Antibiotic residues in non-targeted animal feed – development of a sensitive LC-MS/MS methodology

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Abstract

Antimicrobial substances are used on farms for therapeutic, prophylactic, or growth-promoting purposes. Antibiotic residues can be detected in animal feed due to unauthorised use for prophylaxis and as growth promoters, or as a result of unintentional cross-contamination during feed production. The presence of antibiotic residues in food has been linked to allergic reactions, gut microbiota imbalance, development of antibacterial resistance, or potential toxic effects. A multi-residue method was developed for the detection and quantification of 43 analytes from 9 antibiotic classes (penicillins (2), quinolones (2), macrolides (3), lincosamides (1), phenicols (2), pleuromutilins (2), tetracyclines (4), sulfonamides (25), quinoxalines (2)) in animal feed. The method was designed for feed samples with varying contents and particle sizes. Extraction and purification were based on the addition of organic solvents and the solid-phase extraction (SPE) clean-up method to achieve the best analyte response. Antibiotics were detected by high-performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-MS/MS) within 17 minutes in positive and negative scan modes. Average recovery rates ranged from 75.7% (sulfamethazine) to 121.3% (carbadox) with corresponding relative standard deviations of 9.2% and 49.6%. The method is suitable for screening antibiotic contamination in animal feed in the range from 1 to 50 µg/kg and for confirming substances in the range from 10 to 1000 µg/kg. A significant matrix effect was observed when comparing the analysed signals between different feeds, indicating the necessity of using a matrix calibration curve and a standard addition method.

Key words: *feed; antibiotic residues; cross-contamination; LC-MS/MS*

Introduction

The frequency of antibiotic use in human medicine, as well as in the prevention and treatment of animals, and their use as growth promoters in animals, has contributed to the prevalence of antibiotics in natural ecosystems (Martinez, 2009). The

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genes for antibiotic resistance that were transferred to pathogenic bacteria through horizontal gene transfer originated from naturally occurring bacteria. The contact of bacteria and antibiotics from the human digestive tract, via faeces, with bacteria in sewage and other environmental sources, has further contributed to the spread of existing antibiotic-resistant genes and the emergence of new mechanisms of antibiotic resistance in bacteria (Baquero et al., 2008). Genes responsible for antibiotic resistance can be viewed as pollution of the microbiosphere when antibiotic residues enter natural ecosystems and come into contact with indigenous bacteria, causing the genes responsible for resistance to become an integral part of the bacterial genomic subunit, or when solid waste and sewage contain antibiotic-resistant bacteria.

As human and animal health are closely linked by the One Health approach, joint assessments of antibiotic use and the spread of antibiotic resistance of bacterial species in humans and animals have been carried out in recent years (Varenina et al., 2024). A significant decrease of the consumption of the antimicrobials (AMC) and antimicrobial resistance (AMR) in 28 EU/EEA countries was observed between 2014 and 2021; in 2021, AMC was assessed at 125.0 mg/kg biomass for humans and 92.6 mg/kg biomass for food-producing animals. Between 2014 and 2021, total AMC in food-producing animals decreased by 44%, indicating the efficacy of measures taken at the national level. A study has been observing data related to resistance of carbapenems, third- and fourth-generation cephalosporins, fluoroquinolones and other quinolones, aminopenicillins, polymyxins, macrolides and tetracyclines to *Escherichia coli*, *Campylobacter* and *Klebsiella pneumoniae* (ECDC, EFSA, EMA, 2024).

The Ministry of Agriculture of Croatia releases the list of authorised substances for the medication of animal feed. Approved substances from the antibiotic class include doxycycline, oxytetracycline, florfenicol, and tilmicosin (MA, 2024). Regulation (EU) 2019/4 lays down specific provisions regarding medicated feed and intermediate products, specific maximum levels of cross-contamination for 24 active substances in non-target feed, and methods of analysis for the detection of residues of such active substances (EC, 2019). It also indicates the list of antibiotics administered using animal feed: amoxicillin, amprolium, apramycin, chlortetracycline, colistin, doxycycline, florfenicol, flumequine, lincomycin, neomycin, spectinomycin, sulfonamides, tetracycline, oxytetracycline, oxolinic acid, paromomycin, penicillin, tiamulin, tiamfenicol, tilmicosin, trimethoprim, tylosin, valnemulin, and tylvalosin. During the administration of medicated feed, the withdrawal period specified in the veterinary prescription should be followed.

