Research Article

Development and validation of confirmatory LC–MS/MS method for multi-residue analysis of antibiotic drugs in bovine milk

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Abstract
A confirmatory multi-class LC–MS/MS method have been developed for simultaneous determination of 23 antibiotic drugs from seven different classes in bovine milk. The method was validated in accordance with the criteria prescribed in Commission Decision 2002/657/EC. The linear regression analysis showed good correlation with R2 > 0.9800. LOD are in the range of 0.17–6.94 ng/ml, while the LOQ are in the range of 0.50–22.71 ng/ml. The CCα range from 4.43 to 122.33 ng/mL and CCβ was from 4.88 to 139.78 ng/mL. The recovery of the method ranged from 71.96 to 108.70%. The coefficient of variation for repeatability varied from 1.08 to 20.28% and the coefficient of reproducibility varied from 3.14 to 22.88%. In the present study, 189 bovine milk samples were collected from dairy farms and analyzed using confirmatory multi-class LC–MS/MS method. A total of 14 (7.41%) samples were found positive for antibiotics and sulfonamides. The concentrations of the residues were below the maximum levels established by EU.

Keywords Antibiotics · Bovine · Milk · Validation · LC–MS/MS

1 Introduction

Antibiotics in dairy cattle are widely used to treat or prevent microbial infections or diseases, such as mastitis, diarrhea and pulmonary diseases. Furthermore, antibiotics are used as feed additives to promote growth, improve feed efficiency and to increase of milk production. Moreover, several antibiotics can be added directly in milk to prolong its freshness [1, 8, 14]. Administration of antibiotics in dairy cattle can lead to the presence of antibiotic residues in milk. The presence of antibiotic residues in milk can be a risk for human health because they can cause allergic reactions in hypersensitive individuals, toxic effects, carcinogenic effects or they may result in the development of drug—resistant bacteria. In the dairy industry, the antibiotic residues inhibit the fermentation of bacterial starter cultures and negatively affect the quality of the final product [9, 16, 23]. To ensure and protect the human health it is very important to monitor the presence of antibiotic residues in milk. There are several European legislations for controlling veterinary drug residues which guarantee that foods of animal origin do not include drug residues [20, 21]. The measures to monitor veterinary drug residues in live animals and animal products are prescribed in Council Directive 96/23/EC, while Commission regulation EU 37/2010 set maximum residue limits (MRLs) for veterinary drugs in food [6, 7]. The validation criteria of the methods are prescribed in the Commission Decision 2002/657/EC [5].

For determination of antibiotic residues in milk can be found a lot of screening and confirmatory methods. The most commonly used screening methods for the detection of antibiotics are microbiological, enzymatic and immunological methods. The screening methods

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are rapid, easy to use and handle, economical, able to detect an antibiotic or class of antibiotics, but they have low sensitivity and low specificity [1, 16, 22]. The confirmatory methods are more accurate, more sensitive, more specify and precise, but costly in time, equipments and chemicals. Most frequently applied confirmatory method for detection of antibiotics in milk and tissues is liquid chromatography combined with tandem mass spectrometry (LC–MS/MS method). This type of technique is suitable for determination and identification of more classes of antibiotics in milk [20, 22, 24].

The present work describes the development and validation of LC–MS/MS method for detection of 23 antibiotic drugs from seven different classes: β-lactams, macrolides, tetracyclines, quinolones, sulfonamides, trimethoprim and lincosamide in bovine milk samples.

2 Materials and methods

2.1 Analytical Standards

Amoxicillin (99.6%), Ampicillin (99.8%) and Sulfamethoxazol (99.7%) were from Sigma-Aldrich (Steinheim, Germany), Penicillin G potassium salt (99.3%), Cloxacillin (98.5%), Oxacillin (99.2%), Cefalexin (96.6%), Ceftiofur (98.01%), Cephapirin (98.5%), Enrofloxacin (99.74%), Ciprofloxacin (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).

2.2 Chemicals and reagents

Methanol (LC–MS grade), acetonitrile (LC–MS grade), water (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).

Na_2EDTA-McIlvaine buffer 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.

For solid phase extraction were used OASIS® HLB cartridges 3 cc (60 mg) (Waters, Milford, MA).

2.2.1 Apparatus

The LC–MS/MS system (Instrument model: LC-Acquity BSM, MS-TQD) was purchased from Waters (Waters, MA, USA). The LC–MS/MS system equipped with binary pump, vacuum degasser, thermostated autosampler, thermostated column manager and triple quadruple detector.

The chromatographic separation was achieved on a Kinetex® C18 column (1.7 µM 100A, 50 × 2.1 mm).

For data acquisition and calculation of results for antibiotics in milk MassLynx software version 4.1 was used.

2.3 Standard solutions

Individual stock standard solutions of 1 mg/mL for all standards, except ciprofloxacin and ceftioflur, were prepared in methanol.

