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Original scientific paper

SAMPLE PREPARATION, EXTRACTION PROCESSES AND CLEANUP PROTOCOLS FOR THE ANALYSIS OF ANTIMICROBIAL DRUGS IN MILK BY LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

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Abstract

Antimicrobial drugs are used as chemotherapeutic agents for treatment, prophylactic medication, growth promoters, and the improvement of feed efficiency.
Indiscriminate use of these drugs may leave residues in milk, which could pose a potential threat to human health and have undesirable effects on consumers, including allergic or toxic reactions, carcinogenic effects, bacterial resistance, and imbalance of the gut microfloraTo guarantee the safety of food products as well as public health, monitoring the levels of antimicrobial residues is necessary.
The sample preparation, extraction of different physicochemical residues of milk, preconcentration of the extract, and elimination of any matrix interferences that may affect the overall performance of the analytical methods. SPE extraction with an Oasis HLB column is one of the most commonly used techniques for sample preparation and provides an effective and repeatable method for the selective concentration of target analytes in complex matrices. Different variations of this method, including acetonitrile, methanol, and acetonitrile: methanol (50:50), 20% trichloroacetic acid, and McIlvaine buffer, were optimized.
According to the obtained results, the prescribed criteria for the analytical yield are met only by the extraction method using 20% trichloroacetic acid and Na2EDTA-Mcllvaine buffer.
Chromatographic separation of analytes was achieved on a Kinetex®C18 column and provided satisfactory resolution within the shortest run time. The ESI positive ionization was promoted, and the detection of the compounds was improved with the acidic mobile phase. After optimization of chromatographic conditions, MS/MS conditions, and extraction procedures, the LC-MS/MS method was validated according to the criteria of Commission Decision 2002/657/EC.

Keywords: sample preparation, extraction, antimicrobial residues, optimization, validation LC-MS/MS method.

Introduction

Antimicrobial drugs are widely used to treat microbial infections or diseases but are also administered as a preventive measure. Furthermore, these agents are used as feed additives to promote growth, improve feed efficiency, and increase milk production. The bactericidal or bacteristatical antimicrobial activity (Fig. 1), as well as physicochemical and pharmacokinetic properties are strongly related to drugs chemical structure (Fig. 2).

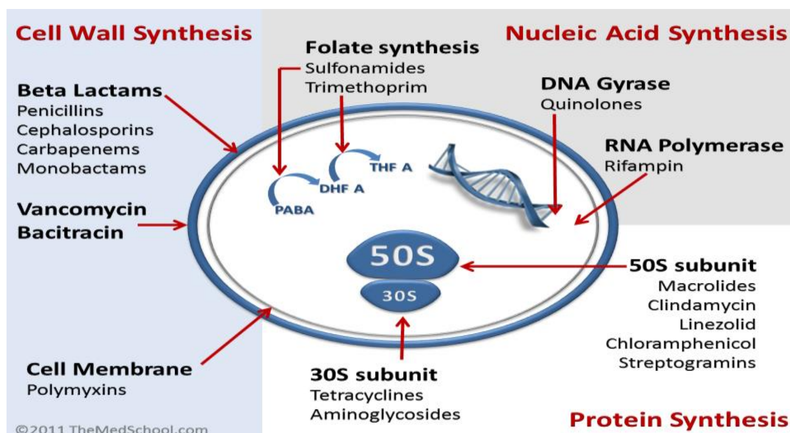

Figure 1. Antimicrobial activity

Illegal use of these drugs may leave residues in milk. Antimicrobial residues are metabolites or compounds with a negative impact on human health, processing, and the quality of milk. In public health, they can cause allergic reactions in hypersensitive individuals, toxic effects, carcinogenic effects, or the development of drug-resistant bacteria. (Alija et al, 2020, Girma et al, 2012, Nisha, 2008).

