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Multiclass method for detecting 41 antibiotic residues in bovine liver, muscle, and milk using LC-Q-Orbitrap-HRMS

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ABSTRACT

This study developed and validated an analytical method for the simultaneous identification and quantification of 41 veterinary antibiotic drugs from 9 different classes, including sulfonamides, quinolones, tetracyclines, penicillins, macrolides, nitroimidazoles, cephalosporins, diaminopyrimidines, and amphenicol, in animal-derived food products. The method used modified QuEChERS and liquid chromatography coupled to quadrupole-Orbitrap high-resolution mass spectrometry (LC-Q-Orbitrap HRMS). The method was validated in accordance with Commission Implementing Regulation (CIR) EU 2021/808 at five different concentrations ranging from 0.075 to 10,000 μg kg^{-1}. The mean recoveries ranged from 58 % to 123 %, while repeatability values were all below 21 %. The calibration curves showed good linearity, with correlation coefficients (R^2) ranging from 0.9905 to 0.9999. The limits of detection (LOD) and limits of quantification (LOQ) were in the range of 0.005–43.0 μg kg^{-1} and 0.008–71.7 μg kg^{-1}, respectively. The decision limit (CCα) and detection capability (CCβ) ranges were 0.37–2347 μg/kg and 0.44–3099 μg/kg, respectively. Out of the 1330 samples collected from Egyptian local markets, 1.20 % had antibiotic residues. The applicability of the developed method was proven through successful four proficiency testing (PT). The proposed method was demonstrated to be reliable for the simultaneous analysis of multiclass veterinary drugs in bovine liver, muscle tissue, and milk.

1. Introduction

Veterinary drugs play a crucial role in animal farming, serving not only as preventive measures and treatments for animal diseases but also as stimulants for animal growth (Zhu et al., 2023; Wang et al., 2014). The most common groups of livestock antibiotics are tetracyclines, penicillins, and sulfonamides. Broad-spectrum antibiotics like tetracyclines kill many bacteria and are often used to treat livestock respiratory and enteric infections. Penicillins, which kill gram-positive bacteria, are used to treat Streptococcus and Staphylococcus infections. Sulfonamides, which also kill many bacteria, are used to treat livestock respiratory and enteric infections (USDA, 2019; FDA, 2021). These drugs are typically administered through intramuscular, intraperitoneal, or intravenous injections, applied to the skin, or added to feed, and are subsequently absorbed and metabolized by the animal's body (Reeves, 2007). However, the use of antibiotics in this setting has been linked to high rates of resistant bacteria in animals' gut flora (Konstantinidis et al., 2020). Consequently, the increased use of antibiotics in livestock production has raised consumer concerns, because residues from these drugs often persist in animal-derived food products (Tian et al., 2016). The utilization of antibiotics has been extensively employed within the livestock industry. Nevertheless, this approach has raised concerns regarding the dissemination of bacteria that are resistant to antibiotics and the transfer of resistant genes to human communities (Eissa & Shehata, 2024). These residues potentially pose health risks to humans, cause allergic reactions in people who are already allergic, and promote the establishment of bacterial strains that are resistant to antibiotics (Dasenaki & Thomaidis, 2015). In order to establish regulations in this context, the European Commission has implemented Maximum Residue Levels (MRLs) for distinct veterinary medications in liver, muscle, and milk (European Commission, 2010). Products such as milk and chicken tissue containing concentrations of these substances that exceed the MRLs are deemed unsuitable for industrial use or human consumption since there are currently no methods to deactivate these substances (Lakew et al., 2022a; Arsand et al., 2016). Consequently, to ensure compliance with these regulations and detect illegal use of banned drugs, there is a need for precise and specialized analytical techniques. Currently, the primary methods employed for identifying veterinary

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drug residues in food samples encompass liquid chromatography UV detection (Lakew et al., 2022b), chromatography-mass spectrometry, immunoassay, molecular blotting, and microbial biochemical detection (Zhu et al., 2023). The QuEChERS method (quick, easy, cheap, effective, rugged, and safe) is a modern technique used to prepare samples for antibiotic analysis (Zhang et al., 2019), which has many benefits over traditional methods such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE). The QuEChERS method offers high recovery and accuracy, quick sample processing, and lower solvent consumption. On the analytical side, Alcántara-Durán et al. (2019) and Park et al. (2022) point to ultra-performance liquid chromatography coupled with tandem mass spectroscopy (UPLC-MS/MS) as the method of choice for fast, targeted analysis in high-throughput environments. Kang et al. (2014) developed a rapid and sensitive method for detecting antibiotic residues in muscle tissue using a modified QuEChERS extraction and HPLC-MS/MS analysis. The authors suggested that their method could be used for routine monitoring of muscle quality and safety. However, when considering the broader applications of mass spectrometry techniques in food safety, Orbitrap mass spectrometry has a substantial advantage over UPLC-MS/MS due to its remarkable high-resolution mass spectra capabilities when combined with a variety of chromatography techniques. Accurate mass measurements, structural information, and the determination of previously identified chemicals in complicated combinations are its strong suits (Yan et al., 2022). Additionally, quadrupole Orbitrap (Q-Orbitrap) and high-resolution mass spectrometry (HRMS) have enhanced the data acquisition speed and resolution significantly (Chiesa et al., 2018; De Paepe et al., 2019; Pugajeva et al., 2019; Mehl et al., 2021; Zhang et al., 2021). These techniques have also improved MS detection, allowing for the identification and quantification of more analytes in complex matrices. When compared to traditional unit-mass-resolution tandem mass spectrometry, HRMS has several advantages. This technology enables the acquisition of full-scan spectra, which allows for a thorough analysis of the sample's composition. Moreover, it enables the detection of compounds without requiring any prior tuning specific to each compound. In our study, we introduce a pre-treatment method based on the QuEChERS procedure. This method is complemented by the development and validation of a quantitative technique for the detection and quantification of forty-one veterinary drugs in liver, milk, and muscle tissue. Our approach utilizes liquid chromatography coupled with quadrupole-Orbitrap high resolution mass spectrometry (LC-Q-Orbitrap HRMS). Furthermore, our research contributes to food safety assurance by gathering a significant quantity of samples from the Egyptian market. This observation contributes to evaluating the health implications of antibiotic residues in widely consumed animal-derived food products. The presence of these residues raises concerns regarding consumer health, which is particularly important given the scarcity of studies conducted in this specific area within the Egyptian context.

2. Materials and methods

2.1. Chemicals and reagents

All the analytical standards of veterinary drugs used in this work were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and were of high purity (95 %). The LC-MS grade acetonitrile (ACN) and methanol (MeOH) were purchased from CARLO ERBA (Milan, Italy) and Merck (Darmstadt, Germany), respectively. The chemicals used in this study were citric acid monohydrate, ammonium hydroxide, ammonium acetate, pure formic acid (99 %), and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), all obtained from Sigma-Aldrich (Darmstadt, Germany). The acquisition of ultrapure water was accomplished by utilizing a MilliQ UF-Plus system, manufactured by Millipore (Darmstadt, Germany). Stock solutions of individual compounds were prepared in ACN (1000 μg/mL), which were then stored in glass bottles at a temperature of −18 °C, within their designated validity period. The mixed standard working solution used in the experiment was diluted and then stored at a temperature of −4 °C.

2.2. Apparatus

The centrifuge was obtained from Hermle (Gosheim, Germany). Similarly, the rotary evaporator was supplied by Heidolph (Schwabach, Germany). The pH-meter was obtained from Mettler Toledo (Greifensee, Switzerland) and calibrated before being used with certified calibration standards, including pH 4, pH 7, and pH 10.

2.3. Chromatographic conditions

The chromatographic separation procedure was performed using a Thermo Scientific Vanquish High Performance Liquid Chromatography system (Thermo Scientific, Bremen, Germany). The separation technique employed a reversed-phase ZORBAX Eclipse Plus C18 column (150 mm length × 4.6 mm inner diameter; 5 μm particle size, Agilent Technologies, Santa Clara, CA, USA). Thermo Hypersil GOLD aQ (100 mm length × 2.1 mm inner diameter; 1.9 μm particle size, Thermo Scientific, Bremen, Germany) and Thermo Accucore VDX (100 mm length × 2.6 mm inner diameter; 1.9 μm particle size, Thermo Scientific, Bremen, Germany) were also used. In the positive mode of HPLC, eluent A consisted of an aqueous solution containing 0.1 % formic acid, while eluent B comprised ACN with 0.1 % formic acid. The gradient process started with 5 % eluent B for 0.3 minutes, followed by a linear increase to 100 % over 7 minutes. This condition was maintained for 4 minutes. The system then reverted to 5 % eluent B in 0.1 minute and was re-equilibrated for 3 minutes, resulting in a cumulative run time of 14 minutes. A flow rate of 0.5 mL/min was used. In the negative mode for HPLC, eluent A was an aqueous solution containing 2 mM ammonium acetate, and eluent B was ACN with 0.1 % formic acid. The gradient procedure started with 20 % eluent B for 0.2 minutes, then increased linearly over the course of 3 minutes to 100 % B. This state persisted for a minute. After 2.5 minutes of re-equilibration, the system returned to 20 % B (total run time: 6.5 minutes). The sample temperature was held at 25 °C, the injection volume was 10 μL, and the column temperature was kept at 40 °C. The flow rate was set at 0.8 mL/min in the negative mode.

