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Original Scientific Paper

Determination of cephalosporin antibiotic residues in milk using liquid chromatography – tandem mass spectrometry (LC – MS/MS) method

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Abstract

The possible presence of cephalosporin residues in milk is one of the key issues for food safety which arouses great public concern. When these classes of drugs are retained in food stuff, this may result in allergic or toxic reactions, alterations in the intestinal flora, bacterial resistance and the inhibition of fermentation in the dairy industry. A modest liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous determination of 3 cephalosporins residues (cephapirin, cefalexin, ceftiofur) in milk. The method was validated in accordance with the criteria defined in Commission Decision 2002/657/EC.

Samples are initially extracted with EDTA-Mcllvain buffer and after that solid-phase extraction clean-up with Oasis HLB cartridge was performed. Chromatographic separation was achieved using a C18 column. From the gained results from the validation study, it can be concluded that the method was linear with coefficient of correlation (R^2) from 0.9812 to 0.9896. Detection capabilities (CCβ) ranged between 10.27 and 11.97 µg/L and decision limits (CCα) ranged between 9.59 and 12.39 µg/L. The recovery values ranged from 84.53 to 95.70 % with relative standard deviations (RSD) no larger than 17.25 %. This validation study provided evidence that the method it's suitable to be applied in routine analysis for the detection of cephalosporin residues in milk.

Keywords: cephalosporin residues, milk, validation, LC-MS/MS method.

1. Introduction

Cephalosporins are semi-synthetic ß-lactam antibiotics, consisting of a six-membered dihydrothiazine ring fused with a four-membered ß-lactam ring which is responsible for the biological activity of the compounds, are shown in Figure 1. Nowadays, five generations of cephalosporins are distinguished reflecting their spectrum of activity, structural similarity and time of introduction. Cephalosporins are highly effective antibiotics in the treatment of bacterial infections of the respiratory tract (Hornish, 2002; Khaskheli, *et al*., 2008; Etebu, & Arikekpar, 2016).

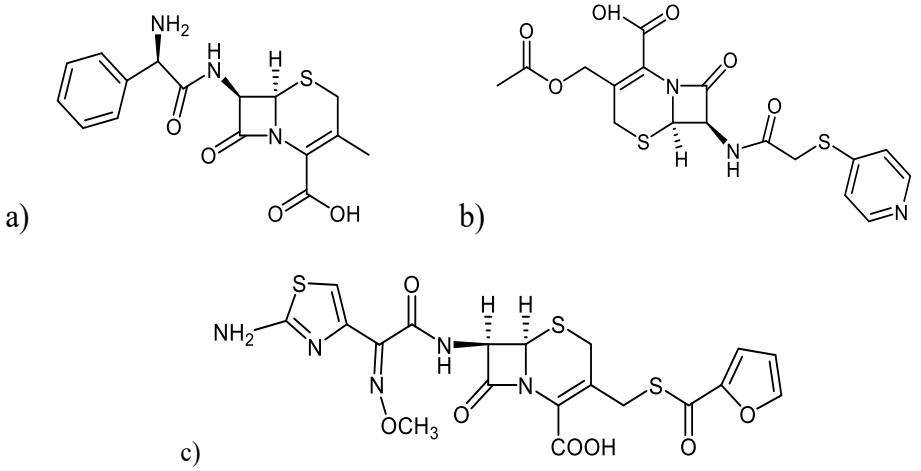

Figure 1. The chemical structure of cephalosporin antibiotics
a) Cefalexin b) Cephapirin c) Ceftiofur

Antimicrobials that can act on the bacterial cell wall: these interfere with the synthesis of the peptidoglycan layer in the cell wall; they eventually cause cell lyses, bind to, and inhibit the activity of enzymes responsible for peptidoglycan synthesis. They interfere with cell wall synthesis by binding to penicillin binding proteins (PBPs), which are located in bacterial cell walls (Bogialli, & Corcia, 2009; Stolker, *et al.*, 2005). The intrinsic antimicrobial activity of natural cephalosporins is low, but the attachment of various R1 and R2 groups has yielded drugs of good therapeutic activity and low toxicity. The excessive and inconsiderate use of antibiotics may lead to the occurrence of drug residues in milk, bearing risk to human health, since they can cause allergic reactions in hypersensitive individuals, or they may result in drug-resistant bacteria, toxicity, teratogenicity, and carcinogenicity (Nirala, *et al.*, 2010; Abdul Rathe, & Al-Shaha, 2017). Therefore, the European Union has established maximum residue levels (MRLs) for most antibiotics in milk and animal tissues. In the EU, the maximum residue limit (MRL) for antibiotics is established according to (EU) 37/2010. Council Directive 96/23/EC contains guidelines for controlling veterinary drug residues in animals and their products with detailed procedures (Council Directive 96/23/EC). Decision 2002/657/EC also establishes the method for the determination of these compounds in milk and animal tissues (Commission Regulation (EU) 37/2010, Commission decision: 2002/657/EC). According to these requirements, the Macedonian legislation was fully aligned with the EU legislation concerning residues of antibiotics in foodstuffs of animal origin in regarding the Council regulation 37/2010/EU (Official gazette of RNM 80/2011). Thus, the antibiotic residues' analysis is important to guarantee food safety. Screening methods are economical, easy to use and have high sample through-put, but are characterized by low sensitivity and low specificity (Ibraimi, *et al*., 2013; Ishraga, *et al.,* 2016; Dimitrieska, *et al.,* 2011). Confirmatory methods have high specificity and provide full information for analysis of antibiotic residues in milk when it is coupled with mass spectrometers (Alija, & Hajrullai-Musliu, 2016; Bohm, *et al.,* 2009; Ortelli, *et al.*, 2009; Karageorgou, *et al.,* 2018; Clark, *et al.,* 2011; Gaugain-Juhel, et al., 2009; Stolker, *et al*., 2008; Bladek, *et al*. 2011; Marilena, 2015; Zhang, *et al*., 2015)
The aim of this study was to develop and validate of LC-MS/MS method for the detection of cephalosporin antibiotics residues in milk.