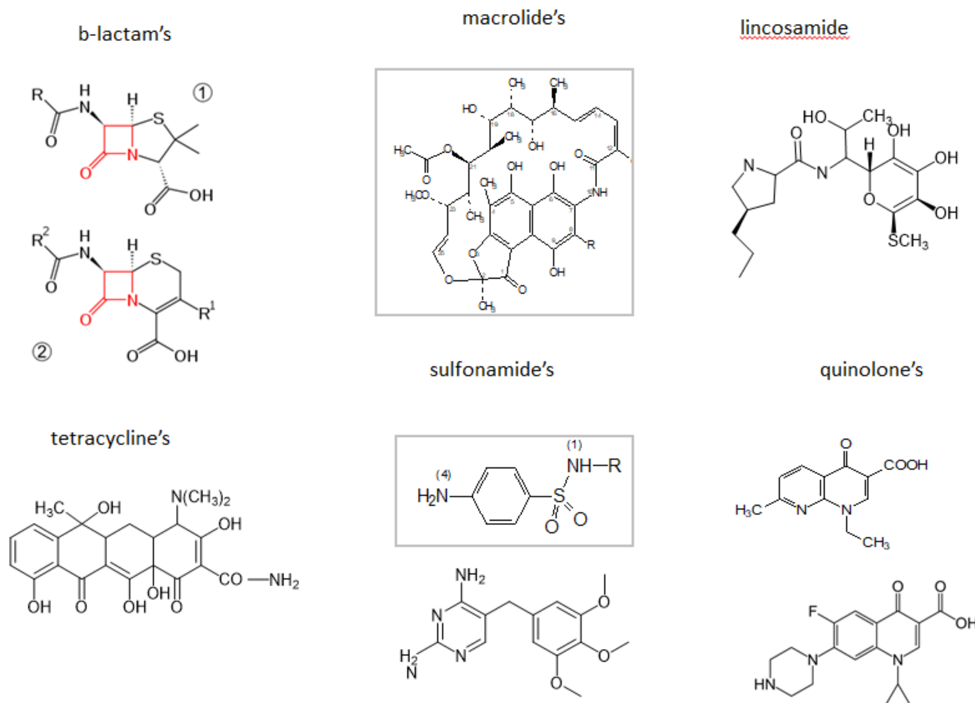

Figure 2. Chemical structure of antimicrobial drugs

Most countries have established official standard documents and legislation for monitoring antimicrobial drug residues, which is necessary to ensure food safety and prevent consumer exposure (Council directive 96/23/EC, Commission Regulation (EU) No 37/2010) Various types of methods have been developed for the detection of antimicrobial residues. Quantitative and qualitative determination of antimicrobial residues in milk involves several steps, from collecting the sample to issuing the final report with the results. In between, there are steps that usually involve the extraction of the analytes with a suitable solvent and then a suitable purification. Sample preparation prior to chromatographic separation has three main goals:

dissolving the analyte in a suitable solvent, removing as many interferences as possible, and concentrating the analyte (Wang, 2012). Solid-phase extraction (SPE) is one of the most widely used techniques for sample preparation that provides an efficient and reproducible method for selective concentration of target analytes in complex matrices (Marilena, 2015).
Kinsella and cop. (2009) in a literature review presented the current trends in the preparation of samples for the isolation of veterinary drugs in different matrices.
For determination of antimicrobial residues in food, there can be found various chemical, microbiological and immunological assays. The most commonly used screening method for the detection of antibiotic residues. They are economical, rapid, easy to use and suitable for high-throughput analysis, but they have low sensitivity and low specificity. The confirmatory methods are more specified, more accurate, more sensitive and precise, but costly in time, equipment and chemicals. These methods provide complementary information for simultaneous analysis of antimicrobial residues of different classes and quantification at the level of interest (Dimitrieska et al., 2011; Alija et al., 2022). The validation criteria of the methods are prescribed in the Commission Decision 2002/657/EC. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become a technique with high specificity, selectivity, sensitivity and for the simultaneous multi-class residue analysis of antimicrobial drugs in milk, only a restricted number of methods are reported in the literature, mainly due to difficulties related to differences in physic-chemical properties between families of compounds (Alija, 2021; Didier et al., 2009).
The aim of this study describes the optimization, sample preparation, extraction and validation of the LC-MS/MS method for multi-residue analysis of 23 antimicrobial drugs from seven different classes: b-lactam’s, macrolide’s, tetracycline’s, quinolone’s, sulfonamide’s, trimethoprim, and lincosamide, in bovine milk.

Materials and Methods

Analytical Standards

Penicillin G potassium salt (99.3%), Cloxacillin (98.5%), Oxacillin (99.2%), Cefalexin (96.6%), Ceftiofur (98.01%), Cephapirin (98.5%), Enrofoxacin (99.74%), Ciprofoxacin (98.0%), Tylosin (87.9%), Trimethoprim (99.5%), Lincomycin (100.3%), Doxycyclin (97.0%), Oxytetracyclin (96.5%), Tetracyclin (96.8%), Chlorotetracyclin (93.3%), Sulfachloropyridazin (99.1%), Sulfadiazin (99.8%), Sulfadimetoxin (99.7%), Sulfadimidin (99.6%) and Sulfafurazol (99.3%) were supplied by Fluka-Vetranal (Steinheim, Germany), Amoxicillin (99.6%), Ampicillin (99.8%) and Sulfamethoxazol (99.7%) were from Sigma-Aldrich (Steinheim, Germany).

Chemicals and reagents

Acetonitrile (LC–MS grade), Water (LC–MS grade), Methanol (LC–MS grade), trichloroacetic acid, disodium hydrogen phosphate dihydrate and disodium salt of ethylenediaminetetraacetic acid were purchased from Carlo Erba (Milan, Italy). Formic acid, dimethyl sulfoxide (DMSO), ammonium hydroxide, sodium chloride, formic acid, citric acid monohydrate were purchased from Sigma-Aldrich (Steinheim, Germany).
Na2EDTA-McIlvaine bufer pH 3.5, was prepared by dissolving 11.80 g of citric acid monohydrate;13.72 g of disodium hydrogen phosphate dihydrate; 33.62 g ethylenediaminetetraacetic acid disodium salt in 1000 mL of water.