2.4. Mass spectrometry conditions

The Orbitrap mass spectrometer utilized in this study was the Q-Exactive model (Thermo Scientific, Bremen, Germany). It was equipped with a heated electrospray ionization (HESI) source, capable of operating in both positive and negative ionization modes. The HESI temperature was set to 350 °C, while the capillary temperature was set to 325 °C. The electrospray voltage was set to 3.75 kV for positive ionization mode and 3.20 kV for negative ionization mode. The value of the S-lens was adjusted to 50 volts. The sheath and auxiliary gas flows were adjusted to 50 and 12 arbitrary units, respectively. The automatic gain control (AGC) was configured to 3.10^6, and the maximum injection time (IT) was set to 200 ms. Full scan data were acquired in both positive and negative ionization modes at a mass resolving power of 70,000 full width at half maximum (FWHM), with an m/z scan range of 120–2000 and Full MS/vDIA as the scan type. The resolution was set at 70,000 for Full MS and 35,000 for vDIA. Data acquisition and processing were performed using TraceFinder (version 4.1) software (Thermo Fisher Scientific, Bremen, Germany).

2.5. Targeted compounds

A total of 41 target analytes are listed in Table 1, which contains detailed information such as compound names, exact precursor masses, characteristic fragment ions, mass accuracy, and molecular formula. The mass differences that were measured experimentally were consistently

Table 1
Molecular formula, retention time, and mass spectrometry parameters for the target 41 veterinary drugs compounds.

Class	Analyte	Retention time	Molecular formula	Adduct	Precursor *m/z*	Product ion *m/z*	Ionization mode
Sulfonamides	Sulfacetamide	6.10	$C_8H_{10}N_2O_3S$	$[M+H]^+$	215.04849	215.12522, 156.01120, 108.04425,	Positive
	Sulfachloropyridazine	7.03	$C_{10}H_9ClN_4O_2S$	$[M+H]^+$	285.02075	156.01122, 108.04426, 92.04934	Positive
	Sulfadiazine	6.20	$C_{10}H_{10}N_4O_2S$	$[M+H]^+$	251.05972	156.01130, 108.04431, 96.05553	Positive
	Sulfadimethoxine[a]	7.13	$C_{12}H_{14}N_4O_4S$[a]	$[M+H]^+$	311.08085	245.10319, 236.03340, 156.07675, 108.04429, 92.04938	Positive
	Sulfadoxine[a]	7.51	$C_{12}H_{14}N_4O_4S$[a]	$[M+H]^+$	311.08085	156.01133, 108.04433, 92.04935	Positive
	Sulfaguanidine	4.93	$C_7H_{10}N_4O_2S$	$[M+H]^+$	215.05972	156.01138, 108.04440, 92.04947, 65.0386	Positive
	Sulfamerazine	6.48	$C_{11}H_{12}N_4O_2S$	$[M+H]^+$	265.07537	190.02805, 156.01132, 110.07124, 108.04432, 92.04938	Positive
	Sulfamethazine	6.68	$C_{12}H_{14}N_4O_2S$	$[M+H]^+$	279.09102	213.11337, 204.04371, 156.01128, 124.08690, 108.04430	Positive
	Sulfamethizol	6.56	$C_9H_{10}N_4O_2S_2$	$[M+H]^+$	271.03179	156.01138, 108.04439, 92.04947	Positive
	Sulfamethoxazole	7.15	$C_{10}H_{11}N_3O_3S$	$[M+H]^+$	254.05939	156.01137, 108.04439, 92.04950	Positive
	Sulfamethoxypyridazine[b]	6.58	$C_{11}H_{12}N_4O_3S$[b]	$[M+H]^+$	281.07029	156.01131, 126.06617, 108.04432, 92.04938	Positive
	Sulfamonomethoxine	6.83	$C_{11}H_{12}N_4O_3S$[b]	$[M+H]^+$	281.07029	215.09267, 156.01126, 126.06613, 108.04429, 92.04934	Positive
	Sulfamoxol[c]	6.40	$C_{11}H_{13}N_3O_3S$[c]	$[M+H]^+$	268.07504	156.01138, 108.04440, 92.04950	Positive
	Sulfanilamide	5.37	$C_6H_8N_2O_2S$	$[M+H]^+$	173.03792	125.04710, 93.05730	Positive
	Sulfapyridine	6.31	$C_{11}H_{11}N_3O_2S$	$[M+H]^+$	250.06447	184.08680, 156.01120, 108.04424, 95.06025, 92.04930	Positive
	Sulfathiazole	6.14	$C_9H_9N_3O_2S_2$	$[M+H]^+$	256.02089	156.01134, 108.04434, 101.01678, 92.0490, 68.04955	Positive
	Sulfisoxazole[c]	7.25	$C_{11}H_{13}N_3O_3S$[c]	$[M+H]^+$	268.07504	268.07480, 156.01131, 113.07119, 108.04472, 92.04997	Positive Positive
Quinolones	Ciprofloxacin	5.78	$C_{17}H_{18}FN_3O_3$	$[M+H]^+$	332.14050	314.12883, 288.15028	Positive
	Enrofloxacin	5.90	$C_{19}H_{22}FN_3O_3$	$[M+H]^+$	360.17180	316.18162, 245.10821, 72.07807	Positive
	Flumequine	8.18	$C_{14}H_{12}FNO_3$	$[M+H]^+$	262.08740	238.05081, 244.07740, 202.03040, 174.03530	Positive
	Oxolinic acid	7.35	$C_{13}H_{11}NO_5$	$[M+H]^+$	262.07100	244.06023, 216.02899	Positive
	Sarafloxacin	6.06	$C_{20}H_{17}F_2N_3O_3$	$[M+H]^+$	386.13107	342.14109, 299.09893	Positive
Tetracyclines	Chlortetracycline	6.26	$C_{22}H_{23}ClN_2O_8$	$[M+H]^+$	479.12157	462.09364, 444.08320, 154.04946, 98.06025	Positive
	Doxycycline[d]	6.31	$C_{22}H_{24}N_2O_8$[d]	$[M+H]^+$	445.16054	428.13400, 410.12333, 339.08620, 321.07561, 154.04983	Positive
	Oxytetracycline	5.85	$C_{22}H_{24}N_2O_9$	$[M+H]^+$	461.15546	337.07019, 226.07076, 154.04972	Positive
	Tetracycline[d]	5.96	$C_{22}H_{24}N_2O_8$[d]	$[M+H]^+$	445.16054	428.13361, 410.12291, 154.04970	Positive
Penicillins	Ampicillin	6.58	$C_{16}H_{19}N_3O_4S$	$[M+H]^+$	350.11690	192.04748, 160.04248, 106.06497	Positive
	Penicillin V	7.80	$C_{16}H_{18}N_2O_5S$	$[M+H]^+$	351.10092	192.06530, 160.04253, 114.03710, 98.02354, 95.04901	Positive
Macrolides	Erythromycin	6.52	$C_{37}H_{67}NO_{13}$	$[M+H]^+$	734.46852	576.37390, 158.11764, 127.07539, 116.07063, 80.04910	Positive
	Tylosin	6.58	$C_{46}H_{77}NO_{17}$	$[M+H]^+$	916.52643	174.11230, 145.08576, 132.10176, 101.05957, 83.04898	Positive
Nitroimidazoles	Dimetridazole	6.34	$C_5H_7N_3O_2$	$[M+H]^+$	142.06110	112.06299, 95.06025, 81.04463, 56.04974	Positive
	Dimetridazole-OH	5.93	$C_5H_7N_3O_3$	$[M+H]^+$	158.05602	158.05602	Positive
	Ipronidazole	7.66	$C_7H_{11}N_3O_2$	$[M+H]^+$	170.09240	140.09435, 124.09949, 109.07600, 84.08073	Positive
	Ipronidazole-OH	6.76	$C_7H_{11}N_3O_3$	$[M+H]^+$	186.08732,	168.07673, 138.07877, 128.04540, 121.07596, 82.06506	Positive
	Metronidazole	5.83	$C_6H_9N_3O_3$	$[M+H]^+$	172.07167	128.04535, 111.04262, 98.04739	Positive