On farms where animals are kept in groups, various bacterial diseases can occur, including respiratory and gastrointestinal tract infections. Antibiotics are used in the EU for therapeutic purposes, but they can also be used for prophylactic and growth-promoting purposes in other parts of the world. Metaphylaxis ensures that treatment covers both clinically ill and potentially infected animals, for which the administration of drugs in feed is most appropriate route. The use of antibiotics as growth promoters has been banned in the EU since January 2006 (EC, 2003). The presence of antibiotic residues in food is associated, in some cases, with allergic reactions, gut microbiota imbalances, and the development of antibacterial resistance. Cephalosporins (third to fifth generation), glycopeptides, macrolides, ketolides, polymyxins, quinolones, and tetracyclines are important antibiotics for human med-

icine and are therefore banned for use in veterinary medicine. They are among the most important antibiotics to monitor in residue analysis (Muaz et al., 2018).

Animal feed may often fail to meet legal requirements due to residues of veterinary drugs resulting from unintentional cross-contamination during feed production or on the farm, or from unauthorised use for prophylaxis and as growth promoters. Therefore, highly sensitive and selective multi-residue methods are necessary to detect possible veterinary drugs that could be added to feed (Borràs et al., 2011). Various methods have been developed to determine antibiotics in medicated feed and antibiotic residues resulting from cross-contamination. Microbiological methods are commonly used, where the main principle is to determine the inhibition of sensitive microorganism growth (Arsène et al., 2022). These methods offer easy application and quick results, but they are prone to frequent false positive results (Przeniosło-Siwczynska et al., 2020). Simple screening methods based on immunochemical tests, such as ELISA (enzyme-linked immunosorbent assay), are more precise and can provide semi-quantitative results. However, each test is specific only a few representatives from a specific antibacterial group. To comprehensively screen the entire group of antibiotics that may contaminate animal feed, analysis would require the use of a range of analytical ELISA kits, which is not economically feasible (Borràs et al., 2011).

In modern analytical chemistry, liquid chromatographic methods are increasingly used in combination with mass spectrometry (LC-MS/MS) to achieve high sensitivity and selectivity. Such multi-methods include different groups of antimicrobial substances and thus enable the screening and confirmation of the analytes to be determined (Varenina et al., 2023). Due to the complexity of the matrix, it is necessary to develop a purification procedure that simultaneously extracts the analyte efficiently and removes the matrix, which can affect sensitivity. By choosing a chromatographic column, the selectivity of the method is improved, and by optimising the mass spectrometric parameters, high sensitivity is achieved, which is crucial for detecting unintentional contamination of feed in production. This article outlines the multi-residue method for determining antibiotic residues in feed, along with the validation procedures and parameters. The method encompasses eight groups of antibiotics, such as penicillins, quinolones, macrolides, phenicols, pleuromutilins, quinoxalines, tetracyclines, and sulfonamides, most of which serve as representatives of unauthorised pharmacologically active substances for prevention and treatment purposes.

Materials and methods

Chemicals and standards

All chemicals were of HPLC or LC-MS grade. UPLC/MS grade acetonitrile and methanol were supplied from Biosolve Chimie (Dieuze, France). Formic acid LC-MS Ultra and dimethyl sulfoxide (DMSO) Chromasolv plus for HPLC were supplied from Honeywell Burdick Jackson (Michigan, USA). Nitrogen 5.0 and 5.5 were acquired from SOL spa® (Monza, Italy). Ultrapure water was produced with the Milli-Q system (Millipore®, Bedford, USA). The solid phase extraction tubes (SPE) Oasis Prime HLB was purchased from Waters (Waters Corporation, Milford, USA).

The standards amoxicillin trihydrate, chlortetracycline hydrochloride, doxycycline hydrochloride, florfenicol, flumequine, lincomycin hydrochloride,

olaquindox, oxolinic acid, oxytetracycline hydrochloride, penicillin V potassium salt, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadoxin, sulfaguanidine, sulfamerazine, sulfameter, sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamethoxypyridazine-D3, sulfamonomethoxine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, sulfabenzamide, sulfacetamide, sulfaclozine, sulfamethizol, sulfamoxole, sulfapyridine, sulfasalazine, sulfatroxazole, sulfisomidine, tetracycline hydrochloride, thiamphenicol, tiamulin, tilmicosin, trimethoprim, tylosin tartrate and valnemulin hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Penicillin V-D5, sulfamethazine D4, tetracycline D6 and tylvalosin were used from Toronto Research Chemicals (North York, ON, Canada). Carbadox was supplied from Dr. Ehrenstorfer LGC Group (Teddington, UK).