Collection of milk samples

A total of 400 bovine milk samples was collected from individual animals from dairy farms in the Republic of North Macedonia, during 2017-2019. The samples were transported to the laboratory on the same day of collection at+4 °C. The samples were kept at − 20 °C until analysis.

Standard solutions

For all substances, Individual stock standard solutions of 1mgmL-1 were prepared in methanol, and storing at -20ºC, but Ciprofloxacin in a mixture of methanol and 2M sodium hydroxide (9:1 v/v), and Ceftiofur a mixture of methanol and DMSO (9:1 v/v). For solid phase extraction were used OASIS® HLB cartridges 3cc (60 mg) were obtained from Waters (Milford, MA). Before spiking the stock solutions were combined in five groups according to MRL values. The standards with the same MRL values were included in a common group, because the spike of the milk was in 3 levels, 0.5; 1.0 and 1.5*MRL. MRL values are given in Table 2.

Apparatus

The LC-MS/MS system was purchased from Waters (Waters, MA, USA). The chromatographic separation was achieved on a Kinetex®C18 (1.7µM100A, LC Column 50x2.1 mm) column. The MS/MS measurements were performed in triple quadruples mass spectrometer (Micromass, Manchester, UK). Instrument control and data processing were carried out by means of Masslynx 4.1 software (Waters). The following spectrometer parameters were used for all substances: source temperature was set at 150°C, capillary voltage of 4.0 kV, nitrogen as desolvation gas at a flow rate of 500L/h, nitrogen as nebuliser gas at a flow rate of 100L/h, desolvation temperature was 400°C.

Chromatographic conditions

The compounds were separated at 40°C, applying a flow rate of 0.4 mL/min and an injection volume of 10 µl. The total run time for each injection was 13 min. Mobile phase A was water with 0.1% formic, and Mobile phase B was acetonitrile with 0.1% formic acid. The Elution gradient program is given in Table 1.
The multiple reaction monitoring (MRM) mode was used for the LC-MS/MS chromatograms acquisition of antibiotics. The conditions are given in Table 2.

Method Validation

The validation procedure followed the described by the EU Commission Decision 2002/657/EEC (COMMISSION DECISION 2002/657/EC). According to those requirements, linearity, decision limit (CCα), detection capability (CCβ), limit of detection (LOD), limit of quantification (LOQ), selectivity, accuracy (expressed as recovery) and precision (repeatability and reproducibility) were determined.

Sample preparation

Various solvents were investigated in order to optimize the extraction process. We used four different extraction processes with acetonitrile, methanol and acetonitrile: methanol (50:50) and 20% trichloroacetic acid and McIlvaine buffer followed by SPE with Oasis HLB column. An aliquot of 5 mL of milk was transferred into a 50 mL plastic centrifugal tube. For the first extraction process we used 10 ml of

acetonitrile, for second extraction process 10 mL methanol and for the third extraction process we used acetonitrile :methanol (50:50) and 2 mL of 20 % aqueous trichloroacetic acid was added of. Ultrasonic bath for 5 minutes, shaken in vortex for 1 minute and centrifuge for 5 minutes at 3000 rpm at + 20ºC . The supernatant was immediately applied to an SPE cartridge. For the fourth extraction processes we used an aliquot of 5 mL of milk was transferred into a 50 mL plastic centrifugal tube. 2 mL of 20% aqueous trichloroacetic acid was added. The samples were shaken for 5 min. After shaking, 20 mL of McIlvaine buffer was added. The samples were vortexed for 1 min, and centrifuged at 4000 rpm for 20 min, at + 4 °C. The supernatant was immediately applied to an SPE cartridge. The progress of the same has continued in the four processes noted below. The cartridge was previously activated with 3 mL of methanol and 2 mL of water. After sample loading, the cartridge was washed with 4 mL of water and dried for 20 min at full vacuum. Antimicrobial residues were eluted with 3 mL of methanol. The samples were evaporated to dryness under a stream of nitrogen at 35 °C. The dry residues were reconstituted in 250 µL of mobile phase and filtered on a 0.22 µm micro filter. 10 µL of the final extract was injected into LC–MS/MS system.