(*continued on next page*)

Table 1 (*continued*)

Class	Analyte	Retention time	Molecular formula	Adduct	Precursor *m/z*	Product ion *m/z*	Ionization mode
	Ronidazole	6.12	$C_6H_8N_4O_4$	$[M + H]^+$	201.06183	140.04541, 110.04742,	Positive
	Tinidazole	6.56	$C_8H_{13}N_3O_4S$	$[M + H]^+$	248.06995	128.04522, 121.03163	Positive
Cephalosporins	Ceftiofur	6.85	$C_{19}H_{17}N_5O_7S_3$	$[M + H]^+$	524.03629	324.05800, 241.03873, 210.02044, 167.02726, 126.01196	Positive
Diaminopyrimidines	Trimethoprim	5.79	$C_{14}H_{18}N_4O_3$	$[M + H]^+$	291.14517	275.11374, 261.09821, 245.10333, 230.11626, 123.06651	Positive
Amphenicol	Florfenicol	3.01	$C_{12}H_{14}Cl_2FNO_4S$	$[M - H]^-$	355.99319	185.02803, 183.01236, 151.96680, 120.05260, 119.04920, 78.98470	Negative
	Chloramphenicol	3.32	$C_{11}H_{12}Cl_2N_2O_5$	$[M - H]^-$	321.00505	257.03405, 194.04440, 176.03558, 152.03360, 141.01610	Negative

Isomers having the same molecular weight.
[a] sulfadimethoxine and sulfadoxine.
[b] sulfamethoxypyridazine and sulfamonomethoxine.
[c] sulfamoxol and sulfisoxazole.
[d] doxycycline and tetracycline.

less than 2 ppm, and all 41 analytes showed either protonated molecules ($[M + H]^+$) in the positive ion mode or deprotonated molecules ($[M-H]^-$) in the negative ion mode.

2.6. Sample collection

In this study, bovine liver, bovine muscle tissue, and bovine milk (n = 1330) were collected from July 2022 to June 2023 from a variety of local markets throughout Egypt, ensuring a representative sampling of products available to consumers. The sampling strategy entailed a randomized selection process to minimize bias. Samples were collected using sterile instruments and containers, labeled, and transferred in an icebox to the laboratory at the same day. Upon arrival, the samples were stored at −20°C to maintain their condition until the time of analysis.

2.7. Sample preparation

All samples were obtained from local markets. For a typical test, 2.00 ± 0.02 g of homogenized samples were placed into 50 mL polypropylene centrifuge tubes. Subsequently, 1.0 mL each of 1.0 M sodium citrate ($Na_3C_6H_5O_7$) and 0.5 M sodium EDTA (Na_2EDTA) was added. The samples were then extracted twice with 20 mL of ACN (divided into two 10 mL portions). They were mechanically shaken for 3 minutes at 700 rpm. Following a 10-minute centrifugation at 4500 rpm at 4°C, the combined supernatants were transferred to a 50 mL flask and evaporated at 40°C using a rotary evaporator. The residue was reconstituted into 2.0 mL of 10 mM ammonium formate at pH 2.78. The final solution was filtered through a disposable 0.45 μm PTFE membrane filter into an amber glass vial and then injected into the HPLC-Orbitrap HRMS for analysis.

2.8. Method validation

The method validation was conducted in accordance with the CIR EU 2021/808 (European Commission, 2021). The validation process involved a thorough examination of key parameters, including linearity, repeatability, reproducibility, recovery, decision limit (CCα), and detection capability (CCβ). Linearity refers to the ability of the analytical method to obtain test results that are directly proportional to the concentration of analyte in the sample within a given range. Linearity was comprehensively assessed in both the solvent and matrix. Calibration curves were designed in accordance with the Minimum Method Performance Requirements (MMPR) at five different concentration levels. Clear linearity was tested across various concentration ranges for different antibiotic groups in all matrices, ranging from 0.075 to 1000 μg kg^{-1} for liver, 0.075–5000 μg kg^{-1} for muscle and milk. Precision encompasses both repeatability and within laboratory reproducibility. Repeatability evaluates the precision of an analytical method under the same operating conditions over a short interval of time. It is assessed by conducting several independent tests on the same samples, with each test using an identical method carried out by the same operator and employing the same equipment, all within the same laboratory and over a brief period. Within-laboratory reproducibility refers to the consistency observed in the results of repeated measurements of the same analytes when the tests are conducted under varied conditions, including different laboratories, operators, instruments, and time periods. For each spike level, six replicates were analysed on the same day, with the use of matrix-matched calibration curves. This process was repeated over three different days, introducing variations in time, operator, and the calibration status of the LC-HRMS/MS equipment. Recovery (trueness), refers to the percentage of an analyte retrieved at the end of an analytical procedure relative to the initial amount of the analyte present in the original sample. Recovery provides an estimate of the accuracy of the method, specifically its capability to measure the analyte concentration without interference from the matrix. The decision limit (CCα) is the threshold value above which a sample can be deemed non-compliant, with an associated error probability of α. Conversely, the value of 1 – α represents the statistical confidence, expressed as a percentage, that the established threshold has been exceeded. Detection capability (CCβ) means the lowest content of the analyte that may be identified or quantified with an error probability of β. According to the European Commission (2021), for analytes with specified MRLs, CCα should be calculated as CCα = MRL + 1.64 s, where MRL represents maximum analyte residue levels and 's' represents repeatability standard deviation. CCβ is calculated as CCβ = CCα + 1.64 * CV, where 'CV' is the coefficient of variation. A blank sample was fortified with a standard solution at a level equivalent to 1 MRL for each analyte. This process was repeated 20 times to calculate CCα. Following this, we conducted 20 repetitions using blank samples to compute the CCβ. Likewise, the limit of detection (LOD), which is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantified. Moreover, the limit of quantitation (LOQ) is the concentration level at which the analyte can not only be reliably detected but also measured with a specified degree of accuracy and precision. LOD is calculated as three times the standard deviation of the fortified blank divided by the slope of the calibration curve, and LOQ is five times the same standard deviation over the slope.

Matrix effects (ME) were conducted to measure the extent of ion suppression or enhancement. The calculation of the ME involved the division of the slopes of the matrix-matched calibration curves (Slope M) by the slopes of the calibration curves produced using ACN solvent (Slope S), as expressed by the following equation: ME % = 100 × ((Slope

M)/(Slope S)) - 1

This quantifies how the matrix affected the analyte analysis. A result of 100 % indicates the absence of any matrix influence. Meanwhile, outcomes over 100 % indicate ion enhancement, while results below 100 % indicate ion suppression. Positive values boost the ion signal, while negative values suppress it. The importance of addressing the ME to accurately measure multiple veterinary drug classes in different matrices. Thus, matched matrix calibration was used in this study. Quantitatively evaluates the matrix effect, which compares solvent and matrix-matched standards (Shin et al., 2021).

2.9. Proficiency testing (PT)

Method validation accuracy was ensured by the analysis of four PT samples from the Food Analysis Performance Assessment Scheme (FAPAS). The sample rounds examined (02464, 02485, 02505, and 02515) targeted specific analytes. Round 02464 aimed to detect enrofloxacin and oxolinic acid. Round 02485 focused on a suite of sulfonamides: sulfamethazine, sulfamethoxazole, sulfapyridine, and sulfathiazole. The analytes for round 02505 included erythromycin, sulfadiazine, sulfamerazine, sulfamethazine, and sulfathiazole. Lastly, round 02515 was dedicated to the detection of chloramphenicol and florfenicol. These proficiency testing samples were crucial in affirming the precision and reliability of the analytical methods used in this study.

