Article

UHPLC-ToF-MS as a High-Resolution Mass Spectrometry Tool for Veterinary Drug Quantification in Milk

Marta Leite [1,2,3], Ana Rita Marques [1,4], Ana Sofia Vila Pouca [1], Silvia Cruz Barros [1], Jorge Barbosa [3], Fernando Ramos [2,3], Isabel Maria Afonso [5] and Andreia Freitas [1,3,*]

1 National Institute for Agricultural and Veterinary Research (INIAV), Rua dos Lágidos, Lugar da Madalena, 4485-655 Vila do Conde, Portugal; marta.leite@iniav.pt (M.L.); ritamarques207@gmail.com (A.R.M.); ana.sofia@iniav.pt (A.S.V.P.); silvia.barros@iniav.pt (S.C.B.)
2 Faculty of Pharmacy, University of Coimbra, Health Science Campus, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal; framos@ff.uc.pt
3 Associated Laboratory for Green Chemistry of the Network of Chemistry and Technology (REQUIMTE/LAQV) R. D. Manuel II, 55142 Porto, Portugal; jmsbarbosa@hotmail.com
4 Escola Superior Agrária, Instituto Politécnico de Viana do Castelo, Rua Escola Industrial e Comercial de Nun'Álvares, 4900-347 Viana do Castelo, Portugal
5 Centro de Investigação e Desenvolvimento em Sistemas Agroalimentares e Sustentabilidade (CISAS), Escola Superior Agrária, Instituto Politécnico de Viana do Castelo, Rua Escola Industrial e Comercial de Nun´Álvares, 4900-347 Viana do Castelo, Portugal; iafonso@esa.ipvc.pt
* Correspondence: andreia.freitas@iniav.pt

Abstract: Milk is one of the most widely consumed foods in the world, despite the increasing consumption of plant-based alternatives. Although rich in nutrients and believed by consumers to be free of undesirable contaminants, milk, whether of animal or plant origin, is not always free from residues of chemical substances, including veterinary medicines. For instance, in intensive livestock production, antibiotics are often used to treat animals or, illicitly, to improve their growth performance, which can lead to their presence in the final food. Additionally, the continuous use of veterinary drugs in intensive animal production can lead to their occurrence in agricultural soils and therefore are absorbed by plants as another source of entering the food chain. An effective and accurate multi-detection quantitative screening method to analyze 89 antibiotics in milk was optimized by ultra-high-performance liquid chromatography coupled with a time-of-flight detector (UHPLC-ToF-MS) and further validated in accordance with the Commission Implementing Regulation (CIR) 808/2021 and the International Council for Harmonization (ICH) guidelines on the validation of analytical procedures. Apart from the specific parameters required by CIR 808/2021, the aim was to access the lower limits of the method, limits of detection (LoD) and quantification (LoQ), regardless of the maximum residue limits (MRLs) defined in the legislation. The method was then applied in the analysis of 32 supermarket samples, resulting in four positive findings, including one plant-based sample. The antibiotics found were from the macrolides and sulphonamides families. Nevertheless, the concentrations detected were below the established maximum residue level (MRL).

Keywords: antibiotic residues; milk; food safety; UHPLC-ToF-MS; screening; validation; occurrence

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1. Introduction

The importance of science and technology in improving the quality of human life is directly related to the need to consume safe and healthy food. Worldwide, several chemical agents are responsible for the contamination of food products, mainly of anthropogenic origin. Veterinary drug medicines are frequently found in residual concentrations in food originating from intensive animal production. Through an increase in the world's population and consequent intensification of agriculture and livestock, the use of veterinary drugs in animal production began to include disease prevention and not exclusively therapeutic purposes. Illicitly, it can also be used to increase feed efficiency and, consequently, result

in the promotion of growth in healthy animals [1,2]. For instance, concerning milk and its derivatives, antimicrobial residues are the most detected [3,4]. Due to its high nutritional composition being rich in water, proteins, lipids, vitamins and minerals, milk is considered one of the most complete and valuable foods. It is, therefore, crucial to guarantee its quality and safety through accurate control measures.

Several factors, such as animal breed, age, feeding quality, lactation phase or infections in the mammary gland, can result in the development of pathogenic microorganisms, which lead to the need for treatment with antibiotics. These treatments are applied as a preventive measure, and the intramammary injection of antibiotics has proven to be an effective way to treat mammary gland infections [5,6]. If the withdrawal period is not respected, the final food product will be contaminated, which can pose risks to human health [6,7] and affect the quality of dairy-fermented products [5]. The health threats to humans may include toxic effects (carcinogenic, mutagenicity, teratogenic, etc.), alterations in the microbiota responsible for gastrointestinal diseases, and allergic reactions, among others [1,4]. Additionally, one of the major threats is the development and transfer of antimicrobial-resistant bacterial strains, which can cause therapeutic failures in the case of infected individuals [8]. This is a problem that has been increasing due to several issues, including excessive and uncontrolled use of antibiotics both in humans and in animals [9–11], leading to an increasingly severe scenario of antimicrobial resistance [12]. In addition, the continuous use of antibiotics in animal livestock breeding and the direct and uncontrolled use of manure as organic fertilizer can cause their dissemination through the environment. In the case of antibiotics, whose absorption by animals is not total, their occurrence in the soil can cause their absorption by plants, leading to their introduction into food chains by contaminated plant-based food products [13–17]. Though the risk is clear, studies concerning the occurrence of antibiotics in plants are scarce, including plant-based milk products.