Results

Chromatographic and MS/MS conditions have been optimised. The optimisation of the MS/MS method in this study was carried out by injecting the standards at a concentration of 10µg/ml in the MS/MS detector and scanning the mass spectrum in ESI+. Table 2 shows a gradient elution program for mobIle phases A and B. An MRM module was used to monitor the main precursor ions, as well as the product ions (daughter ions) of the target analytes (Table 2), and retention times it is also graphically presented

Table 1. A gradient elution program for mobile phase A and B

Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0,4	98.0	2.0
0,75	0,4	98.0	2.0
7,0	0,4	50.0	50.0
11.0	0,4	0.00	100.0
11.5	0,4	98.0	2.0
13.0	0.4	98,0	2.0

Table 2. Parameters of MRM condition, MRL and retention times of the antibiotics

Compound	Formula/Mass		Parent Ion (m/z)	Cone Voltage (v)	Daughter Ions (m/z)	Collision Energy (v)	Retention time (min)	MRL
Amoxicillin	365.4+H+=366.4	1	367.07	28	159.96	16	6.06	4
		2	367.07	28	90.89	40		
Ampicillin	349.4+H+=350.4	1	350.05	26	105.98	20	3.00	4
		2	350.05	26	159.96	12		
Benzylpenicillin	334.4+H+=335.4	1	334.99	44	90.96	42	3.31	4
		2	334.99	44	80.94	52		
Cephalexin	347.4+H+=348.4	1	347.99	22	157.89	8	2.97	100
		2	347.99	22	173.95	16		
Ceftiofur	523.5+H+=524.5	1	523.96	34	241.00	16	5.67	100
		2	523.96	34	125.17	58		
Cephapirin	423.4+H+=424.4	1	423.99	24	291.99	16	2.40	60
		2	423.99	24	151.97	30		
Ciprofloxacin	331.3+H+=332.3	1	332.01	38	245.05	28	3.43	100

		2	332.01	38	230.94	40		
Cloxacillin	435.8+H+=436.8	1 2	435.94 435.94	26 26	159.97 276.96	18 14	7.68	30
Doxycyclin	444.4+H+=445.4	1 2	445.05 445.05	28 28	153.92 97.85	30 44	3.31	100
Enrofloxacin	359.4+H+=360.4	1 2	360.05 360.05	36 36	245.09 72.02	30 36	4.32	100
Lincomycin	406.5+H+=407.5	1 2	407.09 407.09	34 34	126.02 41.79	30 72	2.59	150
Oxacillin	401.4+H+=402.4	1 2	402.05 402.05	24 24	159.96 243.01	10 12	7.51	30
Oxytetracyclin	460.4+H+=461.4	1 2	462.01 462.01	46 46	97.92 153.98	38 30	4.23	100
Sulfachloropyridazin	284.7+H+=285.7	1 2	284.90 284.90	28 28	155.93 91.93	16 34	3.23	100
Sulfadiazin	250+H+=251	1 2	250.97 250.97	28 28	91.93 155.93	30 14	1.71	100
Sulfadimetoxin	310+H+=311	1 2	310.97 310.97	36 36	155.93 91.93	20 32	5.01	100
Sulfadimidin	278.3+H+=279.3	1 2	278.95 278.95	34 34	185.93 91.93	18 36	2.70	100
Sulfafurazol	267+H+=268	1 2	267.97 267.97	26 26	155.95 112.95	16 18	4.81	100
Sulfamethoxazol	253.2+H+=254.2	1 2	253.91 253.91	28 28	92.00 155.94	30 16	3.47	100
Trimethoprim	290.3+H+=291	1 2	291.08 291.08	44 44	122.95 230.06	24 24	3.01	50
Tylosin	916.1+H+=917.1	1 2	916.43 916.43	56 56	174.07 100.97	46 56	7.87	50
Tetracyclin	444.4+H+=445	1 2	445.05 445.05	26 26	410.08 97.92	20 48	4.29	100
Chlorotetracyclin	478.8+H+=479.8	1 2	479.1 479.1	25 25	444 462	25 15	4.75	100

The LCMS/MS multi-residue method was optimized for the simultaneous determination of residues of several different classes of antibiotics and sulfonamides in raw milk samples, including β-lactams (penicillin's and cephalosporin's), sulfonamides, tetracyclines, macrolides, quinolones, trimethoprim, and lincomycin.

The sample preparation is often the most critical step of the method, especially in cases where it is a multiresidues determination. A method that includes antimicrobial drugs from several classes, due to the different physicochemical properties of the compounds, such as polarity and pKa. The extraction processes for the antimicrobial drugs in milk was optimized. The different steps (extraction, separation, isolation and

detection) and different solvents were studied. After optimization of chromatography conditions, MS/MS conditions and extraction processes, the method was validated.
The extraction processes with 20% trichloroacetic acid and McIlvaine buffer followed by SPE with Oasis HLB column was satisfactory for all antimicrobial drugs in this study and the accessed validation parameters were in accordance with the Decision 2002/657/EC. Comparison of the recoveries with the studied four different extraction solvents (acetonitrile, methanol, acetonitrile: methanol (50:50), 20% trichloroacetic acid and McIlvaine buffer) are presented in Table 3. When using these protocols, the analytical yield ranges from 71.96 to 108.74% when using 20% trichloroacetic acid and Na2EDTA-Mcllvaine buffer, from 14.36 to 105.25% when using acetonitrile, from 12.36 to 89.78% methanol, while when using acetonitrile: methanol (50:50) the analytical yield ranged from 20.78 to 108.15 %.