2.10. Methodology optimization

The chromatographic analysis used three different types of columns: Thermo Hypersil GOLD aQ (100 mm × 2.1 mm, 1.9 μm), Thermo Accucore VDX (100 mm × 2.6 mm, 1.9 μm), and the ZORBAX Eclipse Plus C18 column (150 mm × 4.6 mm, 5 μm). Several isomers share identical precursor ions, with values of 311.08085 for sulfadimethoxine and sulfadoxine, 281.07029 for sulfamethoxypyridazine and sulfamonomethoxine, 268.07504 for sulfamoxol and sulfisoxazole, and 445.16054 for doxycycline and tetracycline. This similarity in precursor ions posed challenges in their separation, as illustrated in Figure S1. For the mobile phase optimization procedure, we tested ACN, MeOH, and a 50:50 (v/v) mixture of the two. We explored the addition of ammonium formate and ammonium acetate to the mobile phase, aiming to improve peak shape and intensity for each target compound. To better separate isomers, gradient elution was chosen. Our LC gradient was fine-tuned to efficiently separate all substances. For hydrophilic interference elution, we started the gradient with a 95 % aqueous phase. The aqueous phase was then gradually decreased from 95 % water with 0.1 % formic acid to 100 % ACN with 0.1 % formic acid to clean the column and prevent carry-over. For continuous sample analysis, we returned the aqueous phase to 95 %. We have optimized a modified QuEChERS method, eliminating sample clean-up while adding an evaporation step. This approach involves using an organic solvent, adding salt for partitioning analytes, and removing polar matrix compounds. Unlike methods that use additional clean-up stages like SPE after LLE (Lakew et al., 2022c; Kim and Kang, 2021; Moreno-González et al., 2017; He et al., 2017; Jung et al., 2022). We evaluated the impact of different volumes of ACN - 5 mL, 10 mL, and 20 mL - on the recovery rates of the extracted samples. To enhance our extraction process, we added a 0.5 M Na_2EDTA solution. Sodium EDTA boosts remarkable chelating properties, effectively preventing the formation of insoluble complexes involving divalent and trivalent metal ions. This, in turn, guarantees unhindered tetracycline extraction (Zhou et al., 2009). Notably, Na_2EDTA simultaneously preserves the stability of tetracyclines during extraction by shielding them from potential degradation in the presence of metal ions (Anderson et al., 2005; Shin et al., 2018). This extraction used only 1 mL of 1 M sodium citrate. Sodium hydroxide and citric acid were carefully added to alter the pH of the sodium citrate solution. We avoided mixtures with a pH below 4.0 as they failed to generate dual phases within the concentration range of the phase-forming components (Han et al., 2011). After adding sodium citrate, Na_2EDTA, sodium chloride, and ACN, the sample was agitated. We conducted three distinct trials to ascertain the optimal duration of shaking. The first attempt featured 700 rpm shaking for 1 minute. In the next trial, shaking lasted 2 minutes at the same speed. In the third trial, shaking at 700 rpm was increased to 3 minutes. Subsequently, the samples were subjected to centrifugation to facilitate the separation of the supernatant. This process was carried out for a duration of 10 minutes at a speed of 4500 rpm.

3. Results and discussion

3.1. Optimization of liquid chromatography Conditions

When using the Thermo columns for separation, some compounds displayed reduced response rates, and in some cases, the separation of isomers was challenging. Using the ZORBAX column enabled the complete separation of compounds, effectively resolving the isomeric mixture. After conducting a comparative analysis of the separation of isomeric compounds, achieving baseline separation, and determining retention times (RTs) across the three columns for the 41 compounds, the ZORBAX column consistently exhibited superior performance. The majority of these compounds showed sharper peaks and increased response levels when analysed with the ZORBAX column. Consequently, this column was selected for the present study due to its ability to provide high resolution, allowing for the effective separation of compounds with closely similar properties (Lombardo-Agüí et al., 2012). Fig. 1 displays representative chromatograms.

In high-performance liquid chromatography (HPLC), the column packing material plays a central role. It improves HPLC performance and affects chromatographic separation by interacting with the analyte (Wang et al., 2023). Thus, carefully selecting and packing this material is crucial for the best separation results. The ZORBAX C18 column is composed of silica particles with C18 (octadecyl) hydrocarbon chains (Nováková & Solich, 2005). These chains produce a hydrophobic column. Non-polar molecules interact more strongly with the non-polar stationary phase (C18 chains) and spend more time attached to the column, resulting in prolonged retention. However, polar molecules travel through the column faster and interact less with the stationary phase. The polarity of sulfonamides, tetracyclines, penicillins, macrolides, nitroimidazoles, cephalosporins, and amphenicols ranges from modest to high. When these various groups are placed into a ZORBAX C18 column with a polar mobile phase, each molecule interacts differently with the stationary phase. This separation process achieves the best precision in a short time.

3.2. Optimization of the mobile phase

ACN proved to be more sensitive and had better peak shapes. ACN is used for the extraction of compounds with moderate polarity and non-polar compounds that possess a high degree of lipophilicity (Dasenaki et al., 2016). To improve ionization, we added 0.1 % formic acid to ACN. Previous studies have also used formic acid in ACN as a modifier for the mobile phase to enhance the ionization efficiency and sensitivity of analytes that undergo positive ionization (Kaufmann & Widmer, 2013; De Paepe et al., 2019). Ammonium formate and acetate addition yielded negligible impact on target compound peak shapes. However, chromatographic profiles demonstrated negligible changes to the peak shapes of the target compounds following the addition of these salts. To streamline the experimental process, we decided to exclude salts from the mobile phase. Barreto et al. (2016) used ammonium acetate in the mobile phase to achieve optimal peak shapes and enhance ionization efficiency. Furthermore, we noticed that 2 mM ammonium acetate improved the peak shapes and ionization efficiency of chloramphenicol and florfenicol in negative ionization mode. This decision was critical for identifying and quantifying chloramphenicol and florfenicol residues. Formic acid and ammonium acetate buffered the pH of the mobile

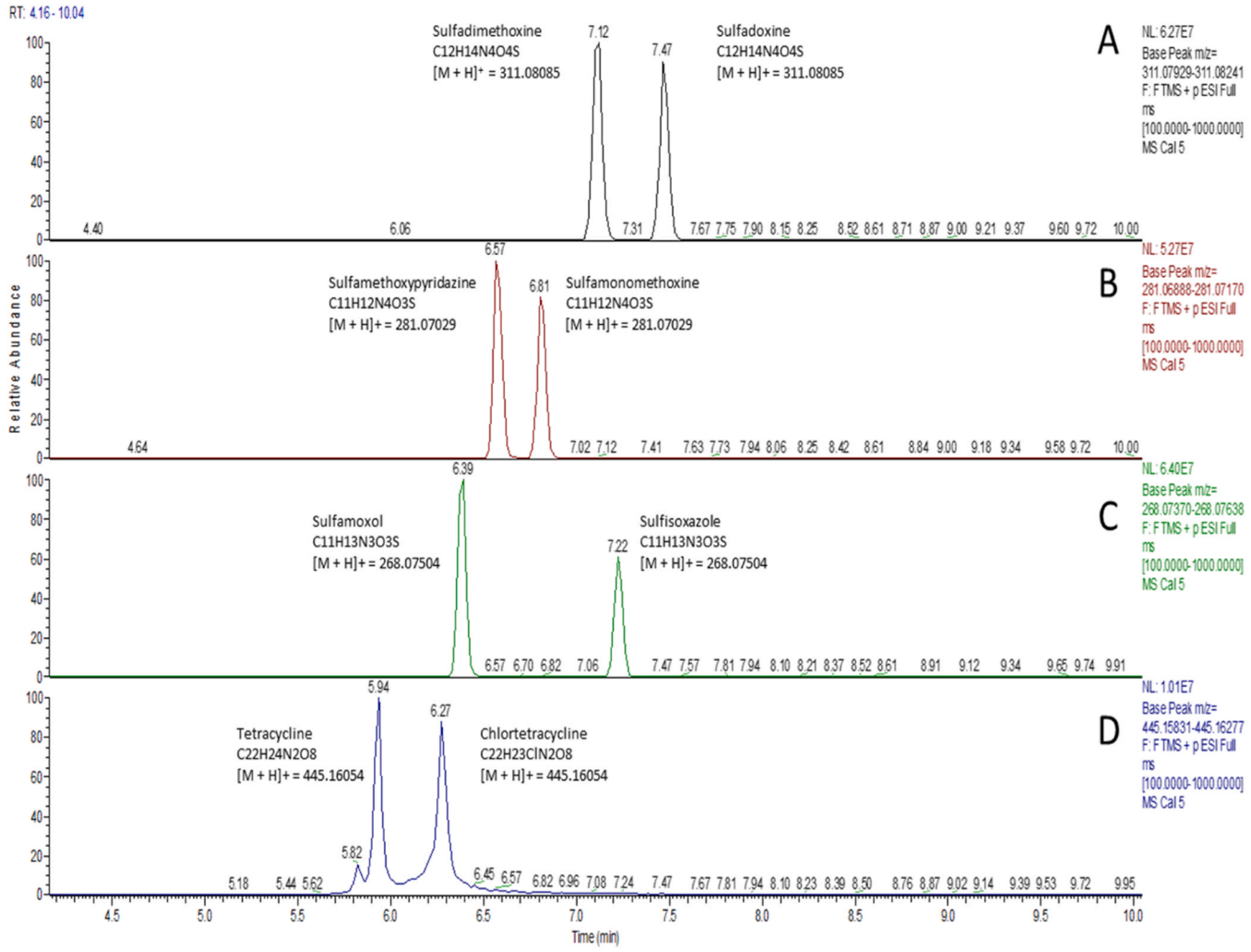

Fig. 1. ZORBAX column enables effective separation of antibiotic isomers having the same *m/z* A) Sulfadimethoxine and sulfadoxine (311.08085), B) Sulfamethoxypyridazine and sulfamonomethoxine (281.07029), C) Sulfamoxol and sulfisoxazole (268.07504), and D) Doxycycline and tetracycline (445.16054).

phase. Since pH changes impact antibiotic ionization states and chromatographic behavior, consistency is essential for ionization and separation.