Ciprofloxacin was prepared in a mixture of methanol and 2 M sodium hydroxide (9:1 v/v), while ceftiofur was prepared in mixture of methanol and DMSO (9:1 v/v). Standard solutions for the calibration curve were prepared in the milk (matrix-match calibration).

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 (Table 4). In the group one was included: Amoxicillin, Ampicillin and Benzylpenicilin, group two: Cloxacillin and Oxacillin, group three: Cephapirin, group 4: Tylosin and Trimethoprim and in group five was included: Sulfamethoxazol, Cefalexin, Ceftiofur, Enrofloxacin, Ciprofloxacin, Lincomycin, Doxycyclin, Oxytetracyclin, Tetracyclin, Chlorotetracyclin, Sulfachloropyridazin, Sulfadiazin, Sulfadimetoxin, Sulfadimidin and Sulfafurazol. MRL values are given in Table 4. The concentration of the standards, which were used for spike of 5 ml milk, for group one and group two was 1000 ng/ml, while the concentration of the standards in group three, four and five was 10 µg/ml. The spiking procedure is given in the Table 1.

2.4 Collection of milk samples

A total of 189 bovine milk samples were collected from individual animals from dairy farms in Republic of North Macedonia, during 2018. The samples were transported to laboratory on the same day of collection at + 4 °C. The samples were kept at − 20 °C until analysis.

2.5 Sample preparation

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.

Table 1 The spiking procedure of milk

Group 1 1000 ng/ml		Group 2 1000 ng/ml		Group 3 10 µg/ml		Group 4 10 µg/ml		Group 5 10 µg/ml	
Spiked level ng/ml	Added volume µl	Spiked level ng/ml	Added volume µl	Spiked level ng/ml	Added volume µl	Spiked level ng/ml	Added volume µl	Spiked level ng/ml	Added volume µl
2	10	15	75	25	12.5	30	15	50	25
4	20	30	150	50	25	60	30	100	50
6	30	45	225	75	37.5	90	45	150	75

*The volume of milk was 5 ml

Table 2 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

After shaking, 20 mL of McIlvaine buffer were 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 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. Antibiotic residues were eluted with 3 mL of methanol. The samples were evaporated to dryness under stream of nitrogen at 35 °C. The dry residues were reconstituted in 250 µL of mobile phase (98% mobile phase A and 2% mobile phase B) and filtered on a 0.22 µm micro filter. 10 µL of the final extract was injected into LC–MS/MS system.

2.6 Chromatographic and MS/MS conditions

Mobile phase A consist of water with 0.1% formic and mobile phase B was acetonitrile with 0.1% formic acid, while the flow rate was 0.4 mL/min. Elution gradient program is given in Table 2. The compounds were separated at 40 °C.

Electrospray ionization in positive mode (ESI +) for all antibiotics was used with the following parameters of the mass spectrometer: source temperature 150 °C, capillary voltage 4.0 kV, nitrogen as desolvation gas at a flow rate of 500 L/h, nitrogen as nebuliser gas at a flow rate of 100 L/h and desolvation temperature 400 °C.

For the chromatograms acquisition was used multiple reaction monitoring (MRM) mode.

2.7 Method validation

The method was validated according to the criteria established by the Commission Decision 2002/657/EC. For each one of the studied antibiotics the following parameters were assessed: 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).

3 Results and discussion

The LC–MS/MS method was developed for the simultaneous determination of 23 antibiotic residues in milk samples. At first the chromatographic and MS/MS conditions were optimized. The conditions are given in the section Chromatographic and MS/MS conditions and Table 2.

To achieve maximum sensitivity for all antibiotics, as well as determination of the precursor and product ions, the individual antibiotics with concentration from 1 µg/ml were analyzed by direct infusion in the MS/MS detector. Additionally, the positive ionization was promoted and detection of the compounds were improved with the acidic mobile phase. Acidic pH of the mobile phase was adjusted with 0.1% of formic acid [9]. Full mass spectra were obtained for all antibiotics, but only the parent ion and two daughter ions were monitored. For each one of the antibiotics the daughter ion with the highest intensity was used for quantification, while the second daughter ion was used for confirmation.

Two antibiotics, tetracycline and doxycycline, have the same molar mass and the same parent ion, but these compounds can be easily distinguished on the basis of retention time: tetracycline at 4.29 min and doxycycline at 3.31 min, as well as according to daughter ions. The daughter ions for tetracycline are 410.08 and 97.92,

while for doxycycline the daughter ions are 153.92 and 95.85. The results are given in Table 3. According to the Commission Decision 2002/657/EC, for the confirmation of antibiotics (substances listed in Group B of Annex I of Directive 96/23/EC), a minimum of 3 identification points should be required (2002/657/EC). This method fulfills the requirements for a confirmatory method, because the monitoring of one parent ion and two daughter ions yields 4 identification points (1 for the parent ion and 1.5 for each daughter ion).