In this sense, monitoring the presence of antibiotic residues in milk intended for human consumption is crucial to the protection of consumers and should comply with the regulations used for that purpose. Reliable, accurate and precise analytical methodologies are therefore needed to ensure efficient control of these anthropogenic contaminants. The European Commission (EC) has determined the rules for that specific control to prevent residues in food, with substances being completely prohibited and others with defined and established maximum residue limits (MRLs) in case of allowed pharmacologically active substances in edible tissues of animal origin [18,19]. Thus, the Commission Implementing Regulation (CIR) 2021/808 [20] defines the mandatory requirements to be met for the development and validation of analytical methods for the determination of drug residues in animal matrices. For the analysis of antibiotics in a multi-detection and multi-class approach, an efficient screening method is very important to ensure the reliability, accuracy and time-effectiveness of the results [21]. Those multi-residue methods are increasingly sought and adopted in routine analyses, improving the relationship between the cost and effectiveness of analytical methodologies by maximizing the number of analyses determined in each sample in one analysis. The most frequent analytical methodologies used for the presence of drug residues in food matrices are microbiological [4], immunochemical [22] and physico-chemical [21,23–25]. However, to fulfill the necessary and mentioned specifications, techniques such as ultra-high-performance liquid chromatography (UHPLC) coupled with mass spectrometry detectors are the golden choice to achieve such accurate and unequivocal identification of target antibiotics [26,27]. Although triple quadrupole mass spectrometry detectors are still one of the main choices for contaminant analysis in food, in recent years, the use of high-resolution mass spectrometry (HR-MS) in the field of multi-detection of a large number of veterinary drug residues has proven to provide the necessary specificity and sensitivity, despite the number of compounds, for target and also untargeted analyses [28–31]. Another valuable characteristic of such technology is the given possibility of performing a retrospective analysis of suspected and untargeted contaminants, considering its linking to computer and data analysis systems [32]. The specific

determination of antibiotics in milk has been described mainly by liquid chromatography coupled with tandem mass spectrometry in order to comply with the necessary requirements implemented by the European Commission, which reflect the very low levels of contaminants to be detected [33]. Notwithstanding, these methods are mostly aimed at a reduced number of target compounds or at one class of compounds due to the loss of the sensitivity of MS/MS detectors when a large number of analytes are analyzed in a single run, affecting parameters such as the limits of detection (LoD) and quantification (LoQ) [34,35]. A few reports using LC-ToF-MS have been applied for this purpose, highlighting the simplicity, time and cost-effectiveness of such a method [33,36–38], being a tool of excellence as a screening method for a high number of analytes (multi-class methods) in a short period of time, allowing to significantly reduce the analyses length, especially in routine analysis, with the added value of a preliminary quantitative assessment in positive cases.

In line with the aforementioned, the present study intends to describe an analytical methodology for the screening of 89 antibiotics from 10 classes, including penicillins, cephalosporins, macrolides, tetracyclines, quinolones, sulfonamides, pleuromutillins, sulfone (dapsone), amphenicols and diaminopyrimidines, in milk matrices. The method performed using HR-MS, namely by UHPLC-ToF-MS, was fully validated, comprising the calculation of LoD and LoQ values to further evaluate the levels of contamination in real milk samples available to consumers. The sampling profile of real samples was defined according to current consumption patterns of this type of food, which included animal milk (raw and processed) and also plant-based milk samples (soya and oat) since the increasing consumer search for healthier food products leads to an increase in such products in the market, which can be contaminated by antibiotic-contaminated soils or water.

2. Materials and Methods

2.1. Reagents and Standards

All solvents and reagents used were of analytical grade or LC-MS grade for the mobile phase preparation, including ultrapure water type I. Acetonitrile, methanol, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and formic acid were acquired from Merck (Germany). For the preparation of the EDTA solution 0.1%, it was dissolved at 29.23 g in water until a final volume of 1 L. Mobile Phase A (formic acid 0.1%) was prepared by diluting 1 mL of formic acid in water to 1 L of the solution. Antibiotic standards, including internal standards (IS), were provided by Sigma-Aldrich (Spain) with a purity of at least 98%. The appropriate amount of each standard was weighted to prepare the individual stock solutions with concentrations of 1 mg mL^{-1} in methanol, except for the beta-lactams, which were prepared in water. These solutions were stored at −20 °C for 6 months and used to prepare the necessary mixtures to be used to spike the blank samples for the matrix calibration curves. Similarly, a mixed solution of IS (demeclocycline, sulfadiazine 13C6, penicillin G d7, cefadroxil d4, erythromycin 13C d3, lomefloxacin, florfenicol d3, trimethoprim d9) with a concentration of 10 µg mL^{-1} was prepared. The mixtures and working solutions were kept for 1 month, also at −20 °C.

2.2. Instrumentation

In addition to the current material in the laboratory, the following equipment was used: analytical balances, Toledo PC200 and AE100 (Greifensee, Switzerland); Heidolph Reax mixer (Schwabach, Germany); Selecta Mater ultrasonic water bath with a controlled temperature (Selecta, Spain); Heraeus Megafuge centrifuge (Hanau, Germany); Turbovap Zymark evaporator coupled with a nitrogen generator (Hopkinton, MA, USA); and Whatman Mini-Uniprep PVDF 0.45 µm filters (Clifton, NJ, USA).

The detection was performed with an UHPLC-ToF-MS comprising an UHPLC Nexera X2 (Shimadzu, Japan) for chromatographic separation coupled with a Triple TOFTM 5600+ (Sciex, Framingham, MA, USA). The UHPLC system consisted of a solvent degasser, a binary pump, an autosampler with a controlled temperature (10 °C), an automatic injector

with a variable volume (10 µL), and an oven for the column (40 °C). The reverse-phase column used was an Acquity UPLC HSS T3 1.8 µm, 2.1 × 100 mm (Waters, Milford, MA, USA). The flow rate was 500 µL.min^{-1} with mobile phases (A) formic acid 0.1% (*v*/*v*) in water and (B) acetonitrile. A gradient program was selected as follows: the first 2 min was kept at 97% (A) and then until 5 min from 97% to 40% (A); 5–9 min from 40% to 0% (A); 9–10 min from 0% back to 97% (A) in a total run time of 11 min.

