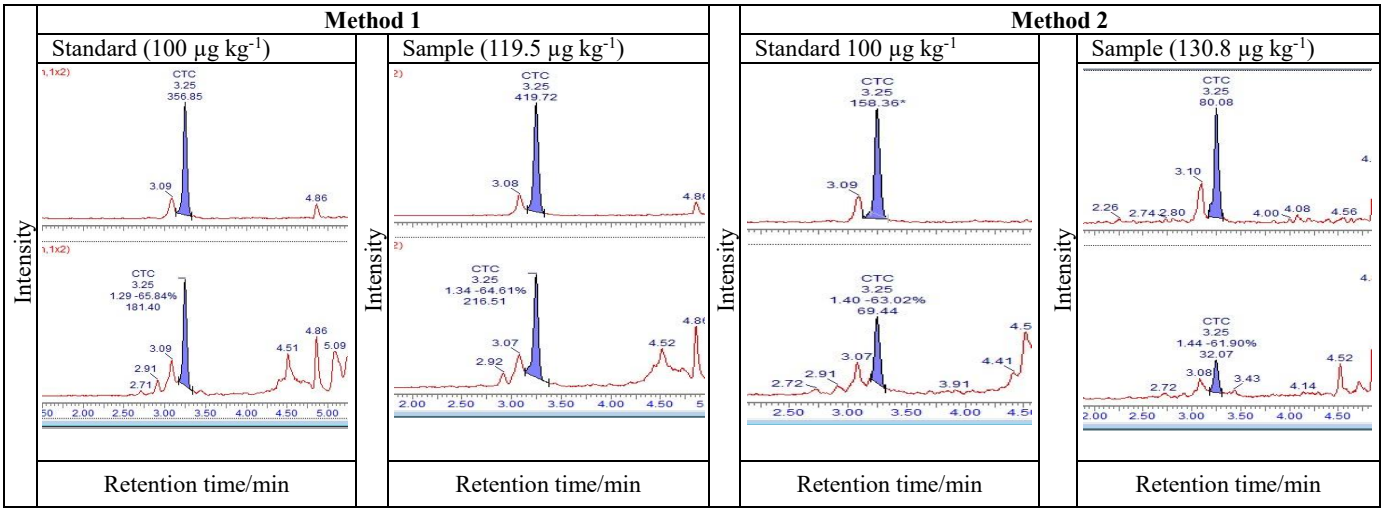
3.4. *Applicability of methods to real samples*

To assess the applicability of the validated methods, 39 poultry feed samples were collected from farms and feed distributors. Information on whether the feed was medicated was not available. The feed used in laying hens and broiler production, specifically in food-producing poultry, was assumed to be non-medicated prior to analysis. A summary of the identified antimicrobials and their concentrations is provided in Supplementary Table 2S. The results from both methods were comparable, considering the associated measurement uncertainties (Table 2). The findings indicated that approximately 41 % of the tested feed contained at least one substance within the scope of the methods, with TYL, LIN, CTC, and SMT identified. Multiple analytes were observed in 32 % of the samples (Table S3), and in 35 % of the samples, the MLs and CCα values were exceeded (Table 2).