Because of the sample preparation is often the most critical step, the extraction procedure for the antibiotics in milk was optimized. For optimization of the extraction procedure were studied different solvents. The extraction with acetonitrile, methanol and acetonitril:methanol (50:50) followed by SPE extraction, [10, 13] was not satisfactory, because the recovery for tetracyclines was very low (< 39.5%). The tetracyclines form a chelate complex with bivalent metal cations and bind with proteins. This is the main reason which can lead to analyte losses during the extraction [2].

The extraction procedure with 20% trichloroacetic acid and McIlvaine buffer followed by SPE with Oasis HLB column was satisfactory for all antibiotics in this study and the accessed validation parameters were in accordance with the Decision 2002/657/EC. The combination of McIlvaine buffer with trichloroacetic acid successfully improve the extraction of tetracyclines from the milk because this combination is deproteinizing agent which eliminate proteins (acid) and stronger chelating agent of cations (EDTA from the McLlvaine buffer) [2, 18, 19]. The Oasis HLB cartridges was chosen to SPE for antibiotics because this type of SPE sorbent provides efficient extraction with optimal recoveries [4, 15].

Comparison of the recoveris with the studied four different exctraction solvents (acetonitrile, methanol, acetonitrile: methanol (50:50), 20% trichloroacetic acid and McIlvaine buffer) are presented in Table 5.

After optimization of chromatographic conditions, MS/MS conditions and extraction procedure, the method was validated. The chromatograms of spiked milk samples at the second level are given in Fig. 1.

3.1 Linearity

The linearity of the calibration curve for all antibiotic standards was evaluated by calculating of coefficient of correlation (R^2). Calibration curve in six points was prepared at concentration levels 0, 0.25, 0.5, 1.0, 2.0 and 3.0* MRL by adding standards of antibiotics to blank milk aliquots. Each calibration standard in each series was injected in triplicate. The calibration standards were prepared in the matrix because this is the most frequent approach to avoid or

Table 3 Parameters of MRM condition 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)
Amoxicillin	365.4+H⁺=366.4	1	367.07	28	159.96	16	6.06
		2	367.07	28	90.89	40	
Ampicillin	349.4+H⁺=350.4	1	350.05	26	105.98	20	3.00
		2	350.05	26	159.96	12	
Benzylpenicillin	334.4+H⁺=335.4	1	334.99	44	90.96	42	3.31
		2	334.99	44	80.94	52	
Cefalexin	347.4+H⁺=348.4	1	347.99	22	157.89	8	2.97
		2	347.99	22	173.95	16	
Ceftiofur	523.5+H⁺=524.5	1	523.96	34	241.00	16	5.67
		2	523.96	34	125.17	58	
Cephapirin	423.4+H⁺=424.4	1	423.99	24	291.99	16	2.40
		2	423.99	24	151.97	30	
Ciprofloxacin	331.3+H⁺=332.3	1	332.01	38	245.05	28	3.43
		2	332.01	38	230.94	40	
Cloxacillin	435.8+H⁺=436.8	1	435.94	26	159.97	18	7.68
		2	435.94	26	276.96	14	
Doxycyclin	444.4+H⁺=445.4	1	445.05	28	153.92	30	3.31
		2	445.05	28	95.85	44	
Enrofloxacin	359.4+H⁺=360.4	1	360.05	36	245.09	30	4.32
		2	360.05	36	72.02	36	
Lincomycin	406.5+H⁺=407.5	1	407.09	34	126.02	30	2.59
		2	407.09	34	41.79	72	
Oxacillin	401.4+H⁺=402.4	1	402.05	24	159.96	10	7.51
		2	402.05	24	243.01	12	
Oxytetracyclin	460.4+H⁺=461.4	1	462.01	46	97.92	38	4.23
		2	462.01	46	153.98	30	
Sulfachloropyridazin	284.7+H⁺=285.7	1	284.90	28	155.93	16	3.23
		2	284.90	28	91.93	34	
Sulfadiazin	250+H⁺=251	1	250.97	28	91.93	30	
		2	250.97	28	155.93	14	1.71
Sulfadimetoxin	310+H⁺=311	1	310.97	36	155.93	20	5.01
		2	310.97	36	91.93	32	
Sulfadimidin	278.3+H⁺=279.3	1	278.95	34	185.93	18	2.70
		2	278.95	34	91.93	36	
Sulfafurazol	267+H⁺=268	1	267.97	26	155.95	16	4.81
		2	267.97	26	112.95	18	
Sulfamethoxazol	253.2+H⁺=254.2	1	253.91	28	92.00	30	3.47
		2	253.91	28	155.94	16	
Trimethoprim	290.3+H⁺=291	1	291.08	44	122.95	24	3.01
		2	291.08	44	230.06	24	
Tylosin	916.1+H⁺=917.1	1	916.43	56	174.07	46	7.87
		2	916.43	56	100.97	56	
Tetracyclin	444.4+H⁺=445	1	445.05	26	410.08	20	4.29
		2	445.05	26	97.92	48	
Chlorotetracyclin	478.8+H⁺=479.8	1	479.1	25	444.0	25	4.75
		2	479.1	25	462.0	15	