The ToF-MS detector was equipped with an electrospray ion source in the positive ionization mode (ESI+) and full-scan acquisition mode in a range from 100 to 900 Da. Four software programs were used, all from the Sciex brand: Analyst® TF for the acquisition; and for the identification and quantification, we used PeakView™, LibraryView™ and MultiQuant™. The criteria of identification followed were based on the CIR 808/2021 requirements: exact mass with an error below 5 ppm (Δppm in Equation (1)) and a maximum variation in relative retention time (RRT) of 1% (ΔRRT Equation (2)).

$$\Delta \text{ppm} = \left(\frac{\text{Mass detected} - \text{Exact mass}}{\text{Exact mass}}\right) \times 10^6 \tag{1}$$

$$\Delta \text{RRT}(\%) = \left(\frac{\text{RRT}_{\text{sample}} - \text{RRT}_{\text{standard}}}{\text{RRT}_{\text{standard}}}\right) \times 100 \tag{2}$$

Quantitatively, and to ensure a reliable identification with an added criterion, the isotope ratio difference was established to be accepted to be lower than 10%. Such a value is automatically generated by the PeakView™ software. Also, to guarantee an accurate mass resolution, the detector was automatically calibrated at every 10 injections.

2.3. Sample Preparation

The method optimized for sample extraction was adapted from a previous method [34]. In summary, to 2 mL of homogenized milk, 60 µL of the IS mixture solution was added. After 10 min of rest in the dark, a liquid–liquid extraction and protein precipitation, with 10 mL of acetonitrile plus 1 mL of 0.1 M of EDTA, was carried out using 20 min of homogenization, 10 min in an ultrasound bath and centrifugation at 3100× *g* for 10 min at 4 °C. The extract was then evaporated under nitrogen steam at 45 °C until 500 µL was left. Then, 200 µL of mobile phase A was added, filtered through a PVDF Mini-Uniprep TM filter (0.45 µm) and injected in the UHPLC-ToF-MS system. Each batch of samples was analyzed with a matrix calibration curve using blank milk samples fortified prior to the sample extraction, with 0.1, 0.25, 0.5, 0.75, 1 and 1.5 MRL or the VL (validation level when no MRLs are established).

For the analyses of real samples, 32 milk aliquots were acquired at Portuguese supermarkets and stored at −80 °C until analysis. The samplings were performed on animal milk, including raw (n = 1), whole (n = 2), semi-skimmed (n = 16), and skimmed (n = 7) milk samples, and plant-based milk, soya (n = 3) and oat (n = 3) milk.

2.4. Validation Procedure

According to the specifications described in the CIR 808/2021 [20], the validation parameters that should be studied for a quantitative screening method are CCβ, trueness (or recovery), precision and selectivity/specificity and the linearity of calibration curves in the 0.1 MRL to 1 MRL range. In addition, the objective of the validation was to determine the LoDs and LoQs [39] for the various antibiotics in milk. It should be noted that for compounds in which there is no established MRL, the lowest MRL value established for another antibiotic in the same family was used as the validation level (VL) in accordance with the cascade MRL's use that can be applied to authorized substances (CIR 470/2018) [19].

Specificity and selectivity were evaluated by analyzing 30 blank samples from different origins (20 from animal origin and 10 from vegetable), and the same 30 samples spiked at the 0.5 MRL (or 0.5 VL). These spiked samples were analyzed over 3 days along with one calibration curve each day (the first and second days with an animal origin milk and

the third day with soy milk), and all data obtained were used to evaluate the recovery, precision, ruggedness and the linearity of the curve. For setting the screening CCβ, samples were analyzed at the levels of 0.1 MRL, 0.25 MRL and 0.5 MRL to access the option able to fulfill the accurate identification. For the *LoD* and *LoQ*, Equations (3) and (4), respectively, were used [39].

$$LoD = \frac{3.3 \times \sigma}{S} \quad (3)$$

$$LoQ = \frac{10 \times \sigma}{S} \quad (4)$$

where σ represents the standard deviation associated with the 30 blank sample analyses, and S is the slope of the calibration curve.

3. Results and Discussion

The main objective of this work was to validate a multi-class method, by UHPLC-TOF-MS, of antibiotics in milk. Although the main purpose is to use the current method in the routine quality control of milk samples, studies of the occurrence of milk at lower concentrations than the MRLs are also considered of huge importance. The growing concerns related to the development and dissemination of antimicrobial resistance are the main driver for the need to evaluate the real levels of contamination that can be presented in food products. For that, the method developed was fully validated in accordance with the CIR 808/2021 [15]. In addition, the evaluation of the analytical thresholds of the method, LoD and LoQ, was performed with the ultimate objective of evaluating the antibiotic levels in milk samples and studying the ability to identify and quantify the target antibiotics independently of the MRLs established in the legislation. The ability to detect and quantify the target antibiotics at concentrations highly below the MRL was proven, as described in detail in the following subsections. Having assessed the applicability of the method through validation, the method was used to analyze 32 supermarket milk samples, including one of plant origin, in the screening of 89 antibiotics from 10 families: penicillins, cephalosporins, macrolides, tetracyclines, quinolones, sulfonamides, pleuromutillins, sulfone (dapsone), amphenicols and diaminopyrimidines.

3.1. Scope of the Method

Even though sample preparation and detection optimizations are easier to develop when working in single-class methods due to the similar chemical characteristics of compounds of the same family, it is consensual that multi-detection and multi-class are the most efficient approaches for monitoring veterinary drugs in food. Some authors are still using the single-family [40] method or multi-class with a limited number of compounds in the same method [29,30] to determine antibiotics in milk [41,42]. The aim of the present work was to obtain a method with a broader number of antibiotics detected than the previous one [34], benefiting from the use of HR-MS, which its sensitivity is independent of the number of compounds analyzed. Another important goal was to maintain the sample extraction as simple as possible in order to have a rapid method able to be used for a high number of samples in a short time period. Other methods available in the literature that also provide multi-class approaches do not always use simple liquid–solid extraction but resort to solid-phase extraction [24,43,44], dispersive solid-phase extraction [43], QuEChERS [45] or the automated turbulent flow cyclone clean-up system [46], resulting in more time-consuming methods with higher consumption of reagents. Previously reported studies also described the development of methods to perform the multi-detection of veterinary drugs in milk, as Stolker et al. [31] presented a method able to detect 100 drugs; however, only half of them were antibiotics. Additionally, the validation process was in accordance with the MRLs established without further studies on the limits of detection. The advantages of mass spectrometry were also stated by other multi-class studies on an LC-MS/MS method basis. This recent report described the optimization and development of 78 veterinary drugs in bovine milk through an efficient and cost-effective procedure, encompassing a

broad scope of analytes with an increase in laboratory productivity of 2- to 3-fold [45]. Nonetheless, the authors stated the importance and future trends in milk analyses using HR-MS technologies towards non-target residue screening.