Table 3. Recovery of the four extraction solvents (acetonitrine, methanol, acetonitrile: methanol (50:50), 20% trichloroacetic acid and McIlvaine buffer

Antimicrobials	Acetonitrile	Methanol	Methanol Acetonitrile	20% trichloroacetic
β-lactams	71.22% - 95.15%	69.36% - 78.46%	75.44% - 108.24%	71.96% - 95.40%
Sulfonamides	75.22% - 105.25%	69.40% - 81.20%	71.22% - 89.14%	82.14% - 108.74%
Tetracyclines	14.36% - 29.44%	12.36% - 27.32%	20.78% - 39.44%	80.89% - 102.15%
Macrolides	71.34% - 75.14%	81.20% - 89.78%	81.45% - 90.36%	92.46% - 97.88%
Quinolones	75.14% - 81.63%	68.34% - 73.14%	74.13% - 88.36%	84.16% - 97.54%
Lincosamides	81.36% - 91.35%	81.40% - 88.36%	36.15% - 85.46%	88.46% - 105.08%
	14.36% - 105.25%	12.36% - 89.78%	20.78% - 108.15%	71.96% - 108.74%

Discussion and Conclusion

The LC-MS/MS method was highly specific and sensitive to detect low levels of drug residues, considering the complexity of obtaining good recovery of all drugs with different physicochemical properties. According to this Decision, at least 3 points for identification are required for the confirmation of substances from group B of Directive 96/23/EC. By determining the main-precursor ions and two product ions, 4 points are got for identification (1 point for precursor ion and 1.5 for each product ion), which fulfills this criterion. We monitored one precursor ion and two product ions for each analyte, where the product ion with the strongest intensity is used as the ion for the quantification of the analyte, and the second product ion is used for the confirmation of the analyte. I gave the optimum fragmentation voltage and collision energies (Table 2).
The results for main-precursor ions and two product ions obtained for antimicrobial drugs in raw milk correspond with literature data from Martins et al., 2016; Han et al., 2007; Freitas et al., 2013; Schwaiger et al., 2018; Amatya, 2010; and they are presented in Table 4.

Table 4. Comparison of main-precursor ions and product ions of antibiotics by different authors

Animicrobial	main-precursor ions	product ions Alija, 2019	Martins and cop., 2016	Han and cop., 2015	Freitas and cop., 2016	Schwaiger and cop., 2018	Amatya, 2010
Amoxicillin	367.07	159.96 90.89	на*	114 208	на	208 114	208 114
Ampicillin	350.05	105.98 159.96	на	106 174	на	192 174	106 192
Benzylpenicillin	334.99	90.96 80.94	на	160 176	на	176 160	176 160
Cloxacillin	435.94	159.97 276.96	на	160 277	на	160 277	160 277
Oxacilin	402.05	159.96 243.01	на	160 243	на	На	160 243
Cephalexin	347.99	157.89 173.95	на	158 140	на	На	158 140
Ceftiofur	523.96	241.00 125.17	на	241 285	на	241 126	на
Cefapirin	423.99	291.99 151.97	на	292 152	на	На	292 181
Ciprofloxacin	332.01	245.05 230.94	288.2 314.0	314 288	288.2	245 314	на
Enrofloxacin	360.05	245.09 72.02	245.2 316.2	316 342	316.3	245 342	316 342
Doxicylin	445.05	153.92 95.85	428.2 153.9	428 321	428.2	428 154	на
Tetracyclin	445.05	410.08 97.92	410.2 153.9	410 427	410.3	410 427	на
Chlorotetracyclin	479.1	444.00 462.00	153.9 97.60	154 462	444.2	444 462	на
Oxytetracyclin	462.01	97.92 153.98	426.2 444.2	На	426.3	426 443	на
Sulfachloropyridazin	284.90	155.93 91.93	107.8 107.8	156 108	92.3	На	на
Sulfadiazin	250.97	91.93 155.93	108.0 108.0	156 108	156.2	92 156	на
Sulfadimethoxin	310.97	155.93 91.93	140.0 140.0	156 108	156.2	156 108	на
Sulfadimidin	278.95	185.93 91.93	123.8 123.8	186 156	156.3	186 156	на
Sulfapurazol	267.97	155.95 112.95	156.0 156.0	На	156.2	На	на

Sulfamethoxazol	253.91	92.00 155.94	155.9 155.9	Ha	156.4	92 156	на
Thrimetoprim	291.08	122.95 230.06	275.2 230.2	230 261	на	261 230	на
Tylosin	916.43	174.07 100.97	на	174 101	174.3	174 101	на
Linkomycin	407.09	126.02 41.79	на	126 359	на	126 359	на

**на – not analyzed*

From the research, it can be concluded that all 23 analytes showed typical fragmentation, and complete mass spectra were obtained for all antimicrobial drugs. From the obtained results, shown in Table 3, it can be noted that two antibiotics from the tetracycline group, tetracycline and doxycycline, have the same molar mass and major ion, but these compounds can be easily distinguished based on retention time and by the product of the ions Tetracycline occurs at 4.29 min., while doxycycline occurs at 3.31 min. The product ions for tetracycline are 410.08 and 97.92, while for doxycycline the product ions are 153.92 and 95.85.