2. Material and methods

2.1 Chemical, reagents and solutions

Antibiotic standards: Cefalexin, Ceftiofur, Cephapirin were supplied by Fluka-Vetranal.
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, formic acid, dim-ethyl sulfoxide (DMSO), ammonium hydroxide, sodium chloride, citric acid monohydrate were of analytical grade from Sigma-Aldrich, formic acid from Helix pomatia.

2.2. Standard solutions

For all substances, individual stock standard solutions concentration of 1 mg/mL were prepared in methanol and kept at –20 °C and Ceftiofur a mixture of methanol and DMSO (9:1 v/v).
Mixtures of working standard solution from 1, 10, and 100 ngmL -1 of all antibiotics were prepared in the mobile phase. Calibration (working) mixed standard solutions with concentration levels 10, 25, 50, 75, 100, and 150.00 ng/mL were obtained from the intermediate solution at 1, 10 and 100 ng/mL.

2.3 Sample preparation

An aliquot of 5 mL of milk added 2 mL of 20% trichloroacetic acid in water and shaken for 5 min. After shaking, 20 mL of McIlvaine buffer (11.80 g of citric acid monohydrate; 13.72 g of disodium hydrogen phosphate dehydrate; 33.62 g ethylene-diamine-tetra-acetic acid disodium salt diluted in 1 liter of water; 0.01

M; pH 3.5). The samples were vortexed for 1 minute and centrifuged at 4000 rpm for 20 min at +4 °C. The supernatant was immediately applied to an SPE HLB Oasis cartridge, previously activated with 3 mL of methanol and 2 mL of water, washed with 4 mL of water and dried for 20 min. while drawing a full vacuum. Antibiotic residues were eluted with 3 mL of methanol. The samples were evaporated to dryness under a stream of nitrogen at 35 °C. The dry residue was reconstituted with 250 mL of the mobile phase and filtered on a 0.22 μm micro filter and 10 μL of the final extract was injected into LC-MS/MS system.

2.4 LC-MS/MS Analysis

The LC-MS/MS system was purchased from Waters. The LC system consisted of a vacuum degasser, thermostated autosampler and thermostated column manager and a binary pump. A triple quadripole mass detector (MS/MS measurements) set in a positive ESI mode was used for the detection of the antibiotics. Instrument control and data processing were carried out by means of Masslynx 4.1 software (Waters). For solid phase extraction were used OASIS® HLB cartridges 3cc (60 mg). The quadruple instrument was operated in the positive ion mode under the following conditions: 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. The values of collision energy, transitions for the SRM mode, are given in Table 1.

Table 1. The MS / MS conditions

Type of ionization	ES+	Desolvatation gas flow (L/Hr)	400
Capilary (kV)	4.0	LM 1 resolution	11
Cone (V)	26	HM 1 resolution	14.7
Extractor (V)	3.0	Ion energy 1	0.5
RF Lens (V)	0.1	Entrance	50
Source temperature °C	150	LM 2 resolution	10.0

2.5 LC-MS/MS Conditions

The mobile phase A: aqueous solution with 0.1% of formic acid (0.1% HCOOH / H2O) and mobile phase B was acetonitrile with 0.1% of formic acid (0.1% HCOOH / CH3CN). The best results were observed at 40 °C, 0.4 mL/min as the flow rate, 10 μL volume of injection and the autosampler temperature was 10°C. To achieve good partitioning of the analyzed components, the method was optimized under laboratory conditions; they are given in Table 2.

Table 2. The liquid chromatography gradient elution method

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

2.6 Method Validation

The method was validated using the regulatory guidelines from the Commission Decision 2002/657/EC. According to those requirements, specificity, recovery, repeatability, reproducibility, decision limit (CCα) and detection capability (CCβ) were determined.