3.3. Optimization of Q-Orbitrap HRMS parameters

LC-MS/MS is widely used in the determination of antibiotics due to its high sensitivity and specificity for complex analyses (Kim and Kang, 2021). The Q-Exactive Orbitrap/MS instrument was operated in Full MS/vDIA scanning mode, utilizing both positive and negative ion modes. The initial full mass scan was employed for screening and quantifying target compounds, as well as for retrospective analysis of unknown substances. To confirm the identity of the target compound, the generation of fragment ions was crucial. When a target compound was identified and its signal intensity surpassed the predefined threshold, it was selected using the quadrupole and directed to the higher-energy collision dissociation collision cell through the C-trap for fragmentation (Zhao et al., 2017). All resulting fragments from the collision cell were gathered within the C-trap and subsequently introduced into the Orbitrap mass analyzer (Jia et al., 2014). This workflow allows for a comprehensive analysis of the compounds. It initiates with a full MS scan, followed by a series of data-independent scans focusing on fragment ions with applied fragmentation energy. Detailed information for the 41 analytes, including compound names, exact precursor masses, characteristic fragment ions, mass accuracy, and molecular formula, is presented in Table 1. All of the 41 analytes displayed either protonated molecules ($[M + H]^+$) in the positive ion mode or deprotonated molecules ($[M–H]^-$) in the negative ion mode, with experimentally measured mass differences of less than 2 ppm.

3.4. Optimization of the preparation procedure

In our multi-residue veterinary drug analysis, the extraction is critical due to the diverse characteristics of the analytes. Our method aimed to use LLE without extra clean-up stages to reduce both costs and analysis time. However, to address the potential damage to the chromatographic column and contamination of the mass spectrometry system due to sample co-extractives, we used smaller injection volumes, which considered a cost-effective and straightforward method that could potentially reduce matrix effects and co-extractives interference (Deventer et al., 2014). In this context, we investigated three injection volumes: 2.0, 5.0, and 10.0 μL. Notably, the injection of a 10.0 μL extract yielded the smallest coefficient of variation (CV) and provided a satisfactory response. This sample preparation method proved to be highly efficient in extracting target compounds, offering a swift and eco-friendly process with reduced solvent residues, distinguishing it from other approaches involving SPE. Using 5 mL of ACN led to low recoveries for most analytes. Increasing the volume to 10 mL improved the recovery of most analytes, but it remained below 60 % for sulfonamides, nitroimidazoles, and penicillins. When the ACN volume was

further increased to 20 mL, it resulted in stable and high recovery rates, with the mean relative standard deviation (RSD) for most analytes remaining below 20 %. Considering the balance between economic and time-efficiency factors, 20 mL of ACN was selected as the extraction solvent (Fig. 2).

Hence, the use of 0.5 M Na_2EDTA in the extraction process enhances the overall efficiency, resulting in higher recovery rates of tetracycline residues from samples. Despite its advantages, the use of Na_2EDTA was not without challenges, such as observed precipitation and clogging of the chromatographic system. The inclusion of Na_2EDTA in the extraction solvent was necessitated due to its powerful metal-chelating ability, which facilitates the complete release of tetracycline from samples (Cháfer-Pericás et al., 2010). As a practical solution to the issues encountered, we restricted the quantity of 0.5 M Na_2EDTA in the extraction solvent to 1 mL while still maintaining highly efficient tetracycline extraction. Based on the literature by Ye et al. (2022), it was found that incorporating sodium citrate into the extraction process yielded the most favorable outcome in terms of recovery. Additionally, we carefully added 1 M sodium citrate to our extraction process. This choice was supported by sodium citrate's chelating, pH buffering, and antibiotic solubility properties. Sodium citrate, a mild acid, kept the extraction pH constant, enhancing antibiotic extraction efficiency. It also increased antibiotic solubility, improving extraction efficiency. According to Frenich et al. (2011), it was found that the primary-secondary amine (PSA) utilized in extraction has the ability to adsorb quinolones and tetracyclines, leading to decreased recoveries. As a result, we decided to exclude the use of PSA in our research. We observed the highest recovery rate when shaking the sample for 3 minutes at 700 rpm yielded the most efficient extraction.

3.5. Method Validation

According to CIR 2021/808 (European Commission, 2021), the method was validated as a quantitative confirmatory method to test the concentration range in which the method would be applicable for quantitative determination.

3.5.1. Identification of Analytes

During both the development and validation stages of this method, as well as the analysis of actual samples, the analytes were positively identified and confirmed when the retention time (RT), precursor ion, and product ion met the established criteria. Four pairs of epimers were successfully discriminated by their differing retention times, despite having identical molecular precursor ions and similar transition products, as shown in Table 1.

3.5.2. Selectivity of Analytes

Analytical methods must distinguish analytes from closely similar compounds. This property depends on the measuring technique, the analyte class, and the matrix. The exact mass accuracy and low background noise of Orbitrap technology improve specificity. Twenty blank liver, muscle tissue, and raw milk samples were analysed for interfering chemicals to verify the method's selectivity. Additionally, the retention times of the target compounds showed no interference peaks. Figure S2 shows no matrix interference in all samples.

3.5.3. Linearity, limits of detection (LODs), and limits of quantification (LOQs)

The matrix-matched standard calibration curves displayed neat linearity across various concentration ranges for different antibiotic groups in all matrices. The correlation coefficients (R^2) for all the matrix-matched standard calibration curves were found to be greater than 0.9905. For the sulfonamides group, the range was 25–500 μg kg^{-1}; for the nitroimidazoles group, it was 0.75–15 μg kg^{-1}; for sarafloxacin, the range was 7.5–150 μg kg^{-1}; and for ampicillin, trimethoprim and penicillin V, the range was 12.5–250 μg kg^{-1}. For florfenicol, the range was 5–100 μg kg^{-1}, and for chloramphenicol, it was 0.075–1.5 μg kg^{-1}. In liver samples, the ranges were 75–1500 μg kg^{-1} for ciprofloxacin, enrofloxacin, chlortetracycline, doxycycline, oxytetracycline, and tetracycline; 125−2500 μg kg^{-1} for flumequine; 37.5–750 μg kg^{-1} for oxolinic acid; 50–1000 μg kg^{-1} for erythromycin; and 25–500 μg kg^{-1} for tylosin. For muscle and milk samples, the ranges were 25–500 μg kg^{-1} for ciprofloxacin, enrofloxacin, oxolinic acid, chlortetracycline, doxycycline, oxytetracycline, tetracycline, and tylosin, except for ampicillin in milk samples, which exhibited a range of 2.5–50 μg kg^{-1}. In muscle samples alone, the ranges were 50–1000 μg kg^{-1} for flumequine and erythromycin. In milk samples, the range was 250–5000 μg kg^{-1} for ceftiofur, as shown in Table 2.

In liver samples, chloramphenicol exhibited the lowest observed LODs and LOQs at 0.005 μg kg^{-1} and 0.008 μg kg^{-1}, respectively, while doxycycline showed higher values with LODs of 43.0 μg kg^{-1} and LOQs of 71.7 μg kg^{-1}. Comparable low values for LOD and LOQ were also achieved for muscle and milk samples, notwithstanding their complex matrices with high lipid and protein content, as shown in Table 2, despite their complex matrices.