Standard solution

To prepare a solution of a mixture of standards for sample enrichment, stock solutions were first prepared for each individual analyte (1 mg/mL). Oxolinic acid was diluted in 1 mL 1M NaOH with the addition of 9 mL MeOH, and flumequine was prepared using DMSO. Macrolides, lincosamides, tetracyclines and sulfonamides stock solutions were prepared in methanol, with the exception of sulfadiazine (1M NaOH) and sulfaquinoxaline (1 mL 0.5 M NaOH and 9 mL MeOH). These stock solutions were stored at -20°C. An intermediate stock solution as a mixture of all antibiotic groups was prepared in methanol at 10 µg/mL. Two working solutions were prepared for standard addition at concentrations of 1 µg/mL and 0.1 µg/mL. The working solution of labelled internal standards was prepared in methanol using stock solutions of enrofloxacin-D5, sulfamethazine-D4, tylosin-D3 and tetracycline-D6 at a concentration of 1 µg/mL. The stock solutions are stable for 1 year. Intermediate and working standard solutions were stable for a one-month period. All solutions were stored at -20°C.

Laboratory equipment

The following equipment was used in sample preparation: Waring Commercial Blender 7011HS (Waring Commercial, CT, USA), IKA® Vortex model MS2 Minishaker (IKA®-WERKE GMBH & CO.KG, Staufen, Germany), multi-tube vortexer (VWR International GmbH, Ulm, Germany), ultrasonic bath Grant (Grant instruments, Cambridge, UK), centrifuge Rotanta 460R (Hettich Zentrifugen, Tuttlingen, Germany), ultracentrifuge (Thermo Fisher Scientific, Waltham MA, USA) and MultiVap 54 automatic evaporation system (LabTech Srl, Sorisole (BG), Italy). The LC-MS/MS system consisted of UHPLC Agilent Technology 1290 Infinity II and Triple Quad LC-MS 6470 mass spectrometer with Jet Stream Technology Ion Source (AJS ESI) (Agilent Technologies, Santa Clara, USA). Instrument control and data processing were performed using MassHunter Workstation 10.0 software developed by Agilent Technologies, Inc. For chromatography, an Acquity UHPLC HSS T3 column (1.8 µm, 2.1x150 mm) was used (Agilent Technologies, Santa Clara, USA).

Validation of the multi-residue method

The validation of the method for the determination of antibiotics in feed using the LC-MS/MS technique was carried out in accordance with EU Regulation 2021/808 using the conventional model (EC, 2021). The following validation parameters were calculated using validation software for comprehensive in-house validation InterVal Plus®: linearity, specificity/selectivity, repeatability and inter-laboratory reproducibility, deci-

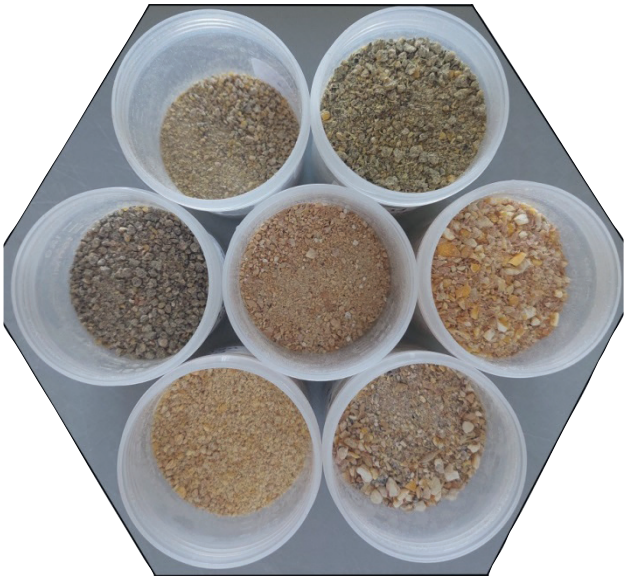

Figure 1. Samples of feedstuffs used in validation with varying granulation and composition

sion limit (CCα) and detection capability (CCβ). To determine the specificity and selectivity, seven types of feed were collected (three lots per feed type) (Figure 1). For the validation, food samples were selected that are most frequently monitored for residues of pharmacologically active substances, i.e., feed from maize and wheat. The validationn was carried out for one month under different conditions (day, operator and type of matrix). The analytical procedure was divided into three different days, and on each of these days, an analyst analysed a total of 40 samples, i.e., four replicates per each matrix, i.e., wheat and maize, at five concentration levels (1, 10, 50, 500 and 1000 μg/kg).