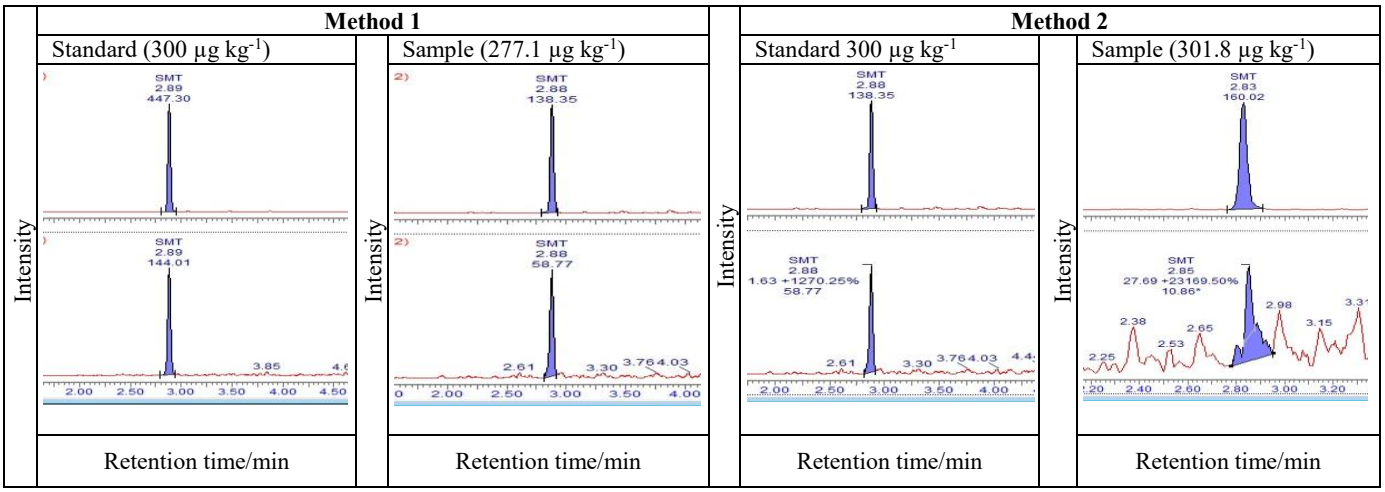
Notably, although the feed samples were assumed to be non-medicated, 12 samples were found to contain TYL or LIN, and one contained SMT at concentrations exceeding the upper range of the established methods (300 μg kg^{-1}). This suggested that some of these feeds were likely medicated. The representative ion chromatograms of the quantification and confirmation ions obtained for samples containing antimicrobial substances are shown in Figure 4. These findings aligned with those of a previous study, which reported 44.4 % contamination of tested samples.[29] Another study reported 17 % contamination of poultry feeds, with tetracyclines being the most prevalent.[24] Tylosin and sulfadiazine were identified as the most frequent findings in a study published by Borràs et al. [33]

The presence of antimicrobial substances at levels exceeding regulatory limits highlighted the necessity for stricter feed control in current agricultural practices and the use of fast, sensitive, and reliable analytical methods that met legislative requirements.[8]

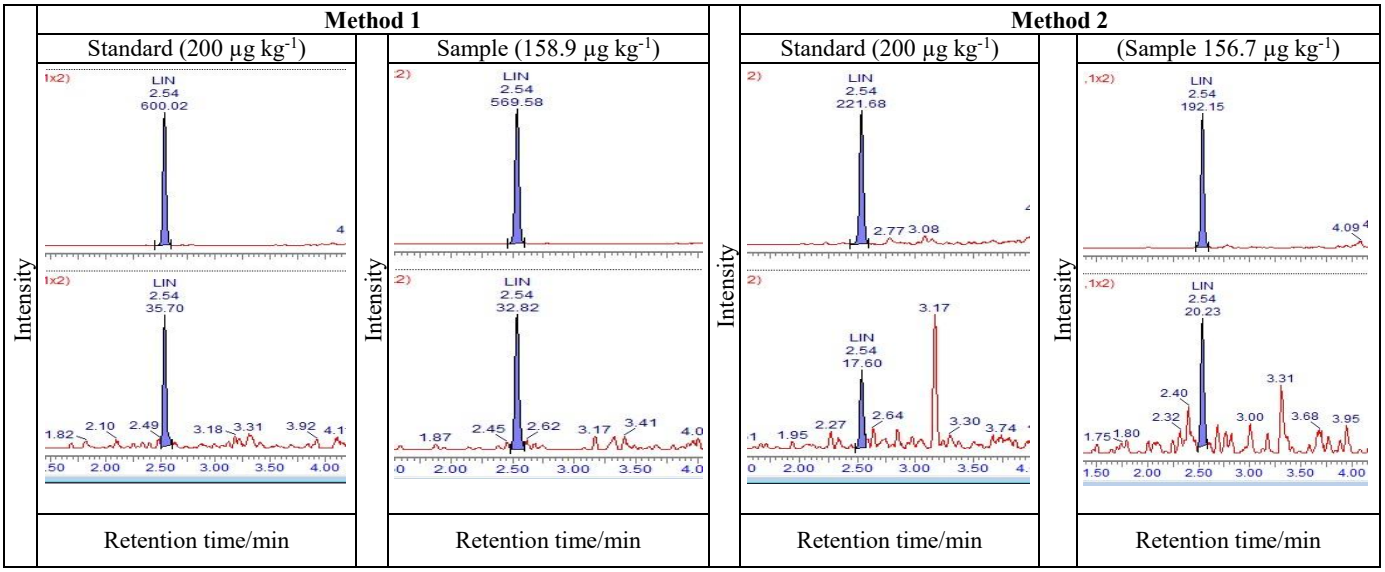
A. Chlortetracycline (sample 73)