3.2. Validation

The validation process allows for the verification that a method is suitable to be used as a quality control tool and is an essential step in developing a method either as screening or confirmatory, qualitative or quantitative. It is important to emphasize that even though some literature presents multi-detection methods for a large number of veterinary drugs, not all provide a complete validation in accordance with the European Regulations for veterinary drug residue analysis [44,47]. The current method, with an extraction procedure based on a previous study [34], provides improvement mainly on the number of compounds analyzed. The broader scope of targeted antibiotics is due to the possibility of using HR-MS as full-scan detection technology, which allows the enhancement of the number of compounds detected without loss in sensitivity. The ionization needed to detect the target compounds was set to be performed by positive electrospray after testing both positive and negative modes. To promote the positive ionization, an acidic mobile phase was adjusted with 0.1% of formic acid, which guarantees the necessary concentration of H^+ to create the positive ions.

Regarding the process of the identification of antibiotics by UHPLC-ToF-MS, it is based on two main factors described in the CIR 808/2021: the identification of the exact mass (with a maximum acceptable error of Δppm = 5) and the variation in the relative retention time (not exceeding 1%). Additionally, to these identification parameters, the overlap of the isotopic profile of each compound, where a maximum of a 10% difference was considered acceptable, was also performed. Such a parameter was internally defined during method validation as a qualitative criterion since there is no official one.

The quantitative screening method was validated according to CIR 2021/808 [20] and for the established MRLs of the target antibiotics. For those that do not have MRLs established, including antibiotics that are not allowed to be used in milk production (doxycycline and oxolinic acid) or are prohibited in food animal production (dapsone), VLs were defined. The optimized method was fully validated, and Table 1 presents the antibiotics and the summary of the validation parameters. The validation, although in accordance with European legislation, included the evaluation of the analytical thresholds, LoD and LoQ, and for all calculations performed, the relative areas were used as a ratio between the areas of the target antibiotics and the corresponding IS. As can be observed in Table 1, all the obtained LoD and LoQ values are below the MRL established in Regulation 37/2010 [13]. For all compounds in which the LoDs were below 0.1 MRL, the achieved screening CCβ was also set as a 0.1 MRL. However, considering the lower level of the calibration curve, 0.1 MRL, the achieved LoD and LoQ for some antibiotics were higher. For instance, amoxicillin, ampicillin and benzylpenicillin had those threshold limits between 1/10 xMRL and 1/2 xMRL. In the class of sulfonamides, sulfanilamide had the worst sensitivity response, and only 50 $\mu g\ kg^{-1}$ for CCβ was acceptable, despite the MRL being 100 $\mu g\ kg^{-1}$. Another example is erythromycin, for which the CCβ obtained was 0.25 MRL, meaning 10 $\mu g\ kg^{-1}$ for an MRL of 40 $\mu g\ kg^{-1}$. In those cases, the lower calibration level considered for the method was defined to be the LoQ calculated.

Table 1. Validation parameters of the target antibiotics.