Sample preparation

Two grams of minced animal feed were weighed into a 50 mL centrifuge tube. All samples, including the blank sample, were spiked with an internal standard at a concentration corresponding to the medium fortification level. Matrix calibration samples were fortified with a working standard mix solution according to Table 1. The extraction process began with the addition 8 mL of 0.2% formic acid in an 80:20 acetonitrile/water solution. Each sample was vortexed for 30 minutes, followed by a mechanical shaker for another 30 minutes. To remove the solid parts of the matrix, the samples were centrifuged at 4500 rpm for 10 minutes. An aliquot of the supernatant was passed through the SPE columns, and the extract was collected using 1-2 psi vacuum on the vacuum manifold. To prevent the extract from drying out during evaporation, 50 uL of DMSO was added to the extract and briefly vortexed. The extract was evaporated until it reached approximately 0.05 mL in a mild stream of nitrogen at 40°C using an automatic evaporation system. The extract residue was diluted by adding 150 uL of 0.1% formic acid and transferred to an HPLC vial.

Chromatographic and mass spectrometry conditions

The analysis of antimicrobial substances in animal feed was carried out using Agilent Technology UHPLC 1290 Infinity II and Triple Quad LC/MS 6470 mass spectrometer with Jet Stream Technology Ion Source (AJS ESI). The analytes were separated using an Acquity UHPLC HSS T3 column (1.8 µm, 2.1x150 mm) with a specific precolumn and thermostating at 40°C. Chromatographic separation and other operating conditions are described in Varenina et al. (2023).

Results and discussion

In accordance with Regulation 2021/808 (EC, 2021), the method was validated using a conventional model at five concentration levels ranging from 1 to 1000 µg/kg, which covers the range in which the analyte may be present as a result of contamination, as well as the range in which certain antibiotics may be used for therapeutic purposes.

To determine specificity and selectivity, 20 different lots of feed samples were analysed and it was concluded that there were no peaks on the retention time of the analyte that could interfere with the analyte peaks (Figure 2). During validation, the parameters of repeatability and intra-laboratory reproducibility were determined, as well as the screening target concentration (STC) and the decision limit of the analyte (CCα).

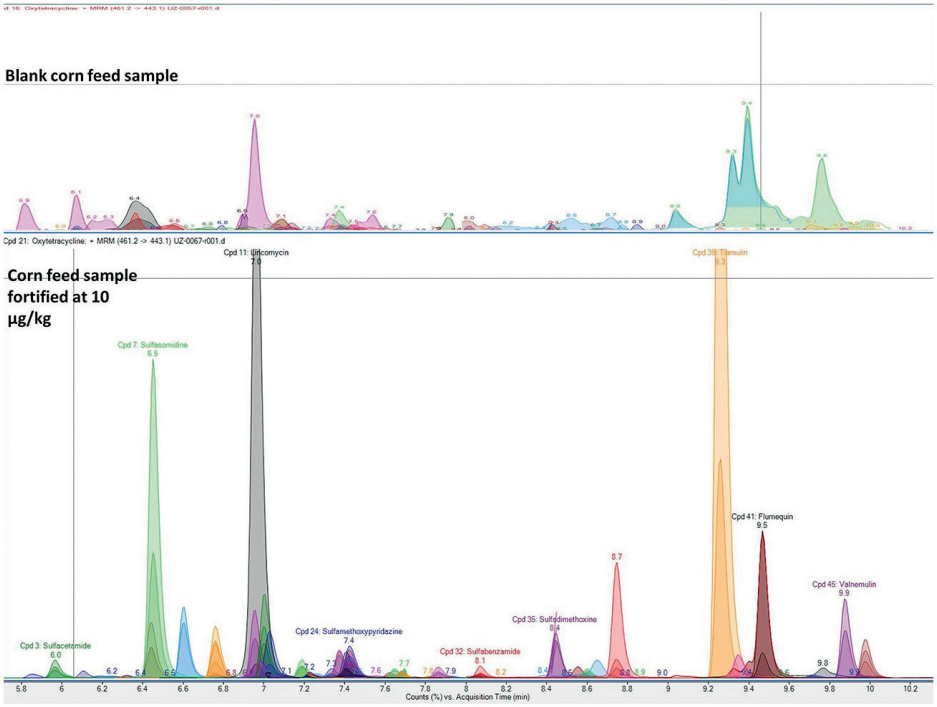

Figure 2. Extracted ion chromatogram of 43 analytes: blank corn sample and corn sample fortified at 10 µg/kg