3.5.4. Matrix effects

Animal-derived food products contain proteins, phospholipids, and various other inherent substances. During sample preparation, these components may mix with analytes, affecting ionization efficiency. Analysis can be skewed by the matrix effect caused by endogenous

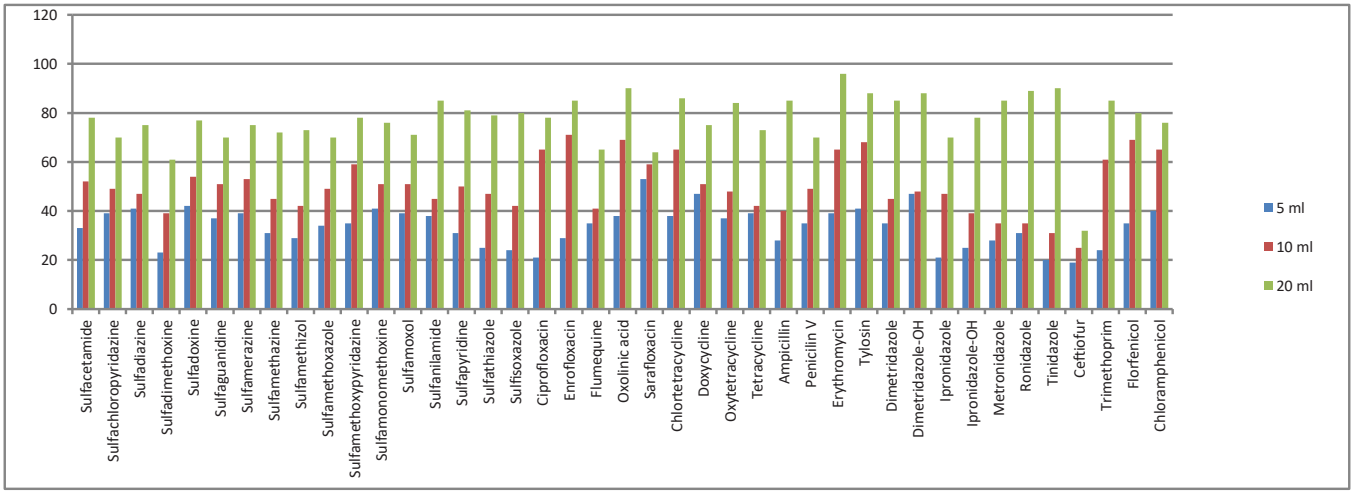

Fig. 2. Effect of different volumes of acetonitrile (5, 10, and 20 mL) on the recovery rates of the 41 veterinary drugs.

Table 2
Linearity, range, limits of detection (LOD) and limits of quantification (LOQ) of the 41 target analytes in bovine liver, muscle, and milk.

Analyte	Liver				Muscle				Milk			
	R^2	Range (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	R^2	Range (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	R^2	Range (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Sulfacetamide	0.9999	25–500	5.38	8.97	0.9988	25–500	4.79	7.99	0.9966	25–500	5.67	9.45
Sulfachloropyridazine	0.9990	25–500	2.58	4.31	0.9975	25–500	3.09	5.15	0.9999	25–500	5.00	8.34
Sulfadiazine	0.9987	25–500	0.95	1.59	0.9985	25–500	2.23	3.73	0.9984	25–500	2.46	4.10
Sulfadimethoxine	0.9905	25–500	3.73	6.22	0.9972	25–500	2.88	4.81	0.9991	25–500	2.32	3.87
Sulfadoxine	0.9991	25–500	3.70	6.16	0.9998	25–500	2.49	4.16	0.9998	25–500	3.32	3.87
Sulfaguanidine	0.9973	25–500	3.27	5.46	0.9975	25–500	1.11	1.86	0.9997	25–500	3.80	6.34
Sulfamerazine	0.9981	25–500	3.19	5.32	0.9985	25–500	2.56	4.42	0.9990	25–500	2.05	3.41
Sulfamethazine	0.9985	25–500	0.82	1.38	0.9982	25–500	1.46	2.43	0.9991	25–500	1.95	3.25
Sulfamethizol	0.9999	25–500	1.64	2.74	0.9980	25–500	1.89	3.15	0.9981	25–500	1.59	2.66
Sulfamethoxazole	0.9998	25–500	1.84	3.07	0.9999	25–500	3.07	5.11	0.9991	25–500	2.29	3.82
Sulfamethoxypyridazine	0.9987	25–500	3.13	5.23	0.9955	25–500	2.39	3.94	0.9955	25–500	1.72	2.88
Sulfamonomethoxine	0.9995	25–500	1.60	2.66	0.9998	25–500	3.27	6.20	0.9998	25–500	1.55	2.58
Sulfamoxol	0.9994	25–500	0.85	1.43	0.9974	25–500	2.98	4.97	0.9994	25–500	1.21	2.01
Sulfanilamide	0.9995	25–500	10.6	17.6	0.9993	25–500	7.00	11.7	0.9984	25–500	2.11	3.52
Sulfapyridine	0.9986	25–500	3.51	5.85	0.9964	25–500	1.66	2.77	0.9972	25–500	3.49	5.82
Sulfathiazole	0.9983	25–500	3.41	5.59	0.9970	25–500	2.61	4.35	0.9953	25–500	3.41	5.69
Sulfisoxazole	0.9993	25–500	1.27	2.12	0.9996	25–500	1.94	3.24	0.9989	25–500	15.1	25.2
Ciprofloxacin	0.9950	75–1500	14.4	24.0	0.9974	25–500	4.07	6.78	0.9985	25–500	2.28	3.81
Enrofloxacin	0.9985	75–1500	14.3	23.8	0.9927	25–500	5.83	9.72	0.9997	25–500	1.66	2.78
Flumequine	0.9966	125–2500	30.0	50.0	0.9974	50–1000	12.5	20.9	0.9978	12.5–250	1.84	3.07
Oxolinic acid	0.9980	37.5–750	1.80	3.01	0.9973	25–500	9.03	15.1	0.9979	25–500	11.0	18.4
Sarafloxacin	0.9998	7.5–150	1.60	2.66	0.9981	7.5–150	1.77	2.96	0.9987	7.5–150	0.72	1.20
Chlortetracycline	0.9997	75–1500	19.1	31.9	0.9990	25–500	3.85	6.42	0.9955	25–500	3.15	5.26
Doxycycline	0.9995	75–1500	43.0	71.7	0.9993	25–500	16.5	27.6	0.9987	25–500	9.60	16.0
Oxytetracycline	0.9987	75–1500	10.7	17.9	0.9989	25–500	10.8	18.0	0.9982	25–500	4.13	6.89
Tetracycline	0.9995	75–1500	27.8	46.3	0.9993	25–500	13.6	22.6	0.9987	25–500	8.93	14.9
Ampicillin	0.9997	12.5–250	3.11	5.18	0.9999	12.5–250	2.32	3.86	0.9993	2.5–50	0.73	1.22
Penicilin V	0.9979	12.5–250	0.90	1.51	0.9993	12.5–250	8.08	13.5	0.9994	12.5–250	4.21	7.01
Erythromycin	0.9990	50–1000	3.87	6.45	0.9971	50–1000	0.17	0.28	0.9980	25–500	1.05	1.76
Tylosin	0.9999	25–500	24.0	39.9	0.9958	25–500	8.37	14.0	0.9998	25–500	2.96	4.93
Dimetridazole	0.9996	0.75–15	0.12	0.21	0.9982	0.75–15	0.23	0.39	0.9983	0.75–15	1.09	1.81
Dimetridazole-OH	0.9997	0.75–15	0.38	0.63	0.9966	0.75–15	0.40	0.66	0.9983	0.75–15	0.15	0.25
Ipronidazole	0.9999	0.75–15	0.07	0.12	0.9998	0.75–15	0.09	0.15	0.9997	0.75–15	0.10	0.17
Ipronidazole-OH	0.9978	0.75–15	0.14	0.24	0.9996	0.75–15	0.36	0.60	0.9996	0.75–15	0.19	0.32
Metronidazole	0.9954	0.75–15	0.21	0.35	0.9990	0.75–15	0.29	0.49	0.9987	0.75–15	0.31	0.51
Ronidazole	0.9993	0.75–15	0.11	0.19	0.9986	0.75–15	0.76	1.27	0.9990	0.75–15	1.53	2.56
Tinidazole	0.9994	0.75–15	0.39	0.66	0.9993	0.75–15	0.19	0.33	0.9972	0.75–15	0.34	0.56
Ceftiofur	0.9995	500–10000	13.1	28.1	0.9994	250–5000	8.54	29.6	0.9993	250–5000	18.1	30.2
Trimethoprim	0.9971	12.5–250	0.82	1.36	0.9994	12.5–250	0.56	0.94	0.9999	12.5–250	1.70	2.84
Florfenicol	0.9996	5–100	0.03	0.06	0.9984	5–100	0.02	0.03	0.9997	5–100	0.02	0.04
Chloramphenicol	0.9950	0.075–1.5	0.005	0.008	0.9977	0.075–1.5	0.008	0.013	0.9974	0.075–1.5	0.005	0.009

substances. Within matrices, the findings revealed that within a liver matrix, there was an enhancement for three analytes ranging between 1 % and 17 %, and suppression for 38 analytes, with values ranging from −38 % for demetridazole-OH to −1 % for sulfamethizol. In the muscle matrix, 7 analytes showed enhancement, with the range being 1–14 %. Meanwhile, suppression was observed for 34 analytes, with values ranging from −33 % for trimethoprim to −1 % for dimetridazole. In the milk matrix, enhancement was seen for 11 analytes within the 1–17 % span, while suppression occurred for 30 analytes, ranging from −43 % for tylosin to −2 % for sulfaguanidine.