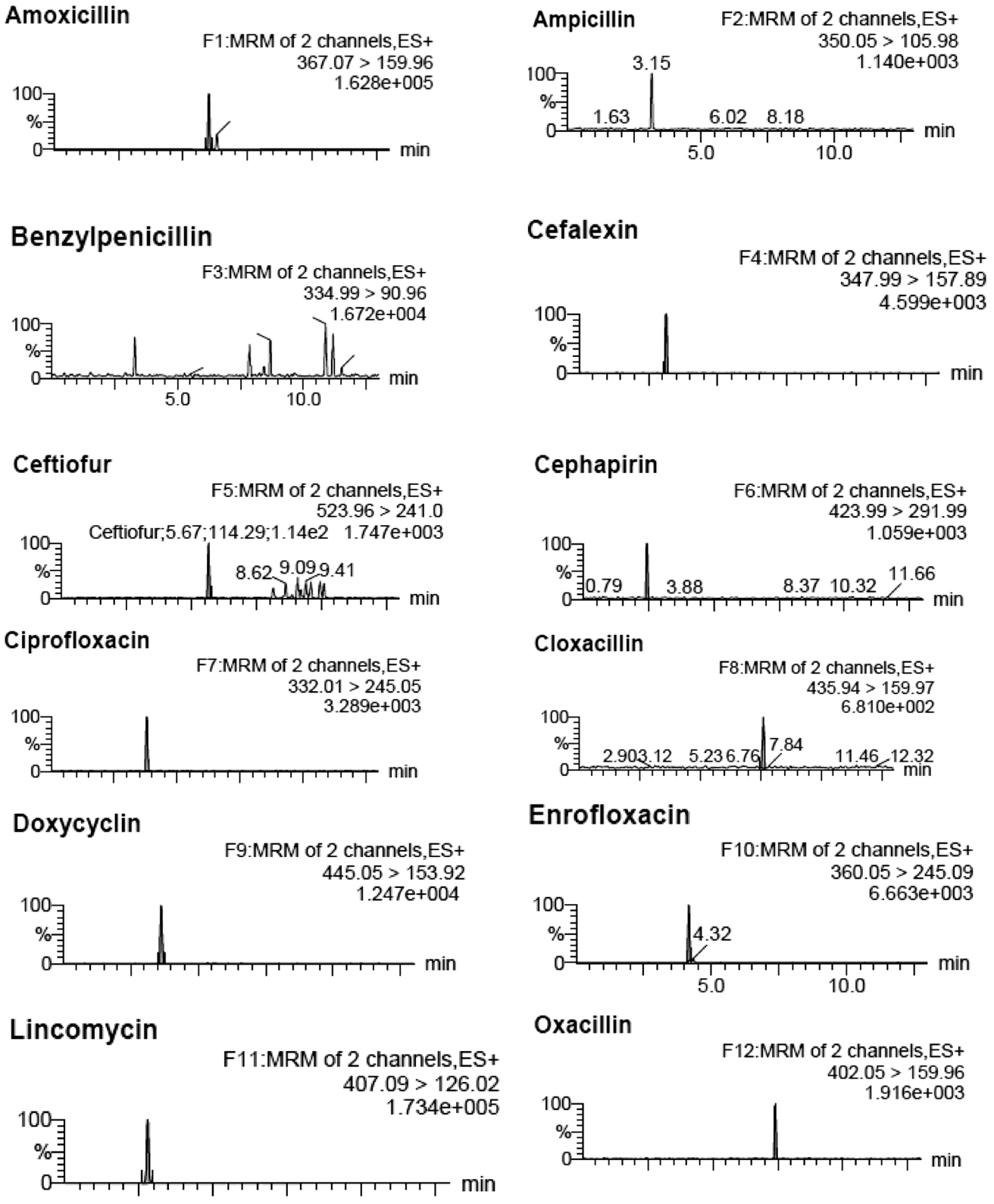

Fig. 1 Chromatograms of spiked milk samples and the second level (the concentration of the antibiotics for second level are given in Table 4)