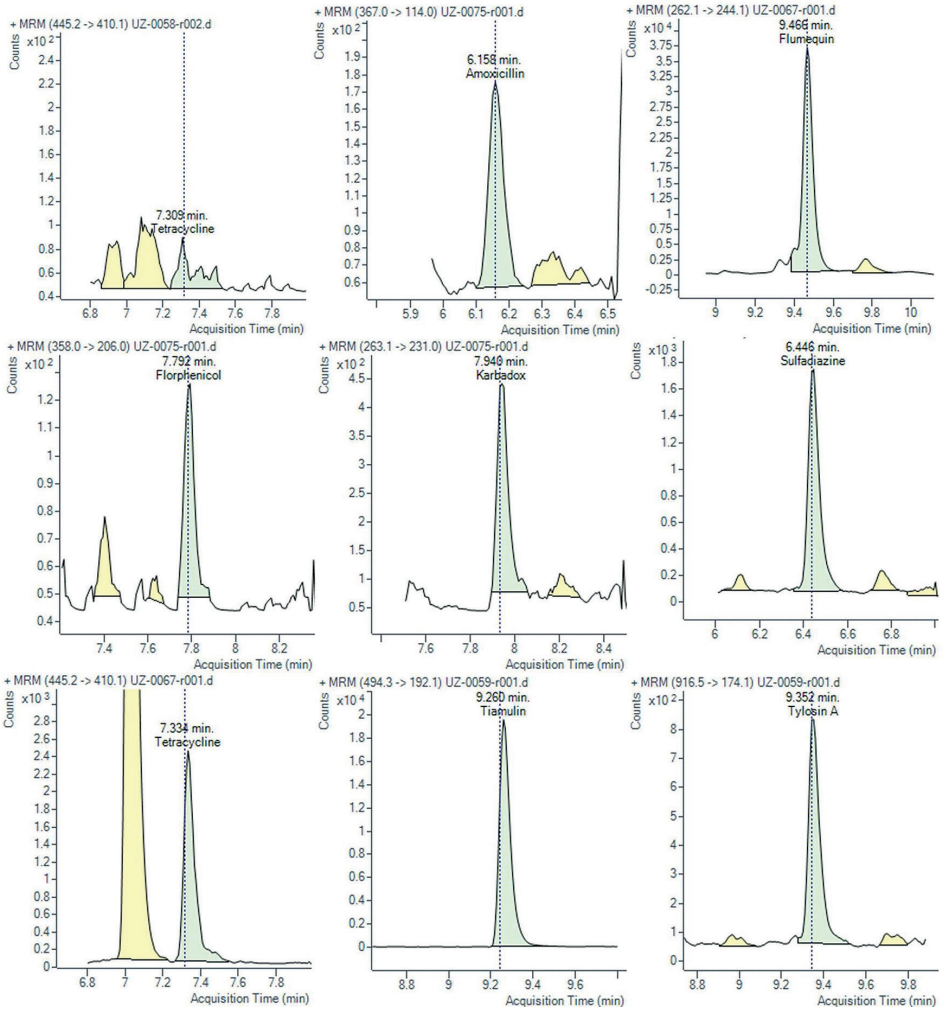

Figure 3. Chromatograms of the blank corn sample and fortified corn samples at cross-contamination levels: amoxicillin 50 µg/kg, flumequine 10 µg/kg, florphenicol 1 µg/kg, carbadox 50 µg/kg, sulfadiazine 1 µg/kg, tetracycline 10 µg/kg, tiamulin 1 µg/kg, tylosin A 1 µg/kg

To ensure a reliable basis for determining sample compliance, the detection capability (CCβ) for the screening step was estimated by analysing 20 fortified blank materials at concentration levels at and above the STC. CCβ equals the concentration where only ≤ 5% false compliant results are present. Good sensitivity has been observed for most analytes with a detection capability at 1 or 10 µg/kg, which is satisfactory for detecting unintentional contamination (Figure 3). For nine analytes, the matrix influence and suppression of ionisation of the precursor ions were more intense, and the screening level was set at 50 µg/kg.