Antibiotic	Molecular Formula	Mass (Da)	$[M+H]^+$ (m/z)	Max. Δppm	RT (min)	MRL or (*)VL (μg kg^{-1})	CCβ (μg L^{-1})	LoD (μg L^{-1})	LoQ (μg L^{-1})	Recovery (%)	Precision		Linearity (R^2)
											Intra-Day (%)	Inter-Day (%)	
Acetyltylosin, 3-O-	C48H79NO18	957.52971	958.53675	0.96	5.36	50	12.5	0.03	0.10	105.6	5.5	8.1	0.9995
Amoxicillin	C16H19N3O5S	365.10454	366.11182	1.49	3.6	4	2	0.67	1.22	84.3	15.5	20.6	0.9978
Ampicillin	C16H19N3O4S	349.10963	350.11690	0.60	4.2	4	2	0.31	1.04	96.1	10.6	15.9	0.9967
Bacitracin	C66H103N17O16S	1421.74894	711.88226	0.76	4.75	100	25	1.41	4.27	111.8	6.4	11.6	0.9968
Baquiloprim	C17H20N6	308.17494	309.18200	−4.54	3.3	30	7.5	0.09	0.28	106.5	20.3	28.8	0.9951
Benzylpenicillin	C16H18N2O4S	334.09873	335.10601	−0.24	4.4	4	2	0.53	1.76	83.1	8.9	13.4	0.9953
Cefacetrile	C13H13N3O6S	339.05251	340.05978	−3.70	4.9	125	12.5	2.42	8.06	85.8	11.3	17.0	0.9988
Cefalonium	C20H18N4O5S2	458.07186	459.07927	0.81	4.3	20	10	0.08	0.23	103.9	10.63	13.6	0.9990
Cefapirin	C17H17N3O6S2	423.05588	424.06316	0.79	4.0	60	6	0.06	0.21	114.5	7.0	10.4	0.9958
Cefazolin	C14H14N8O4S3	454.03002	455.03729	3.77	4.6	50	5	0.06	0.18	129.4	13.0	19.4	0.9973
Cefoperazon	C25H27N9O8S2	645.14240	646.14968	−0.72	4.9	50	5	0.87	2.88	80.7	14.7	25.0	0.9800
Cefquinome	C23H24N6O5S2	528.12496	529.13224	−3.93	3.9	20	2	0.38	1.28	122.0	13.0	16.5	0.9925
Ceftiofur	C19H17N5O7S3	523.02901	524.03629	2.59	5.2	100	10	0.003	0.010	96.2	8.9	13.3	0.9906
Cephalexin	C16H17N3O4S	347.09398	348.10125	0.65	4.2	100	10	0.94	3.15	117.8	10.2	15.3	0.9842
Chlortetracyclin	C22H23ClN2O8	478.11429	479.12157	−0.10	4.6	100	10	0.07	0.24	101.0	10.7	16.1	0.9865
Cinoxacin [(a)]	C12H10N2O5	262.05897	263.06625	−0.30	5.0	30*	3	0.01	0.02	92.1	11.7	17.5	0.9984
Ciprofloxacin	C17H18FN3O3	331.13322	332.14050	0.95	4.4	100	10	0.08	0.28	108.0	11.0	16.4	0.9996
Clindamycin [(a)]	C18H33ClN2O5S	424.17987	425.18745	0.71	5.9	100*	10	0.15	0.44	106.4	5.0	8.6	0.9995
Cloxacillin	C19H18ClN3O5S	435.06557	436.07285	0.65	5.9	30	3	0.03	0.11	99.1	4.1	4.7	0.9992
Danofloxacin	C19H20FN3O3	357.14887	358.15615	1.34	4.4	30	3	0.13	0.42	116.6	10.3	15.5	0.9989
Dapsone [(b)]	C12H12N2O2S	248.06195	249.06923	−0.99	4.8	10*	2.5	0.47	1.58	109.1	7.1	10.7	0.9975
Desacetylcephapirin	C15H15N3O5S2	381.04531	382.05275	0.10	2.7	60	6	0.36	1.09	108.6	8.1	11.7	0.9984
Dicloxacillin	C19H17Cl2N3O5S	469.02660	470.03387	−0.81	6.2	30	3	0.13	0.43	107.7	5.1	7.6	0.9986
Difloxacin	C21H19F2N3O3	399.13944	400.14648	0.80	4.7	30	7.5	0.04	0.13	104.2	5.6	7.7	0.9998
Doxycycline [(c)]	C22H24N2O8	444.15327	445.16054	1.00	4.9	4*	2	0.28	0.95	117.6	13.4	20.1	0.9875
Enoxacin [(a)]	C15H17FN4O3	320.12847	321.13575	1.70	4.3	30*	3	0.02	0.08	107.0	7.9	10.4	0.9982

Table 1. *Cont.*

Antibiotic	Molecular Formula	Mass (Da)	$[M+H]^+$ (m/z)	Max. Δppm	RT (min)	MRL or (*)VL (μg kg^{-1})	CCβ (μg L^{-1})	LoD (μg L^{-1})	LoQ (μg L^{-1})	Recovery (%)	Precision		Linearity (R^2)
											Intra-Day (%)	Inter-Day (%)	
Enrofloxacin	C19H22FN3O3	359.16452	360.17180	0.10	4.5	100	10	0.17	0.56	114.4	11.9	17.8	0.9992
epi-Chlortetracyclin	C22H23ClN2O8	478.11429	479.12157	−0.36	4.4	100	10	0.17	0.58	118.2	11.0	16.6	0.9988
epi-Oxytetracyclin	C22H24N2O9	460.14818	461.15546	0.38	3.9	100	10	0.26	0.86	95.2	8.5	12.8	0.9996
epi-Tetracyclin	C22H24N2O8	444.15327	445.16054	−0.15	4.3	100	10	0.14	0.48	112.7	8.2	12.3	0.9954
Erythromycin	C37H67NO13	733.46124	734.46852	0.90	5.2	40	10	1.75	5.82	110.3	7.5	11.3	0.9988
Florfenicol	C12H14Cl2FNO4S	357.00046	358.00741	3.94	4.5	50	5	1.77	5.35	103.4	9.3	12.2	0.9981
Florfenicol amine	C10H14FNO3S	247.06784	248.07521	−2.89	1.35	50	12.5	0.17	0.52	103.4	5.5	9.3	0.9995
Flumequine	C14H12FNO3	261.08012	262.08740	0.77	5.7	50	5	0.01	0.05	107.5	5.3	7.9	0.9998
Gamithromycin	C40H76N2O12	776.53982	777.54708	−0.10	4.8	50	12.5	0.05	0.14	102.8	6.3	8.4	0.9996
Josamycin	C42H69NO15	827.46672	828.47402	1.03	5.6	50	12.5	0.02	0.05	106.4	4.5	8.6	0.9990
Lincomycin	C18H34N2O6S	406.21375	407.22145	1.35	4.1	150	12.5	0.07	0.21	106.6	6.0	8.7	0.9991
Marbofloxacin	C17H19FN4O4	362.13903	363.14631	1.51	4.3	75	7.5	0.14	0.47	101.3	5.6	8.5	0.9997
Nafcillin	C21H22N2O5S	414.12494	415.13222	0.84	6.0	30	3	0.07	0.22	105.4	5.1	7.7	0.9994
Nalidixic acid [(a)]	C12H12N2O3	232.08479	233.09207	0.29	5.6	30*	3	0.01	0.04	106.3	5.6	7.2	0.9996
Neospiramycin	C36H62N2O11	698.43536	699.44297	0.47	4.7	200	100	0.11	0.34	113.7	12.4	19.9	0.9930
Norfloxacin [(a)]	C16H18FN3O3	319.13322	320.14050	0.96	4.3	30*	3	0.02	0.06	114.9	9.4	14.2	0.9984
Novobiocin	C31H36N2O11	698.43536	699.44199	−0.28	7.0	50	12.5	0.46	1.40	110.1	8.4	12.1	0.9969
Ofloxacin [(a)]	C18H20FN3O4	361.14378	362.15106	0.59	4.3	30*	3	0.01	0.04	110.8	8.8	13.1	0.9994
Oleandomycin	C35H61NO12	687.41938	688.42640	0.35	5.1	50	12.5	0.11	0.33	105.5	6.6	8.6	0.9996
Ormetoprim [(a)]	C14H18N4O2	274.14298	275.15025	0.97	4.4	50*	5	0.13	0.45	103.2	4.3	3.4	0.9981
Oxacillin	C19H19N3O5S	401.10454	402.11182	0.84	5.8	30	3	0.18	0.61	111.2	8.0	11.9	0.9985
Oxolinic acid [(c)]	C13H11NO5	261.06372	262.07100	0.96	5.2	30	3	0.001	0.004	107.9	8.4	12.7	0.9974
Oxytetracycline	C22H24N2O9	460.14818	461.15546	0.51	4.1	100	10	0.09	0.29	106.0	6.2	9.3	0.9994
Phenoxymethylpenicillin	C16H18N2O5S	350.09364	351.10084	0.77	5.7	4	2	0.08	0.24	128.8	12.6	20.9	0.9876
Pirlimycin	C17H31ClN2O5S	410.16422	411.17164	0.46	4.7	100	10	0.29	0.89	106.2	5.0	7.7	0.9998