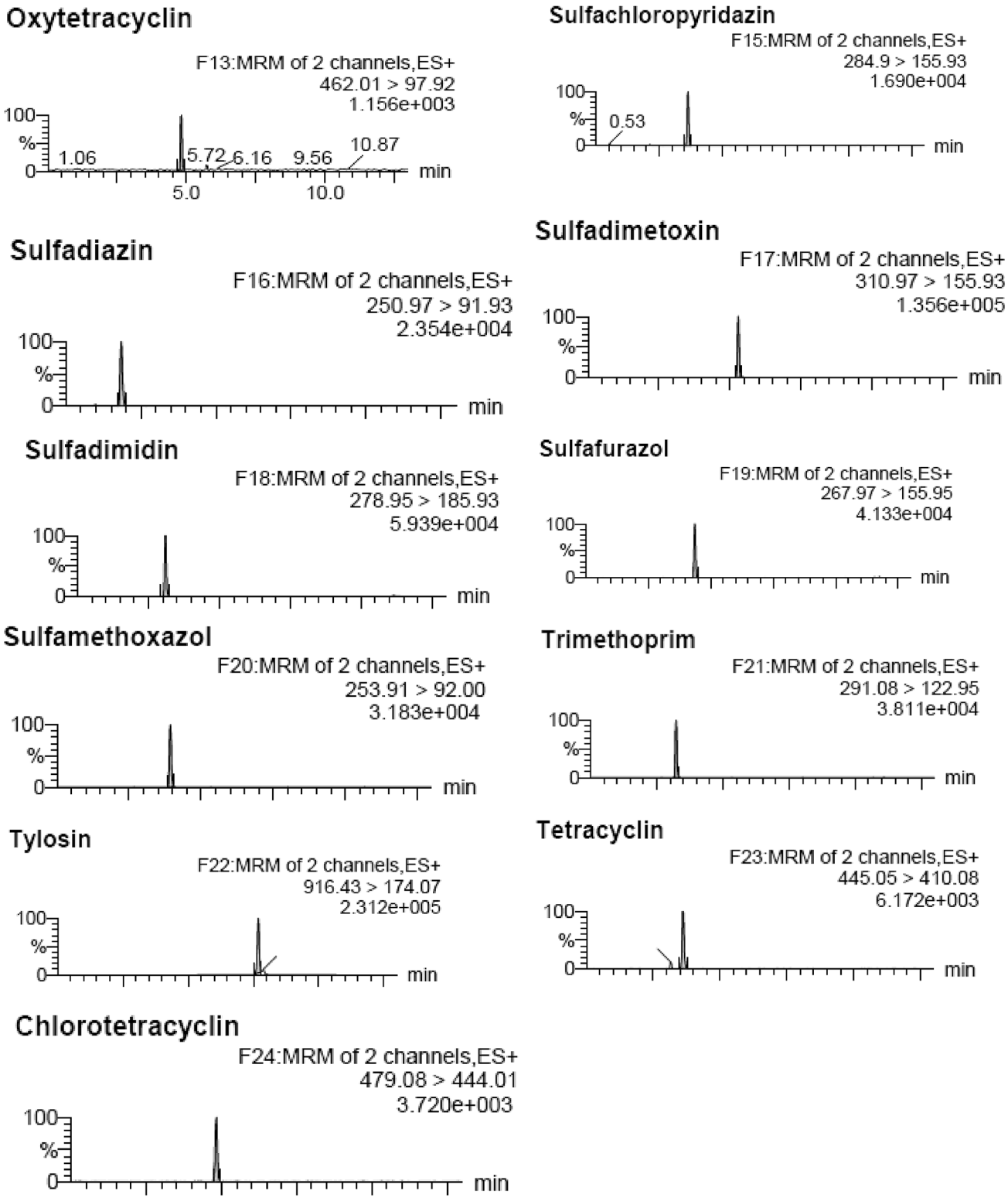

Fig. 1 (continued)

Table 4 CCα, CCβ, MRL for antibiotics in bovine milk, linearity of the method (R^2)

No.	Antibiotic	LOD (ng/ml)	LOQ (ng/ml)	CCα (ng/ml)	CCβ (ng/ml)	R^2	MRL (ng/ml)
1	Amoxicillin	0.23	0.76	4.43	4.88	0.9941	4
2	Ampicillin	0.29	0.98	4.49	4.92	0.9831	4
3	Benzylpenicillin	0.17	0.50	4.58	5.10	0.9900	4
4	Cloxacillin	2.14	7.06	33.77	36.83	0.9834	30
5	Oxacillin	1.78	5.87	32.31	35.58	0.9800	30
6	Trimethoprim	2.36	7.80	54.45	59.63	0.9846	50
7	Tylosin	2.57	8.48	54.97	61.70	0.9991	50
8	Cephapirin	3.24	10.70	68.73	75.78	0.9848	60
9	Cefalexin	5.23	17.25	111.28	125.88	0.9804	100
10	Ceftiofur	4.46	14.72	109.83	123.86	0.9917	100
11	Enrofloxacin	5.46	18.04	113.17	121.50	0.9817	100
12	Ciprofloxacin	4.01	13.23	107.12	111.35	0.9946	100
13	Tetracyclin	4.12	13.60	115.34	121.57	0.9880	100
14	Oxytetracyclin	5.32	17.54	109.46	115.23	0.9801	100
15	Chloroteracyclin	6.86	22.23	106.14	109.44	0.9874	100
16	Doxycyclin	5.51	18.18	122.33	139.78	0.9940	100
17	Lincomycin	6.31	20.82	114.22	129.54	0.9877	100
18	Sulfachloropyridazine	5.15	17.00	104.13	108.22	0.9936	100
19	Sulfafurazol	3.98	13.13	107.33	115.47	0.9991	100
20	Sulfadiazine	5.56	18.31	106.22	112.00	0.9974	100
21	Sulfadimidin	6.94	22.71	117.38	134.66	0.9964	100
22	Sulfamethoxazole	4.22	14.05	112.36	124.33	0.9935	100
23	Sulfadimetoxin	5.92	19.55	109.22	118.15	0.9812	100

minimize matrix effect [12, 17]. The results for coefficient of correlation varied from 0.9800 (oxacilin) to 0.9991 (tylosin and sulfafurazol) (Table 4). It can be concluded that the coefficient of correlation for all standards was satisfactory and the method was linear.