Repeatability and intra-laboratory reproducibility were determined by analysing eight samples (four wheat samples and four maize samples) at five different

Table 1. Method validation parameters for nine antimicrobial classes in feed

Analyte	CCβ µg/kg	CCα µg/kg	Analyte	CCβ µg/kg	CCα µg/kg
Penicillins			***Sulfonamides***		
Amoxicillin	50	98.4	Sulfabenzamide	1	27.1
Penicillin V	50	298	Sulfacetamid	10	42.1
Quinolones			*Sulfachloropyridazine*	50	176
Flumequin	10	112	Sulfadiazine	50	117
Oxolinic acid	10	105	Sulfaclozine	50	110
Macrolides and lincosamiydes			Sulfadimethoxine	50	95.6
Lincomycin	50	110	Sulfadoxin	10	57.1
Tilmicosin iso mix	10	20.2	Sulfaguanidine	10	25.9
Tylosin A	10	23.2	Sulfamerazine	50	131
Tylvalosin	10	103	Sulfamethazine	50	56.2
Phenicols			Sulfameter/ Sulfamethoxydiazine	50	159
Florphenicol	50	93.8	Sulfamethizol	50	103
Tiamphenicol	500	693	Sulfametoxazole	10	49.7
Pleuromutilins			Sulfametoxypyridazin	50	58.8
Tiamulin	10	27.6	Sulfamoxole	10	29.6
Valnemulin	50	89.9	Sulfanilamide	10	29.7
Tetracyclines			Sulfaquinoxaline	10	48.0
Chlortetracycline	50	219	Sulfamonomethoxine	10	51.4
Doxycycline	1	27.3	Sulfapyridine	50	58.1
Oxytetracycline	10	18.9	Sulfasalazine	50	89.6
Tetracycline	10	83.2	Sulfisomidine	50	58.9
Quinoxalines			Sulfatroxazole	10	25.6
Carbadox	10	88.4	Sulfathiazole	10	43.9
Olaquindox	10	27.4	Sulfisoxazole	500	628
			Trimethoprim	50	112

levels and repeating the analyses under different conditions (on three different days and by three different operators). Inter-laboratory reproducibility, expressed as coefficient of variation (%), ranged from 9.2 to 49.6% and includes deviations in the variation factor of matrix type, different analysts and environment circumstances. For confirmatory analyses, the decision limit (CCα) was calculated, taking into account the variation of all factors determined during validation. In this way, the measurement uncertainty of the analyte is taken into account when reporting the

results (Table 1). Minimum trueness of quantitative methods was satisfactory for all analytes.

Due to the very complex matrices of feed that can have variable contents of fibre, carbohydrates, lipids, amino acids, vitamins, and minerals, the extraction procedure is the most crucial part of the method. Previous methods described the complex ultrasonic-assisted extraction combined with dispersive solid-phase extraction and achieved good sensitivity for nine classes of antibiotics (Boscher et al., 2010).

Liquid chromatography, as a separation technique, can be combined with various detectors. Liquid chromatography methods with a DAD detector are less sensitive, and the matrix effect can often affect selectivity. However, such methods can be used as an analytical tool for determining antibiotic residues in medicated feed, with concentrations typically exceeding 1 mg/kg (Butovskaya et al., 2024).

LC-MS methods are most commonly used for the analysis of antibiotic residues in food and feed and are recognised as confirmatory methods due to their precision and selectivity. The development of such methods is complex due to the different chemical structures of the various antibiotic classes. Because of the demanding optimisation of the extraction procedures and the broad concentration range that the methods have to cover, they are often only suitable for the analysis of a single group of antibiotics (Gavilán et al., 2015, 2016; Long et al., 2018; Patyra et al., 2018; Przeniosło-Siwczyńskaet al., 2020; Schwake-Anduschus and Langenkämper, 2018). Simple extraction methods are generally unable to effectively remove the matrix from the sample extracts. If this significantly impacts the precision and recovery of the method, such methods may only be suitable for screening purposes (Boscher et al., 2010). Recently, successful multi-methods covering more than three antibiotic classes have been published, with the matrix-matched calibration curve providing the highest accuracy and precision (Boscher et al., 2010; Jank et al., 2017; Patyra and Kwiatek, 2024).