B. Sulfamethazine (sample 94)



C. Lincomycin (sample 73)



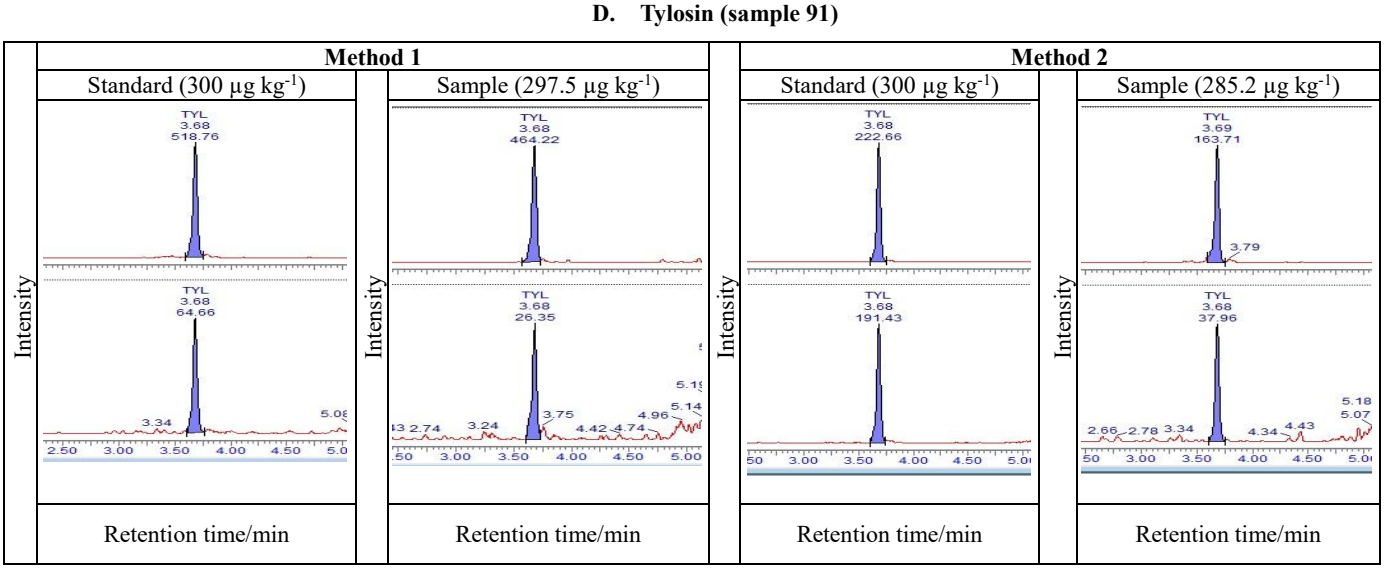


Fig. 4. Representative chromatograms for quantification and confirmation ions for some for selected detected antimicrobials: A. Chlortetracycline, B. Sulfamethazine, C. Lincomycin, and D. Tylosin

4. CONCLUSION

This study successfully established and validated two sensitive and reliable multi-class UHPLC-ESI-MS/MS methods for the analysis of 26 antimicrobial substances in poultry feed at cross-contamination levels. Both methods, without (Method 1) and with SPE purification (Method 2), demonstrated satisfactory performance in terms of sensitivity, selectivity, linearity, limits of quantification, precision, and accuracy. The only deviation from the regulatory requirements was observed for lincomycin when Method 2 was used. This study highlights significant MEs in feed analysis, emphasizing the importance of matrix-matched calibration for accurate quantification. A short-term stability study confirmed the reliability of results when instrumental analysis was performed within two days after sample preparation. Additional validation experiments were necessary to assess the applicability of the established methods for various feed types.

Applying these methods to real poultry feed samples revealed that approximately 41 % contained at least one antimicrobial substance, with some of them exceeding the cross-contamination maximum levels. This underscores the need for routine feed monitoring to prevent uncontrolled use of antimicrobials in poultry production. These validated methods provided valuable tools for regulatory authorities and the feed industry to ensure compliance with cross-contamination limits and support efforts to combat antimicrobial resistance.

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Conflict of interest. We declare that we have no conflicts of interest regarding the submitted manuscript.

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