The LC gradient was established from two mobile phases: mobile phase A was water (LC-MS grade) with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The mobile phase used shows maximum sensitivity and resolution, as well as satisfactory separation of all analytes. The addition of 0.1% formic acid allows for the best sensitivity of the method during positive ionization (Zhan et al., 2012).

From the obtained results, it was concluded that optimal conditions were achieved at a flow rate of 0.4 mL/min and a column temperature of 40 °C, due to the fact that under these conditions a satisfactory resolution was provided, the best separation with a retention time between 1.71 minutes for sulfadiazine and 7.87 minutes for tylosin, and a total analysis time of 13 minutes. Sample preparation before chromatography separation is a key procedure in modern instrumental analysis and includes a number of steps: dissolving the analyte in a suitable solvent, extracting the analyte from the sample, removing multiple interferences, and concentrating the analyte. Free residues and conjugates are easily extracted with organic solvents or buffers, and protein denaturation with acid treatments (Wang et al., 2012, Alija et al., 2020). Trichloroacetic acid (TCA) 20% solutions are effective for protein precipitation. Proteins, which are in cationic form at low pH, form insoluble salts with acids. It is generally sufficient and the best results can be obtained by using reagents that have been cooled to a low temperature. Mcllviane /Na2EDTA buffer is a solvent that denatures proteins and gives a cleaner extract, that is, it facilitates the extraction of residues of antimicrobial drugs that are bound to proteins. (Freitas et al., 2013).

In this research, acetonitrile, methanol, acetonitrile: methanol (50:50) and 20% trichloroacetic acid were used for the three aforementioned extraction methods. The obtained results were not satisfactory for the determination of antibiotics in raw milk, because tetracycline drugs as well as beta-lactam drugs were not extracted at all, while the other antibiotics from the beta-lactam group had a very low analytical yield. Only sulfonamides, quinolones, macrolides, trimethoprim and lincomycin were extracted with this extraction. According to Kinsella et al., 2009, acetonitrile does not extract highly polar components, such as beta-lactams, because these compounds are unstable in this solvent and easily degrade under certain conditions of temperature and pH. Many literature data indicate that trichloroacetic acid is a frequently used extraction solvent, because it has a role in protein removal, but on the other hand it inhibits the ionization efficiency of the analytes. Although 20% trichloroacetic acid was used in this case, the tetracyclines were not extracted. Tetracyclines form a chelate complex with bivalent cations, and bind to proteins. Disruption of these interactions is commonly achieved by adding EDTA to the extraction solution (Anderson et al., 2005).

Extraction solvents acetonitrile and methanol they removed interferences of antimicrobial drug residues in milk. Methanol extracted many compounds from the samples, but no pure extract is obtained. On the other hand, acetonitrile did not extract polar analytes and complex formed compounds at all or sufficiently. For

these reasons it was necessary to use a combination of several solvents. However, acetonitrile did not sufficiently extract the bipolar compounds due to the high milk protein content and degradation. Procedure, such as extraction with acetonitrile, methanol, acetonitrile: methanol (50:50) at that after the initial extraction solid-phase extraction with Oasis HLB columns was used for purification (Berendesen, 2013).

β-lactams belong to the group of bipolar components. These antibiotics are unstable and heat labile. The four-membered β-lactam ring allows easy degradation of these compounds by various solvents and heat. Therefore, temperature and pH during sample preparation play an important role in the stability of these antibiotics. In most of the published methods, regarding the determination of betalactams, solid-phase extraction columns have been used to extract these antibiotics from matrices (Jank et al., 2012)

Tetracyclines form chelate complexes. They are soluble in polar organic solvents, acids and bases, but do not dissolve in saturated hydrocarbons which are strong chelating agents, since chelation of the divalent metal ion is necessary for their antimicrobial activity. These characteristics make it difficult to extract the analytes from the matrix, that is, it is the main reason that can lead to the loss of the analytes during extraction (Anderson et al., 2005).

Macrolides and lincosamides are soluble in methanol and, with isolated or conjugated double bonds, exhibit a hydrophobic profile. They are unstable in acid and are usually extracted from alkalized matrices. Both classes of compounds are isolated using organic solvent and aqueous buffer mixtures. Conventional extraction procedures for quinolone residues are with acidic, aqueous or polar organic solvents (methanol or acetonitrile). Sulfonamides are poorly soluble in water and non-polar solvents, but readily soluble in polar organic solvents due to amphoteric molecules containing different pKa values. During the extraction process it is important to adjust the pH of the aqueous phase in order to obtain higher analytical yield values. This is due to the ionic nature of sulfonamides, which is caused by the inductive effect of the SO2 group (Kinsella et al., 2009).