3.2 Selectivity

For evaluation of the selectivity of the method 20 blank bovine milk samples were analyzed. In all tested blank milk samples the interfering peaks were not detected at the retention times of the target antibiotics. A good separation of peaks without interferences and overlapping between peaks of the analytes, reduce matrix effect and minimize the risk of false positive results [11].

3.3 Sensitivity

The LOD was estimated for a S/N of 3 from the chromatograms of spiked milk samples at the concentration 0.05*MRL. Similarly, the LOQ was determined for a S/N of 10. The data in Table 3 show that the results for LOD and LOQ are lower that the MRL. LOD are in the range of 0.17–6.94 ng/ml, while the LOQ are in the range of 0.50–22.71 ng/ml.

3.4 Critical concentrations CCα and CCβ

CCα values for all antibiotics were determined with fortification of 20 blank bovine milk samples with antibiotic standards at the MRLs values, while CCβ were determined with fortification of 20 blank bovine milk samples with antibiotic standards at the CCα values (the MRLs for all antibiotics are given in Table 4).

The calculation of CCα and CCβ was according to the criteria for substances with established permitted limit (MRL) prescribed in Commission Decision 2002/657/EC. The CCα was calculated as the concentration at the MRL plus 1.64 times the corresponding standard deviation equal the decision limit, while the CCβ was calculated as the value of the decision limit plus 1,64 times the corresponding standard deviation equals the detection capability (2002/657/EC).

Obtained results for CCα and CCβ are summarized in Table 4. The results indicated that the method is relevant and reliable for determination of antibiotics in milk samples.

Table 5 Recovery of the four extraction solvents (acetonitrine, methanol, acetonitrile:methanol (50:50), 20% trichloroacetic acid and McIlvaine buffer)

Antibiotic	Added concentration (ng/mL)	Aceto-nitrile	Metha-nol	Acetonitrile:methanol (50:50)	20% trichloroacetic acid and McIlvaine buffer
Amoxicillin	2.0	74.56	75.26	85.15	73.00
	4.0	71.22	70.48	72.11	81.25
	6.0	78.13	70.56	104.15	80.33
Ampicillin	2.0	81.34	72.11	91.36	76.15
	4.0	87.84	75.88	84.13	94.50
	6.0	75.30	71.13	97.22	86.17
Benzylpenicillin	2.0	78.34	72.68	108.24	71.96
	4.0	95.15	74.35	103.15	85.25
	6.0	81.35	77.18	88.64	74.33
Cloxacillin	15	71.65	71.36	88.35	83.20
	30	77.21	70.18	92.44	95.40
	45	80.15	78.25	79.18	85.51
Oxacillin	15	81.36	69.36	91.90	86.40
	30	88.54	74.18	101.36	87.53
	45	91.15	72.11	88.14	80.36
Trimethoprim	25	90.12	71.45	91.36	107.23
	50	81.45	77.12	88.88	95.93
	75	88.35	75.14	94.17	102.37
Tylosin	25	71.34	82.55	90.36	92.46
	50	75.14	89.78	81.45	97.88
	75	70.34	81.20	85.15	95.43
Cephapirin	30	81.26	71.22	75.44	83.08
	60	74.13	74.15	81.33	91.88
	90	85.14	72.18	81.54	90.77
Cefalexin	50	84.56	72.15	94.13	83.50
	100	91.45	78.14	77.87	88.38
	150	86.18	70.46	79.14	88.10
Ceftiofur	50	84.36	78.46	97.88	92.30
	100	89.15	77.15	91.46	87.46
	150	80.32	70.26	92.18	94.10
Enrofloxacin	50	78.61	68.34	82.17	97.54
	100	75.14	70.25	77.46	92.54
	150	77.22	73.14	85.12	96.77
Ciprofloxacin	50	81.36	70.36	88.36	85.76
	100	79.54	72.55	81.55	84.16
	150	76.33	69.34	74.13	90.81
Tetracyclin	50	29.34	26.38	34.41	81.56
	100	31.15	22.15	31.48	84.15
	150	26.54	24.36	37.89	80.89
Oxytetracyclin	50	15.22	22.11	31.46	83.56
	100	18.17	24.13	24.15	88.25
	150	21.35	27.32	22.45	97.43
Chloroteracyclin	50	14.36	12.36	22.15	84.88
	100	21.48	15.46	29.64	102.15
	150	21.55	17.13	20.78	98.32