Table 1. *Cont.*

Antibiotic	Molecular Formula	Mass (Da)	$[M+H]^+$ (m/z)	Max. Δppm	RT (min)	MRL or (*)VL (µg kg^{-1})	CCβ (µg L^{-1})	LoD (µg L^{-1})	LoQ (µg L^{-1})	Recovery (%)	Precision		Linearity (R^2)
											Intra-Day (%)	Inter-Day (%)	
Rifaximin	C43H51N3O11	785.35236	786.35924	1.25	6.5	60	15	0.63	1.91	107.1	17.1	25.6	0.9962
Roxithromycin	C41H76N2O15	836.52457	837.53246	0.68	5.5	50	5	0.05	0.15	107.6	4.4	8.9	0.9994
Sarafloxacin [(a)]	C20H17F2N3O3	385.12380	386.13107	−0.22	4.7	30*	3	0.09	0.30	104.9	4.5	6.8	0.9999
Spiramycin	C43H74N2O14	842.51401	843.52128	−0.37	4.6	200	20	0.27	0.90	119.2	10.1	12.2	0.9911
Sulfabenzamide	C13H12N2O3S	276.05686	277.06438	−0.47	5.2	100	10	0.11	0.33	95.9	7.9	16.8	0.9952
Sulfacetamide	C8H10N2O3S	214.04121	215.04849	−0.85	3.5	100	10	7.22	24.06	108.4	12.9	19.3	0.9976
Sulfachloropyridazine	C10H9ClN4O2S	284.01348	285.02075	−0.26	4.9	100	10	0.11	0.36	112.4	13.0	19.6	0.9947
Sulfaclozine	C10H9ClN4O2S	284.01348	285.02075	0.27	5.3	100	10	0.14	0.48	99.0	3.1	4.7	0.9999
Sulfadiazine	C10H10N4O2S	250.05245	251.05972	0.80	4.0	100	10	0.39	1.30	81.6	8.1	12.1	0.9895
Sulfadimethoxine	C12H14N4O4S	310.07358	311.08085	1.58	5.3	100	10	0.04	0.14	104.9	4.1	6.1	0.9973
Sulfadimidin	C12H14N4O2S	278.08375	279.09102	0.96	4.6	100	10	1.02	3.39	107.7	5.7	8.6	0.9945
Sulfadoxine	C12H14N4O4S	310.07358	311.08085	0.75	5.0	100	10	0.04	0.15	114.0	13.3	19.9	0.9992
Sulfaguanidin	C7H10N4O2S	214.05245	215.05972	0.78	1.2	100	10	1.10	3.65	98.1	6.0	9.0	0.9967
Sulfamerazine	C11H12N4O2S	264.06809	265.07566	1.21	4.3	100	25	0.16	0.48	98.4	5.9	8.7	0.9995
Sulfamethizol	C9H10N4O2S2	270.02452	271.03180	0.61	4.6	100	10	1.23	4.10	107.7	6.6	10.4	0.9991
Sulfamethoxazole	C10H11N3O3S	253.05211	254.05939	0.67	5.0	100	10	0.20	0.66	105.7	12.1	18.2	0.9968
Sulfamethoxypyridazine	C11H12N4O3S	280.06301	281.07029	0.96	4.6	100	10	0.59	1.98	100.0	2.2	3.3	0.9996
Sulfamonomethoxine	C11H12N4O3S	280.06301	281.07029	0.76	4.8	100	10	0.84	2.81	99.5	3.3	4.9	0.9996
Sulfamoxol	C11H13N3O3S	267.06776	268.07504	0.82	4.5	100	10	0.61	2.03	102.9	3.2	4.8	0.9986
Sulfanilamide	C6H8N2O2S	172.03065	173.03793	−0.85	1.4	100	50	9.13	30.45	109.2	7.2	10.8	0.9927
Sulfapyridin	C11H11N3O2S	249.05720	250.06447	0.78	4.2	100	10	0.24	0.81	102.3	8.3	12.5	0.9951
Sulfaquinoxaline	C14H12N4O2S	300.06810	301.07537	0.87	5.3	100	10	0.43	1.42	104.1	4.8	7.2	0.9940
Sulfasalazine	C18H14N4O5S	398.06849	399.07577	0.83	5.5	100	10	0.11	0.36	101.2	3.7	5.5	0.9995
Sulfathiazole	C9H9N3O2S2	255.01362	256.02090	0.90	4.2	100	10	0.18	0.59	101.9	6.6	10.0	0.9994
Sulfisomidine	C12H14N4O2S	278.08375	279.09102	0.62	3.9	100	10	2.82	9.40	120.8	11.3	17.0	0.9966