The combination of Na2EDTA-Mcllvaine buffer with trichloroacetic acid improves the extraction of tetracyclines from milk, giving better performance, because this combination allows protein precipitation and reduction of analyte loss during extraction. EDTA has a higher affinity for cations than tetracyclines, causing improved analytical yields of tetracyclines when added to the extraction solution (Anderson et al., 2005, Alija et al.,2020).

In SPE, the analyte is retained on the solid phase, while the sample passes through it, that is, with selective elution of the analyte and a suitable solvent, SPE can be considered as a simple type of extraction that is suitable for chromatographic analysis (Berendsen, 2013), but Oasis HLB have a very broad selectivity for polar compounds and lead to a reduction of ion suppression effects caused by matrix interferences. Also they do not contain free silanols to which the target compounds can bind directly or via metal ion complexes (Bitas et al., 2018). The Oasis HLB cartridges was chosen to SPE for antibiotics because this type of SPE sorbent provides efficient extraction with optimal recoveries (Alija et al.,2020)

After optimization of chromatographic conditions, MS/MS conditions and extraction procedure, the method was validated.

When using these protocols, the analytical yield ranges from 71.96 to 108.74% when using 20% trichloroacetic acid and Na2EDTA-Mcllvaine buffer, from 14.36 to 105.25% when using acetonitrile, from 12.36 to 89.78% methanol, while when using acetonitrile :methanol (50:50) the analytical yield ranged from 20.78 to 108.15 % (Table 3).

Gaugain-Juhel et al., (2009) presented two parallel extraction methods of antibiotics and sulfonamides in milk analysis. Acetonitrile was used for the extraction of beta-lactams, macrolides and sulfonamides, while 5% trichloroacetic acid was used for the extraction of tetracyclines, aminoglycosides, lincosamides and quinolones. The results obtained when comparing different solvents for the extraction of 58 antimicrobial agents were not always in full agreement with the expected results taking into account the physicochemical properties of the different analytes.

A different approach in terms of extraction is presented by Martins and cop (2016) for the determination of 25 antimicrobial compounds, belonging to different classes (quinolones, sulfonamides, macrolides, tetracyclines and bromexins) in milk samples. Extraction methods used: acidified ACN (0.1% formic acid) was used for the first extraction for quinolones. The second time they extracted sulfonamides and trimethoprim with acidified ethanol (ethanol: acetic acid) (96: 4). Extraction procedure Tetracyclines were extracted in the same mode as described for sulfonamides, but including the addition of EDTA. The analytical yield ranged from 62 to 108%, and the coefficient of variation was lower than 15% at enrichment levels from 0.25 MRL to 2 MRL.

Schwaiger et al., 2018 developed a unique extraction procedure for the analysis of antibiotic residues in milk and other matrices. Na2EDTA-Mcllvaine buffer and acetonitrile were used for the extraction, purification was done with different polymer sorbents C18 and dSPE and 30 substances from different compound groups (quinolones, macrolides, lincosamides, b-lactams, sulfonamides, derivatives) were analyzed by UHPLC-MS/MS method of diamino-pyrimidine and tetracyclines). The obtained results showed that only 20 out of 30 substances were detected and for those compounds the analytical yields ranged from 70 to 120%

In this study accurate, precise and sensitive multi-class LC–MS/MS method for simultaneous determination of 23 drug residues from seven different classes of antimicrobics in bovine milk was developed and validated. From this research we can conclude that acetonitrile is more effective for a larger number of compounds with high polarity compared to methanol or the combination of the two solvents, while the addition of a chelating agent (EDTA) gives better performance for tetracyclines, compounds that form complexes with polyvalent cations present in the sample extraction solution where loss of compounds occurs.

According to the obtained results, the prescribed criteria for the analytical yield in Commission Decision 2002/657/EC are met only by the extraction method using 20% trichloroacetic acid and Na2EDTA-Mcllvaine buffer. Therefore, this method was chosen as a suitable method for the extraction of several classes of antibiotics from raw milk

References

[1]. Anderson R.C., Rupp S.H., Wu W.H, 2005. Complexities in tetracycline analysis—chemistry, matrix extraction, cleanup, and liquid chromatography. J Chromatogr A 1075:23–32.

[2]. Alija Gj. Hajrulai-Musliu Z. Uzunov R. 2020 Development and validation of confirmatory LC–MS/MS method for multi-residue analysis of antibiotic drugs in bovine milk Journal SN Applied Sciences DOI:10.1007/s42452-020-03361-2

[3]. Alija Gj. 2021 Multiresidue analysis of antibiotics in milk using the LC-MS/MS method. PhD thesis. Ss. Cyril and Methodius University in Skopje. N.R.M.