Table 5 (continued)

Antibiotic	Added concentration (ng/mL)	Aceto-nitrile	Metha-nol	Acetonitrile:methanol (50:50)	20% trichloroacetic acid and McIlvaine buffer
Doxycyclin	50	22.45	21.48	39.40	86.78
	100	17.34	17.35	38.42	89.12
	150	18.55	23.44	36.15	95.03
Lincomycin	50	81.36	88.36	82.54	105.08
	100	82.15	78.48	85.46	88.46
	150	91.35	81.36	90.17	102.88
Sulfachloropyridazine	50	91.36	70.12	81.84	108.70
	100	92.54	74.36	85.86	97.88
	150	87.46	72.14	79.12	102.09
Sulfafurazol	50	92.36	75.76	81.88	95.56
	100	98.77	81.38	85.14	92.15
	150	102.15	80.46	82.17	94.22
Sulfadiazine	50	91.94	70.13	80.14	84.88
	100	90.15	72.15	81.56	97.48
	150	79.14	69.40	89.14	96.77
Sulfadimidin	50	90.36	71.38	75.14	100.92
	100	92.11	70.32	79.36	108.74
	150	87.56	73.18	71.22	99.43
Sulfamethoxazole	50	103.18	75.46	80.25	89.32
	100	105.23	78.23	74.33	82.14
	150	92.11	81.14	77.18	91.68
Sulfadimetoxin	50	75.22	74.22	81.33	85.50
	100	81.36	79.46	75.17	98.14
	150	80.12	71.55	81.46	89.57

3.4.1 Accuracy and precision

The accuracy and precision of the method, in the absence of certified reference material, were determined with fortification of blank milk samples at concentration levels of 0.5, 1.0 and 1.5 times the MRLs (the MRLs for all compound are given in Table 4).

The blank bovine milk samples were spiked at 18 replicates (6 replicates per level) and carried out in three different days, with different operators. The spiked milk samples were allowed to equilibrate for 20 min before extraction procedure. Recovery of the method varied between 71.96% (for benzylpeniciline at concentration of 2.0 ng/ml) and 108.70% (for sulfachloropyridazine at concentration of 50 ng/ml). The results for the recovery were in the accepted range in Commission Decision 2002/657/EC (Table 5).

The precision of the method was expressed as coefficient of variation (CV). The CV for repeatability varied from 1.08% (tylosin at concentration of 50 ng/ml and lyncomicin at concentration of 100 ng/ml) to 20.28% (amoxicillin at concentration of 2.0 ng/ml), while the CV for reproducibility varied from 3.14% (lyncomicin at concentration of 100 ng/ml) to 22.88% (oxytetracycline at concentration of 50 ng/ml). The results for CV did not exceed the acceptable values from the Horwitz equation. They are in compliance with the requirements from Commission Decision 2002/657/EC (2002/657/EC): the results for precision demonstrates a good repeatability and reproducibility of the method. The results for accuracy and precision are summarized in Table 6.

The validation results give a clear indication of the suitability of the detection and identification of the several classes of antibiotics in milk and the method can be used in routine practice for detection of antibiotics in milk samples.

3.4.2 Applicability

To evaluate the applicability of the method, 189 raw bovine milk samples were collected and analyzed. A total of 14 (7.41%) samples were found positive for antibiotics and sulfonamides. Detected antibiotic residues, number and percentage of positive samples, concentration ranges,

Table 6 Accuracy and precision of the method

Antibiotic	Added concentration (ng/mL)	Recovery (%)	Repeatability (CV_r, %)	Reproducibility(CV_R, %)
Amoxicillin	2.0	73.00	20.28	22.17
	4.0	81.25	8.14	10.56
	6.0	80.33	12.46	14.78
Ampicillin	2.0	76.15	19.24	21.23
	4.0	94.50	15.36	17.66
	6.0	86.17	3.54	6.12
Benzylpenicillin	2.0	71.96	13.46	17.11
	4.0	85.25	8.12	12.03
	6.0	74.33	12.56	16.04
Cloxacillin	15	83.20	17.45	22.17
	30	95.40	15.22	19.14
	45	85.51	6.12	9.66
Oxacillin	15	86.40	8.14	11.14
	30	87.53	12.06	18.05
	45	80.36	7.08	13.55
Trimethoprim	25	107.23	2.06	7.11
	50	95.93	4.40	9.12
	75	102.37	4.20	8.66
Tylosin	25	92.46	1.08	4.06
	50	97.88	3.02	7.12
	75	95.43	4.06	9.88
Cephapirin	30	83.08	17.45	20.99
	60	91.88	12.55	17.48
	90	90.77	10.32	15.11
Cefalexin	50	83.50	12.02	15.36
	100	88.38	15.06	21.12
	150	88.10	8.46	13.51
Ceftiofur	50	92.30	18.22	22.88
	100	87.46	12.03	17.14
	150	94.10	11.06	15.22
Enrofloxacin	50	97.54	3.04	6.87
	100	92.54	2.16	6.02
	150	96.77	5.12	11.64
Ciprofloxacin	50	85.76	7.08	11.45
	100	84.16	4.06	8.18
	150	90.81	7.55	11.08
Tetracyclin	50	81.56	15.06	20.05
	100	84.15	14.38	21.13
	150	80.89	6.14	9.12
Oxytetracyclin	50	83.56	20.09	22.88
	100	88.25	18.46	21.14
	150	97.43	11.38	15.11
Chloroteracyclin	50	84.88	16.22	19.54
	100	102.15	15.46	19.68
	150	98.32	9.18	14.02
Doxycyclin	50	86.78	8.04	11.56
	100	89.12	13.41	20.11
	150	95.03	7.15	13.51