Table 1. *Cont.*

Antibiotic	Molecular Formula	Mass (Da)	$[M+H]^+$ (m/z)	Max. Δppm	RT (min)	MRL or (*)VL (μg kg^{-1})	CCβ (μg L^{-1})	LoD (μg L^{-1})	LoQ (μg L^{-1})	Recovery (%)	Precision		Linearity (R^2)
											Intra-Day (%)	Inter-Day (%)	
Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	267.06776	268.07504	0.69	5.1	100	10	0.09	0.29	112.2	12.7	19.0	0.9965
Tetracycline	$C_{22}H_{24}N_2O_8$	444.15327	445.16054	0.88	4.5	100	10	0.42	1.40	97.7	3.4	5.1	0.9959
Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	355.00480	356.01230	−1.40	4.5	50	5	1.18	3.58	106.5	8.3	12.5	0.9989
Tiamulin [(a)]	$C_{28}H_{47}NO_4S$	493.32258	494.32986	0.47	5.4	50*	5	0.07	0.24	103.3	4.2	6.2	0.9998
Tildipirosin	$C_{41}H_{71}N_3O_8$	733.52412	734.53192	0.94	4.0	50	5	0.01	0.02	110.8	8.0	15.2	0.9986
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	868.56604	869.57332	−0.86	4.9	50	25	0.23	0.84	99.5	10.2	13.5	0.9986
Trimethoprim	$C_{14}H_{18}N_4O_3$	290.13789	291.14517	1.57	4.3	50	5	0.17	0.57	108.7	4.7	10.1	0.9998
Tulathromycin	$C_{41}H_{79}N_3O_{12}$	805.56638	806.57344	0.20	4.3	50	5	0.05	0.16	115.6	8.4	15.1	0.9962
Tylosin A	$C_{46}H_{77}NO_{17}$	915.51915	916.52643	−0.36	5.3	50	5	0.02	0.07	117.8	9.1	13.6	0.9984
Tylvylosin	$C_{53}H_{87}NO_{19}$	1041.58723	348.20294	1.70	5.5	50	5	0.03	0.08	105.1	5.4	8.8	0.9998
Valnemulin [(a)]	$C_{31}H_{52}N_2O_5S$	564.35970	565.36697	−0.89	5.6	50*	5	0.01	0.03	118.6	9.4	14.1	0.9998
Virginiamycin M1	$C_{28}H_{35}N_3O_7$	525.24750	526.25497	1.10	5.8	50	5	0.84	2.55	103.9	6.4	8.7	0.9999
Virginiamycin S1	$C_{43}H_{49}N_7O_{10}$	823.35409	824.36136	0.56	6.4	50	5	0.07	0.23	106.1	4.7	7.7	0.9986

[(a)] No MRL defined; [(b)] prohibited; [(c)] not allowed for milk production.

Overall, the lowest LoD and LoQ, 0.001 and 0.004 μg kg^{-1}, respectively, were obtained for oxolinic acid from the fluoroquinolones antibiotic class and considered not allowed for milk production. On the other hand, the highest values were found for sulfanilamide, as already described, with, respectively, 9.13 and 30.45 μg kg^{-1}. In the work by Jadhav et al. (2019) [45], the LoQ range found for 78 veterinary drugs' minimum limits was higher than in our study (0.02 μg kg^{-1}), though a maximum value of 25 μg kg^{-1} was obtained. Similar ranges were found on a validation procedure for 25 veterinary drugs, including quinolones, fluoroquinolones, tetracyclines, sulphonamides, trimethoprim and bromhexine, also using tandem mass spectrometry as a detection technology. LoDs were found between 0.2 and 10 μg kg^{-1}, and LoQs between 2.5 and 25 μg kg^{-1}, though a considerably lesser number of analytes was determined when compared to our study [33]. Analyses of quinolones, penicillins and cephalosporins in cow's milk were also performed in a comparative study using LC-MS/MS and UHPLC-MS/MS techniques [35]. The limit values ranged from 0.03 to 0.5 μg kg^{-1} for the LoD values and from 0.1 to 1.25 μg kg^{-1} for the LoQ values by using LC; and, with UPLC, the range of LOD values was 0.02–0.75 μg kg^{-1} and was 0.1–9 μg kg^{-1} for the LOQ values.

Criteria of acceptance for recovery and precision are directly related to the range of concentrations and are well described in the CIR 808/2021 [20]. For recovery, the tightest range of acceptance is between 80% and 120%. The inferior limit was completely fulfilled, being the cefoperazone compound with a lower recovery of 80.7%. However, for the upper limit, four of the compounds have exceeded 120%, namely: sulfisomidine at 120.8%, cefquinome at 122%, phenoxymethylpenicillin at 128.8% and cefazolin at 129.4%. It can be assumed that the matrix effect has a major influence on the detection of those compounds. For instance, in an aforementioned study of veterinary drugs in milk samples, recoveries were within the range of 70–120% for over 90% of the compounds analyzed at and above the VLL, and tough, very low recoveries were found for amoxicillin, tetracycline, oxytetracycline, chlortetracycline and doxycycline, ranging from 19 to 59% [45]. When it comes to the precision evaluation, the criteria defined are calculated in accordance with the Horwitz equation, which leads to the maximum value acceptable for reproducibility. Two-thirds of the same value provide the criterion for repeatability. Precision is calculated as the coefficient of variation intra-day for repeatability and inter-day for reproducibility. The higher reproducibility obtained was for cefoperazone, 25%, which is the higher limit of acceptance for the range of concentrations evaluated for this compound. On the other hand, for repeatability, the higher coefficient of variation achieved was for baquiloprim, with 20.3% being 20% the limit for the range of concentrations inferior to 10 μg kg^{-1} (MRL is 30 μg kg^{-1}). Essentially, the acceptance criteria established for precision through the Horwitz equation was fully achieved for all molecules.

Concerning the specificity of the method, in all 30 blank samples (20 of animal origin and 10 of plant-based origin), it was observed that no interfering peaks at the retention time of the target antibiotics were present. In addition, the same 30 samples were spiked at half of the validation level, and the identification criteria were all guaranteed, regardless of the composition of the milk sample. Overall, it can be assumed that the matrix effect does not interfere with the detection of the 89 antibiotics in those different types of beverage products.