3.5.5. Trueness and precision

These samples were spiked at five different levels, with a total of 18 samples spiked per level. Recovery rates for liver matrices ranged from 60 % to 95 %, and the coefficients of variation (CVs) did not exceed 19 %. In the muscle matrix, recovery rates varied between 58 % and 123 %, with CVs remaining below 21 %. For the milk matrix, recoveries were within the 70–94 % range, and CVs did not surpass 17 %. The data, consolidated in the corresponding Table 3, underscores the method's high degree of accuracy.

3.5.6. The decision limit (CCα) and detection capability (CCβ)

In this work, the values of CCα and CCβ for every target compound were also estimated, and Table 4 summarises the results. CCα values ranged from 0.40 to 2347 μg kg^{-1}, while CCβ values were between 0.44 and 3099 μg kg^{-1} for liver samples. For muscle samples, the respective ranges were 0.40–1450 μg kg^{-1} and 0.46–2210 μg kg^{-1}. In the case of milk samples, the ranges spanned from 0.37 to 121 μg kg^{-1} and 0.47–165 μg kg^{-1}. These ranges demonstrate the suitability of the developed method for detecting the drug in animal-derived food products.

3.6. Real samples analysis

To verify the feasibility of the established technique, an analysis was conducted on a total of 1330 samples, including 572 liver, 627 muscle, and 129 milk samples from different local markets in Egypt, to detect veterinary drug residues. Out of the 41 antibiotics, 10 were found in 1.20 % of the samples, as shown in Table 5. The detection frequencies varied from 0.15 % to 2.32 %. The mean concentration (μg kg^{-1}) and detection frequency (%) of the most frequently detected antibiotics in the samples were as follows: chlortetracycline (27 μg kg^{-1} and 2.32 %), ciprofloxacin (158 μg kg^{-1} and 0.31 %), and enrofloxacin (120 μg kg^{-1} and 0.31 %). The difficulty in determining the optimal dose of these antibiotics has resulted in veterinary drug misuse as well as failure to adhere to their withdrawal periods, posing a risk to human health and the environment (Kang et al., 2018).

Table 3
Recovery, coefficient of variation (CV) pooled, and matrix effect (ME) in bovine liver, muscle, and milk.

Analyte	Liver				Muscle				Milk			
	Recovery (%)	CV (%) Repeatability	CV (%) Reproducibility	ME (%)	Recovery (%)	CV (%) Repeatability	CV (%) Reproducibility	ME (%)	Recovery (%)	CV (%) Repeatability	CV (%) Reproducibility	ME (%)
Sulfacetamide	80	13	14	-11	76	11	12	10	75	10	11	4
Sulfachloropyridazine	70	13	14	-13	77	6	8	4	72	8	8	14
Sulfadiazine	74	9	9	-17	73	11	14	-22	74	9	10	-22
Sulfadimethoxine	60	11	10	-6	87	9	8	-13	79	9	11	-12
Sulfadoxine	77	11	12	5	83	8	8	14	87	8	9	17
Sulfaguanidine	70	13	14	17	66	10	11	2	74	9	10	-2
Sulfamerazine	76	11	12	-23	89	9	8	-19	74	9	11	-6
Sulfamethazine	72	13	14	-23	88	9	10	-16	83	10	14	-9
Sulfamethizol	73	11	13	-1	71	8	9	-10	74	8	10	-25
Sulfamethoxazole	70	11	12	-2	80	7	11	4	76	7	9	-24
Sulfamethoxypyridazine	78	15	14	-20	87	10	14	-4	89	12	13	-4
Sulfamonomethoxine	76	13	15	-13	79	7	10	1	74	10	12	-14
Sulfamoxol	71	14	16	-8	82	9	10	-17	75	10	11	-9
Sulfanilamide	86	14	15	-33	84	12	12	-29	71	9	9	-19
Sulfapyridine	81	14	14	-30	84	10	11	-3	86	12	11	-22
Sulfathiazole	79	10	12	-25	61	11	12	-3	73	11	13	-32
Sulfisoxazole	79	12	11	-4	76	8	9	-9	83	9	10	1
Ciprofloxacin	76	15	16	-12	108	13	14	-2	75	11	14	-8
Enrofloxacin	83	13	14	-10	101	17	14	-7	87	13	12	-14
Flumequine	66	12	14	-17	97	12	13	-14	72	11	14	-9
Oxolinic acid	89	16	15	-36	123	20	18	-10	82	15	14	-12
Sarafloxacin	64	12	11	-13	83	9	10	-10	88	10	11	1
Chlortetracycline	86	13	14	-7	64	11	12	-8	80	12	13	-5
Doxycycline	75	13	13	-5	74	11	13	-17	82	12	13	-5
Oxytetracycline	84	13	14	-21	76	12	14	-22	58	14	15	-12
Tetracycline	73	13	15	-11	58	2	7	-11	78	11	12	-33
Ampicillin	83	16	13	-11	89	12	13	-6	71	9	11	11
Penicilin V	69	15	14	-16	81	13	16	-2	91	11	12	3
Erythromycin	95	5	9	-28	90	3	6	-11	93	5	7	13
Tylosin	86	11	12	-36	81	9	10	-20	91	8	11	-43
Dimetridazole	84	10	11	-25	95	8	9	-1	70	12	13	1
Dimetridazole-OH	88	13	12	-38	83	12	13	-4	77	12	14	10
Ipronidazole	63	10	10	-16	85	8	10	-20	77	9	10	15
Ipronidazole-OH	75	9	12	-15	89	7	11	-26	83	10	11	-6
Metronidazole	82	13	14	-4	86	10	12	-2	84	13	15	-12
Ronidazole	83	11	12	-22	87	11	14	7	88	11	12	-10
Tinidazole	86	12	13	1	92	10	11	-5	94	10	11	-8
Ceftiofur	81	13	15	-9	79	9	11	-11	71	11	13	-4
Trimethoprim	82	13	14	-3	58	13	14	-33	85	8	10	-26
Florfenicol	78	17	18	-16	82	10	11	-6	76	15	16	-20
Chloramphenicol	71	13	15	-31	85	10	13	-19	91	12	14	-17

Table 4
Maximum Residue Limits (MRL), decision limits (CCα) and detection capability (CCβ) in bovine liver, muscle, and milk.