[4]. Alija Gj., Hajrullai M. Z. , Nuhii N., Havziu Y. D. 2022 Analytical screening method for detecting residues of fluoroquinolone antibiotics in milk Acta Medica Balkanica International Journal of Medical Sciences pp 94-102 ISSN: 2545-4110 Vol. 7 | No. 13-14

[5]. Amatya R. 2010. MultiClass, Multi Residue Method for Determination of Penicillins, Cephalosporins and Quinolones in Cow Milk and Validation in Accordance with Commission Decision 2002/657/E C. European Masters in Quality in Analytical Laboratories

[6]. Berendsen B.J.A. 2013. LC-MS residue analysis of antibiotics, what selectivity is adequate? PhD thesis, Wageningen University, Wageningen, NL

[7]. Bitas D., Kabir A., Locatelli M., Samanidou V., 2018 Food sample preparation for the determination of sulfonamides by high-performance liquid chromatography: State-of-the-art. Separations, 5, 31;

[8]. Commission of the European Communities (2002) Commission Decision 2002/657/EC of 12th August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. OJEC, L221

[9]. Commission of the European Communities (1996) Council directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187EEC and 91/664/EEC. OJEC, L125, 10–32

[10]. Commission of the European Communities (2010) Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classifcation regarding maximum residue limits in foodstufs of animal origin. OJEC. L15, 1–72

[11]. Didier O., Cognard E., Jan Ph., Edder P. 2009 Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra- performance liquid chromatography coupled to time of flight mass spectrometry. Journal of Chromatography B, 877, 2363–2374. DOI: 10.1016/j.jchromb.2009.03.006.

[12]. Dimitrieska-S. E., Hajrulai-Musliu Z., Stojanovska-Dimzoska B., Sekulovski P., Uzunov R., 2011 Screening of veterinary drug residues in milk from Macedonia Mac. Vet. Rev. 34 (1), 5 – 13

[13]. Freitas A., Barbosa J., Ramos F. 2013. Development and validation of a multiresidue and multiclass ultra-highpressure liquid chromatography-tandem mass spectrometry screening of antibiotics in milk. International Dairy Journal. doi: 10.1016/j.idairyj.2013.05.019.

[14]. Girma K. Z. Zenebe H. Disassa, Tolsa T. 2014 Review on Detection of Antimicrobial Residues in Raw Bulk Milk in Dairy Farms. African Journal of Basic & Applied Sciences 6 (4): 87-97. DOI: 10.5829/idosi.ajbas.2014.6.4.8642.

[15]. Gaugain-Juhel M., Delépine B., Gautier S., Fourmond M.P., Gaudin V., Hurtaud-Pessel D., Verdon E. and Sanders P. 2009. Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: a qualitative approach, Food Additives & Contaminants, vol. 26, pp. 1459-1471

[16]. Han R.W., Zheng N., Yu Z.N., Wang J., Xu X.M., Qu X.Y., Li S.L. , Zhang Y.D.,. Wang Q. 2015 Simultaneous determination of 38 veterinary antibiotic residues in raw milk by UPLC–MS/MS. Food Chemistry 181, 119–126.

[17]. Jank L., Hoff R.B., Tarouco P.C., Barreto F., Pizzolato T.M. 2012. β-lactam antibiotics residues analysis in bovine milk byLC-ESI-MS/MS: a simple and fast liquid–liquid extractionmethod. Food AdditContam: Part A 29(4):497–507

[18]. Kinsella B., O'Mahony J., Malone E., Moloney M., Cantwell H., Furey A., Danaher M. 2009. Current trends in sample preparation for growth promoter and veterinary drug residue analysis. Journal of Chromatography A, 1216(46), 13, 7977-8015

[19]. Marilena D. 2015. Development of methods for the determination of veterinary drugs in food matrices by Liquid Chromatography – Mass Spectrometry. Doctoral thesis. National and Kapodistrian University of Athens, Greece

[20]. Martins M.T., Barreto F., Hoff R.B., Jank L., Arsand J.B., Motta T.M.C., Schapoval E.E.S. 2016 Multiclass and multi-residue determination of antibiotics in bovine milk by liquid chromatographyetandem mass spectrometry: Combining efficiency of milk control and simplicity of routine analysis. International Dairy Journal, 59, 44-51.

[21]. Nisha A.R.2008. Antibiotic Residues - A Global Health Hazard. Veterinary World 1(12): 375-377

[22]. Schwaiger B., König J., Lesueur C. 2018. Development and Validation of a Multi-class UHPLC-MS/MS Method for Determination of Antibiotic Residues in Dairy Products Food Analytical Methods 11, 1417–1434

[23]. Wang J., Macneil J., Kay J.F. 2012. Chemical analysis of antibiotic residues in food. John Wiley & Sons, Inc., Hoboken, New Jersey. Canada

[24]. Zhan J., Yu X.J., ZhongY.Y., Zai-ting Zhang, Cui X.M., Peng J.F., FengR., Liuc X.T., Zhua Y. 2012. Generic and rapid determination ofveterinary drug residues and othercontaminants in raw milk byultra-performance liquid chromatography–tandemmassspectrometry.J Chromatogr B 906:48–57