In terms of linearity, the coefficient of determination R^2 was evaluated for all compounds in the spiked matrix calibration curve, and the internal acceptance criteria of $R^2 > 0.95$ was fulfilled. The farthest value achieved was for cefoperazone, with an R^2 of 0.98.

3.3. Analysis of Real Samples

After a complete validation and, consequently, limits of quantification (LOQ) and detection (LOD) were established, the analytical method was applied to a total of 32 real milk samples from different sources and types. The sampling was therefore performed on animal milk, including raw (n = 1), whole (n = 2), semi-skimmed (n = 16), and skimmed

(n = 7) milk samples, and plant-based milk, namely soya (n = 3) and oat (n = 3) milk. In Table 2, data on the sample analyses for antibiotic residue determination is shown.

Table 2. Antibiotics detected in raw milk samples.

Origin	Milk Source	Types of Milk	Detected Compounds	Antibiotic Group	Concentration (µg kg^{-1})
Animal	Cow	Raw	-	-	-
Animal	Cow	Semi-skimmed milk	Gamithromycin Tilmicosin	Macrolides	10.70 <LOQ
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk	-	-	-
Animal	Cow	Semi-skimmed milk (Fresh milk)	-	-	-
Animal	Cow	Skimmed milk	Tildipirosin	Macrolides	3.33
Animal	Cow	Skimmed milk	-	-	-
Animal	Cow	Skimmed milk	-	-	-
Animal	Cow	Skimmed milk	Tildipirosin	Macrolides	2.89
Animal	Cow	Skimmed milk	-	-	-
Animal	Cow	Skimmed milk	-	-	-
Animal	Cow	Skimmed milk	-	-	-
Animal	Cow	Whole milk	-	-	-
Animal	Cow	Whole milk	-	-	-
Animal	Grazing cow	Semi-skimmed milk	-	-	-
Plant-based	Oats	-	-	-	-
Plant-based	Oats	-	-	-	-
Plant-based	Oats	-	-	-	-
Plant-based	Soya	-	-	-	
Plant-based	Soya	-	Sulfamerazin	Sulfonamides	4.25
Plant-based	Soya	-	-	-	-

In most of the samples, no traces of the targeted antibiotics were observed, with only four samples (approximately 13%) presenting antibiotic residues in their composition at low concentrations. Three of the samples presented one antibiotic, with only one presenting a co-occurring antibiotic profile of two macrolides, gamithromycin and tilmicosin. The highest concentration was found in this semi-skimmed cow milk sample, at a value of 10.70 µg kg^{-1}. Tilmicosin was also found, though it was not possible to quantify since the value obtained was between the LOD and LOQ of the validated method for this compound. Two samples of skimmed milk also presented the macrolide tildipirosin at concentrations of 3.33 and 2.89 µg kg^{-1}. One of the samples of plant-based origin also presented one contaminant of the sulfonamide family, sulfamerazine, at a concentration level of 4.25 µg kg^{-1}. Despite the occurrence of antibiotic residues found, it can be concluded that these samples do not pose a risk of toxicity to the consumer since all were far below the established MRLs.

Although the occurrence studies available in the literature are difficult to compare due to the geographic different origins of milk samples and the scope of the analytical methods used (in terms of antibiotics and detection limits), there are a few worth mentioning. Nearest to our collection area, Castilla-Férnandez et al. [43], in Spain, after comparing two sample extractions, collected 24 milk samples from local supermarkets and detected traces of danofloxacin in two of them, both below the MRL. Another occurrence study,

performed in a city in India [45], obtained a higher rate of contaminations, despite all being at low concentrations. In 1000 milk samples analyzed for different veterinary drugs, 80% presented at least one contaminant from antibiotics (sulfonamides, tetracyclines and fluoroquinolones) and anthelmintics. Wang et al. [48] also presented a method to detect only 20 antibiotics (tetracyclines, fluoroquinolones, macrolides, beta-lactams and amphenicols) and afterwards collected 106 samples from Shanghai markets of edible animal origin tissues, including milk, which was found to reach 10.6% of the positive findings in milk. More recently, also in China [24], milk samples acquired in local markets were analyzed, and the antibiotics mostly found were tetracyclines, macrolides and trimethoprim, all below MRLs. In Brazil [49], a study of more than 1000 milk samples resulted in the detection of tilmicosin, cloxacillin and ceftiofur in three samples at concentrations higher than the MRL.

Overall, the occurrence studies that were performed and are available in the literature agree with the results achieved in this work concerning macrolides' detection in animal milk samples. Thus, the presence of antibiotics in milk is verified as a reality scenario in different types of milk, supporting the need to implement accurate farm and industrial production measures to minimize this exposure. On a wide systematic review study on this theme of antibiotics in milk, it was observed that the highest number of published works occur in Europe (n = 105), with bovine milk being the mostly used matrix worldwide (193), followed by ovine (n = 19) and caprine (n = 14) (Sachi et al., 2019), though this is still a trim down number of occurrence studies to fully comprehend the exposure range in this widely consumed food product. The specific case of the contaminated plant-based milk samples can also provide a promising insight into the arising issue of antibiotic absorption in plant and vegetable crops from contaminated soil or water. Future work should therefore focus on a wider range of types of milk, with a representative number of samples for each category, by also adding new veterinary drugs that represent a risk to public health, namely anti-inflammatory and antiparasitic agents.

4. Conclusions

The analytical strategy presented can be considered an efficient tool in terms of the food safety control of milk by combining new technology with the required sensitivity and the efficiency of a rapid, handy and easy approach. Another feature that should be highlighted is the possibility of revisiting the results in the future for presently untargeted molecules. The use of HR-MS and the full-scan acquisition provides that possibility in the mass range of the method. The presence of antibiotic residues in milk samples also emphasizes the need to establish assessment and management strategies to minimize or eliminate such a presence in a widely consumed food product, especially by vulnerable age population groups. Though it was proven that the safety of consumers could be perceived as not being compromised, combined toxicology is still an unexplored field that ought to be of utmost concern in the field of anthropogenic contaminants in food.

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