Analyte	Liver			Muscle			Milk		
	MRL (μg kg^{-1})	CCα (μg kg^{-1})	CCβ (μg kg^{-1})	MRL (μg kg^{-1})	CCα (μg kg^{-1})	CCβ (μg kg^{-1})	MRL (μg kg^{-1})	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
Sulfacetamide	100	113	132	100	143	174	100	107	124
Sulfachloropyridazine	100	109	120	100	112	128	100	106	125
Sulfadiazine	100	112	128	100	114	137	100	110	118
Sulfadimethoxine	100	111	128	100	113	128	100	108	130
Sulfadoxine	100	108	122	100	113	120	100	105	121
Sulfaguanidine	100	117	131	100	114	133	100	111	125
Sulfamerazine	100	109	130	100	113	126	100	107	129
Sulfamethazine	100	110	129	100	116	130	100	108	135
Sulfamethizol	100	110	120	100	113	126	100	106	131
Sulfamethoxazole	100	111	122	100	113	137	100	113	134
Sulfamethoxypyridazine	100	112	124	100	116	126	100	116	144
Sulfamonomethoxine	100	109	116	100	114	121	100	121	140
Sulfamoxol	100	115	128	100	120	130	100	113	132
Sulfanilamide	100	133	166	100	119	199	100	119	153
Sulfapyridine	100	110	122	100	115	127	100	109	125
Sulfathiazole	100	112	124	100	116	127	100	115	135
Sulfisoxazole	100	115	126	100	114	140	100	109	129
Ciprofloxacin	300	325	377	100	144	177	100	112	131
Enrofloxacin	300	326	395	100	135	173	100	113	122
Flumequine	500	529	581	200	223	289	50	55	69
Oxolinic acid	150	174	203	100	136	265	No MRL	113	139
Sarafloxacin	30	32	35	30	38	48	No MRL	36	45
Chlortetracycline	300	333	403	100	125	170	100	111	132
Doxycycline	300	322	394	100	176	249	No MRL	111	144
Oxytetracycline	300	342	378	100	114	254	100	113	129
Tetracycline	300	343	409	100	330	400	100	108	165
Ampicillin	50	57	71	50	59	74	4	5	6
Penicilin V	50	59	79	50	71	101	4	5	6
Erythromycin	200	236	242	200	105	128	40	41	45
Tylosin	100	115	143	100	112	173	50	54	59
Dimetridazole	No MRL	3	4	No MRL	3	4	No MRL	4	5
Dimetridazole-OH	No MRL	4	5	No MRL	4	6	No MRL	4	5
Ipronidazole	No MRL	3	4	No MRL	3	4	No MRL	3	4
Ipronidazole-OH	No MRL	3	4	No MRL	3	4	No MRL	4	5
Metronidazole	No MRL	4	5	No MRL	3	4	No MRL	3	4
Ronidazole	No MRL	4	5	No MRL	4	5	No MRL	4	5
Tinidazole	No MRL	4	4	No MRL	3	5	No MRL	4	5
Ceftiofur	2000	2347	3099	1000	1450	2210	100	109	125
Trimethoprim	100	57	60	100	65	82	50	54	61
Florfenicol	3000	23	27	200	23	27	No MRL	25	30
Chloramphenicol	No MRL	0.40	0.44	No MRL	0.40	0.46	No MRL	0.37	0.47

Table 5
Veterinary drug residues in bovine liver, muscle, and milk samples (n = 1330; positive samples = 16) collected from Egyptian retail markets.

Species	Sample number	Detected number	Analyte	MRL (μg kg^{-1})	Range (μg kg^{-1})		Mean	SD	Frequency[d]	
					Minimum	Maximum			No.	(%)
Liver	574	0	-	-	-	-	-	-	-	-
Muscle	627	5	Chlortetracycline	100	LOQ[a]	LOQ[a]	-	-	1	0.15
			Ciprofloxacin	100	72.3	243	158	120	2	0.31
			Enrofloxacin	100	22.1	219	120	139	2	0.31
			Flumequine	200	178	-	-	-	1	0.15
			Oxytetracycline	100	259	-	-	-	1	0.15
			Oxolinic acid	100	LOQ[b]	LOQ[b]	-	-	1	0.15
Milk	129	11	Chlortetracycline	100	17.2	42.3	27	14	3	2.32
			Ciprofloxacin	100	34.3	-	-	-	1	0.77
			Enrofloxacin	100	21.2	-	-	-	1	0.77
			Oxytetracycline	100	17.5	25.2	21	5	2	1.55
			Sarafloxacin	No MRL	LOQ[c]	LOQ[c]	-	-	1	0.77
			Tetracycline	100	15.5	47.5	32	23	2	1.55

[a] Limit of quantification (LOQ) for chlortetracycline in muscle is 6.42 μg/kg.
[b] For oxolinic acid in muscle is 15.05 μg/kg.
[c] for sarafloxacin in milk is 1.20 μg/kg.
[d] Frequency (%) indicates the proportion of samples in which the compound was detected, out of the total number of samples analysed.

3.7. Application to a previously analysed PT sample

As part of the Food Analysis Performance Assessment Scheme (FAPAS), four proficiency testing (PT) sample rounds (02464, 02485, 02505, and 02515) were conducted. In round 02464, the optimized assay verified the presence of enrofloxacin and oxolinic acid at

concentrations of 148 μg kg^{-1} and 43.8 μg kg^{-1}, respectively. These findings concurred with the results obtained using the established method and reported in the round 02464 results report. For round 02485, the assay confirmed the presence of sulfapyridine, sulfamethoxazole, sulfathiazole, and sulfamethazine at concentrations of 56.7 μg kg^{-1}, 42.1 μg kg^{-1}, 38.2 μg kg^{-1}, and 27.7 μg kg^{-1}, respectively. These results aligned with those obtained using the accepted method and reported in the round 02485 results report. In the third PT round, 02505, the optimized assay confirmed the presence of erythromycin, sulfadiazine, sulfamerazine, sulfamethazine, and sulfathiazole at concentrations of 191 μg kg^{-1}, 31.27 μg kg^{-1}, 83.7 μg kg^{-1}, 63.1 μg kg^{-1}, and 87.5 μg kg^{-1}, respectively. These findings were consistent with those obtained using the accepted method and reported in the round 02505 results report. In the final PT round, 02515, the assay confirmed the presence of chloramphenicol and florfenicol at concentrations of 0.24 μg kg^{-1} and 210 μg kg^{-1}, respectively. These results were in harmony with those obtained using the accepted method and reported in the round 02515 results report, as shown in Table 6. All results produced satisfactory outcomes within the acceptable range of z-scores, where the absolute value of z is less than (2,−2). The z-score was computed for the obtained results and was found to be within the acceptable range of $|z| < ((2,-2)$as specified by the FAPAS reports.

To the best of our knowledge, no prior research studies have validated their results using four distinct rounds of proficiency testing (PT) analysis in the multiclass residue of veterinary drugs.

4. Conclusions

The study presents LC-Q-Orbitrap HRMS technology for quantifying 41 antibiotics in liver, muscle tissue, and raw milk using a simplified pretreatment method based on QuEChERS. The developed method proved to be highly reliable for the Full MS/vDIA scanning mode, accurately determining the masses of parent ions and fragment ions. The analytical separation and detection procedure significantly reduced analysis time and increased sample throughput, while maintaining high method sensitivity and the ability to detect residues ranging from 0.075 to 10,000 μg kg^{-1}. The validation procedure included assessments of selectivity, linearity, LOD, LOQ, trueness, repeatability, reproducibility, CCα, and CCβ. All validation parameter values met the intended use and established criteria. The developed method was successfully applied to analyse four proficiency testing (PT) samples as well as real samples. Out of 1330 samples, 16 positive samples were detected. This method provides an integrated strategy for effectively screening and quantifying of multiclass veterinary drugs. Continuous monitoring studies should be conducted regularly to determine the presence of veterinary drug residues in animal-derived food products, identify their sources, and implement preventive and remedial strategies.

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CRediT authorship contribution statement

Mostafa Nagi: Writing – review & editing, Supervision, Investigation. **Lamia Ryad:** Writing – review & editing, Supervision, Resources, Methodology, Data curation. **Omar Khaled:** Writing – original draft, Methodology, Investigation, Formal analysis. **Fawzy Eissa:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 6
Outcomes of the Food Analysis Performance Assessment Scheme (FAPAS), proficiency testing (PT) utilizing the validated method for bovine liver, muscle, and raw milk analysis.

FAPAS round	Analyte	Assigned value (μg kg^{-1})	Found (μg kg^{-1})	Z-score	Observations
02464	Enrofloxacin	150	148	-0.1	Satisfactory
	Oxolinic Acid	50	43.8	-0.9	Satisfactory
02485	Sulfamethazine	34.1	27.7	-0.3	Satisfactory
	Sulfamethoxazole	51.8	42.2	0.1-	Satisfactory
	Sulfapyridine	50	56.8	0.3	Satisfactory
	Sulfathiazole	48.4	38.2	-0.4	Satisfactory
02505	Erythromycin	200	191	-0.1	Satisfactory
	Sulfadiazine	26.8	31.3	0.2	Satisfactory
	Sulfamerazine	100	83.7	-1.9	Satisfactory
	Sulfamethazine	73.7	63.1	-0.9	Satisfactory
	Sulfathiazole	100	87.5	-1.2	Satisfactory
02515	Chloramphenicol	0.3	0.24	-0.9	Satisfactory
	Florfenicol	200	210	0.8	Satisfactory

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106299.

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