During the development of multimethods, special attention should be paid to aminoglycosides, which are often used in combination with beta-lactams against gram-negative bacteria. From a chemical perspective, aminoglycosides are highly polar molecules that form polycations at physiological pH. Therefore, it is essential to devise a distinct method with specific conditions (Ferrari et al., 2024).

Research into the use of fertilisers on agricultural land and the uptake of antibiotic residues from fertilisers in plants is of great ecological importance. Experiments have shown that tetracyclines persist in the compost-soil-plant chain at a higher rate than the sulfonamide group (Kim et al., 2022). The presence of antibiotics in drinking water and animal waste at the farms originates mainly from feed, but there have also been cases of the presence of antibiotics after using medicated feed in animal husbandry (including injections and other oral routes) (Liu et al., 2022). The management of antibiotic residue control should start from the production of animal feed to the control of use of antibiotics for therapeutic purposes and the processing and selection of manure (Chung et al., 2017).

Conclusions

Due to significant human overpopulation and the high demand for food, the use of antibiotics is inevitable, and thus human activities contribute the most to the development of antibiotic resistance. When using antibiotics in animal hus-

bandry, special attention must be paid to unintentional cross-contamination, which can occur during the production of feed and on the farm. Consequently, the entire ecosystem is consistently exposed to low concentrations of antibiotics, leading to the development of antibiotic resistance. Analytical laboratories are tasked with developing methods that are sensitive and selective enough to detect all antibiotics used and that are suitable for various matrices. Presently, research is focused on detecting antibiotic concentrations in a range of cross-contamination scenarios where antibiotic resistance does not occur. This approach could help establish permissible limits for cross-contamination and adapt analysis methods accordingly. Feed manufacturers and farmers have access to sensitive and precise multi-methods conducted by authorised laboratories. Monitoring the feed used in animal husbandry ensures food safety for humans and the compliance of animal products, which is also a responsibility of each country.

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Rezidue antibiotika u neciljanoj hrani za životinje – razvoj osjetljive LC-MS/MS metodologije

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Antimikrobne se tvari koriste na farmama u terapeutske, profilaktičke svrhe, ali i kao promotori rasta. Ostatci antibiotika mogu se detektirati u hrani za životinje kao rezultat neovlaštene uporabe u profilaksi ili kao rezultat nenamjerne kontaminacije tijekom proizvodnje hrane za životinje. Prisutnost ostataka antibiotika u hrani u nekim slučajevima može izazvati alergijske reakcije, neravnotežu crijevne mikrobiote, razvoj antibakterijske rezistencije, ali može imati i toksične učinke. Razvijena je multi-metoda za detekciju i kvantifikaciju rezidua 43 antibiotika u hrani za životinje, koji su prema svojoj kemijskoj strukturi podijeljeni u 9 skupina (penicilini (2), kinoloni (2), makrolidi (3), linkozamidi (1),

fenikoli (2), pleuromutilini (2), tetraciklini (4), sulfonamidi (25), kvinoksalini (2). Metoda je razvijena za uzorke hrane za životinje proizvedene od različitih sirovina te različitog stupnja granulacije. Ekstrakcija i pročišćavanje temeljila se na dodavanju organskih otapala i SPE metodi pročišćavanja da bi se postigao najbolji odziv analita. Antibiotici su detektirani tekućinskom kromatografijom visoke učinkovitosti i tandemnom masenom spektrometrijom s elektrosprej raspršivačem (UHPLC-MS/MS) tijekom 17 minuta u pozitivnom i negativnom načinu snimanja. Vrijednosti iskorištenja su se kretale od 75,7 % (sulfametazin) do 121,3 % (karbadoks) s odgovarajućom relativnom standardnom devijacijom od 9,2 % i 49,6 %. Metoda je prikladna za detekciju kontaminacije antibioticima u hrani za životinje u rasponu od 1 μg/kg do 50 μg/kg i za potvrdne analize u rasponu od 10 μg/kg do 1000 μg/kg. Uočen je snažan utjecaj matrice pri usporedbi analiziranih signala analita dodanog u matricu i otapalo, čime je u analizama za kvantitativno određivanje analita potrebna primjena kalibracijske krivulje na matriksu i metode standardnog dodavanja.

Ključne riječi: *hrana za životinje, zaostatci antibiotika, unakrsna kontaminacija, LC-MS/MS*