Table 6 (continued)

Antibiotic	Added concentration (ng/mL)	Recovery (%)	Repeatability (CV_r, %)	Reproducibility(CV_R, %)
Lincomycin	50	105.08	4.03	9.12
	100	88.46	1.08	3.14
	150	102.88	2.15	7.15
Sulfachloropyrida-zine	50	108.70	6.15	12.53
	100	97.88	6.22	10.66
	150	102.09	8.14	14.02
Sulfafurazol	50	95.56	12.56	15.21
	100	92.15	7.13	9.14
	150	94.22	9.56	13.51
Sulfadiazine	50	84.88	3.02	7.08
	100	97.48	3.04	10.21
	150	96.77	1.12	4.16
Sulfadimidin	50	100.92	3.18	8.81
	100	108.74	5.66	9.15
	150	99.43	2.02	6.64
Sulfamethoxazole	50	89.32	12.26	17.78
	100	82.14	9.15	14.46
	150	91.68	7.78	13.11
Sulfadimetoxin	50	85.50	3.15	5.88
	100	98.14	6.66	10.14
	150	89.57	5.81	10.08

LOQ and MRL's for detected antibiotics and sulfonamides are presented in Table 7. The concentration of the residues were above LOQ, but below the maximum levels established by EU. The obtained results are in agreement and comparable with the data, published in other scientific studies [3, 14, 17, 25]. The study from Jayalakshmi et al. [14] is a review that describes the detected antibiotic residues in animal products (milk, muscle, liver, kidney, diaphragm), with different techniques, from different authors. Martins et al. [17], used the LC–MS/MS method for the detection of antibiotics in bovine milk, but sample preparation is different for each class of antibiotics. The authors used three types of extraction procedures (first extraction procedure for quinolones and fluoroquinolones, second for sulphonamides, trimethoprim, bromhexine and third extraction procedure for tetracyclines). Also, the extraction procedures are different from the present study. Moreover, the study didn't include the β-lactams, tylosin, lincomycin and cephalosporines.

The different extraction procedures, with EDTA–Na2 and ethanol–acetonitrile (1:5, v/v), for determination of veterinary drug residues and other contaminants in raw milk by LC–MS/MS method are published from Zhan et al. [25]. Also, in this study aren't included ampicillin, benzylpenicillin, cephalexin and ceftiofur. Bilandžić et al. [3] published the study for concentrations of veterinary drug

Table 7 Results from routine analysis of milk samples

Antibiotic residues	Number of positive samples(n = 189)	% of positive samples	Concentration range (ng/ml)	LOQ (ng/ml)	MRL (ng/ml)
Oxytetracycline	1	0.53	20.06	17.54	100
Ceftioflur	1	0.53	23.55	14.72	100
Ciprofloxacin	1	0.53	15.38	13.23	100
Sulfametoxazol	1	0.53	21.67	14.05	100
Tetracycline	2	1.06	15.90–28.01	13.60	100
Doxycicline	2	1.06	24.14–48.86	18.18	100
Tylosin	2	1.06	18.09–45.02	8.48	50
Sulfadimetoxin	4	2.12	34.96–36.22	19.55	100

residues in milk in Croatia. The authors analyzed several classes of antibiotics with the Immunoassay method and they use 7 different Enzyme Immunoassay (EIA) kits. Each kit is specific to one antibiotic or one class of antibiotics. The sample preparation step is different for all EIA kits.

4 Conclusion

In this study accurate, precise and sensitive multi-class LC–MS/MS method for simultaneous determination of 23 veterinary drug residues from seven different classes of antibiotics in bovine milk was developed and validated.

In the beginning the LC–MS/MS condition and extraction procedure were optimized. After that, the method was completely validated according to the criteria prescribed in Commission Decision 2002/657/EC.

The validation results for the linearity, selectivity, LOD, LOQ, CCα, CCβ, accuracy and precision are in accordance with criteria established in this document.

According to the validation results it can be concluded that the method is applicable for routine analysis of antibiotic residues in bovine milk samples.

Consequently, the method was successfully applied in routine analysis of 189 raw milk bovine samples.

The antibiotics were detected in a total of 14 (7.41%) bovine milk samples, but the concentration was below MRL.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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