The linearity of the method was determined by the coefficient of correlation (R^2) from the calibration curves for each component. For the determination of CCα and CCβ the milk was enriched with the standards below the MRL value, and during this were prepared 18 replicates. The CCa equals the corresponding concentration at the intercept plus 2.33 times the standard deviation of the intra-laboratory reproducibility of the intercept. CCβ was calculated as the decision limit CCα plus 1.64 times the corresponding standard deviation (β = 5%), supposing that the standard deviation at the MRL is similar to that obtained at the CCα level. The recovery of an analytical method is defined as the parameter that measures the efficiency that the method has in the analytes extraction process. The precision of the method was evaluated in terms of repeatability (single day - intraday precision) and intermediate precision (different days - interday precision).

3. Results

The LCMS/MS method for the simultaneous determination of 3 cephalosporin antibiotics residues in milk samples was developed.

The analytes were extracted with solvent trichloroacetic acid 20% and Na_2EDTA-McIlvaine buffer followed by an SPE Oasis HLB cartridge clean-up procedure. Chromatographic separation was achieved using a C18 column (50 x 2.1 mm, 1.7µm) and a mobile phase composed by acidified with 0.1% formic in acetonitrile and 0.1% formic in water. The results were given in Table 2. ESI mass spectra were initially analyzed by LC-MS with direct injection 10 µL into the column to select the monitoring ion for each compound. All 3 analytes were measured in MRM mode. We monitored one precursor ion and two product ions for each analyte. The results are presented in Table 3.

Table 3. Parameters of MRM condition and retention times of cephalosporin antibiotics

Compound	Formula/Mass		Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode	Retention time
Cefalexin	347.4+H^+=348.4	1 2	347.99 347.99	22 22	157.81 173.93	81 61	ES+	2.97
Ceftiofur	523.5+H^+=524.5	1 2	523.96 523.96	34 34	241.0 4 125.17	16 58	ES+	4.84
Cephapirin	423.4+H^+=424.4	1 2	423.99 423.99	24 24	291.99 151.97	16 30	ES+	2.40

The obtained data confirmed that the methods were appropriate for detection of residues at the concentration level of interest. Linear calibrations were established for all the analytes and the calibration curves showed excellent linearity in the wide range with correlation coefficient (R^2) of 0.9812 to 0.9896. The decision limits CCα were from 9.59 µg/l to 12.39 µg/L and detection capability CCβ varied within the range from 10.27 µg/ L to 11.97 µg/ L. The results are presented in Table 4.

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

Nr.	Antibiotic	CCα µg/L	CCβ µg/L	R^2	MRL µg/L
1	Cephapirin	10.5	11.25	0.9848	60
2	Cefalexin	9.59	10.27	0.9812	100
3	Ceftiofur	12.39	11.97	0.9896	100

The recoveries of all analyses were 84.53 to 95.70 % with relative standard deviations RSD < 17.25 %. The results are presented in Table 5.

Table 5. Validation parameters

Nr.	Antibiotic	Level of spike ng/mL	Recovery %	RSD_r %	RSD_R %
1	Cephapirin	50	84.53	2.69	1.62
2	Cefalexin	50	92.70	8.79	9.23
3	Ceftiofur	50	95.70	14.54	11.44

4. Discussion

Solvent trichloroacetic acid and Na_2EDTA-McIlvaine buffer were found to be sufficiently effective for the extraction of cephalosporin antibiotics and further SPE cleanup was performed using an Oasis HLB cartridges (Karageorgou, *et al*., 2013; Martins-JúniorI, *et al.* 2007; Freitas, *et al.,* 2013). The extraction with solution of TCA, compounds from all tested classes of antibiotics were isolated with variable recovery (Junza, *et al*., 2009; Gaugain-Juhel, *et al*., 2009; Bladek, *et al*., 2011). Mcllvain buffer is widely used in aqueous extraction to minimize interaction with chelating complexes and for the isolation of the selected residues from milk (Tomáš, *et al*., 2016). For determination of precursor ion and product ions for each analyte and a full-scan spectrum was collected in scan mode for the mass range *m/z* 50-1000. The mass spectrometer was operated in electro spray positive ionization mode (ESI+) (Marilena, 2015; Ortelli, *et al*., 2009; Stolker, *et al*., 2008; Zhang, *et al*., 2015).

The calibration curves for detection of the analytes were obtained by performing a linear regression analysis showed good correlation with R^2 from 0.9812 for Cefalexin to 0.9896 for Ceftiofur on samples spiked with the analytes before the extraction. Linearity of the method was performed according to Decision 2002/657/EC. For the calculation of CCα and CCβ, all compounds were treated as banned substances since the goal of the developed method is to quantitatively screen for cephalosporin antibiotics in the lowest possible level. The CCα ranged from 9.59 µg/l to 12.39 µg/L, and the CCβ ranged from 10.27 µg/ L to 11.97 µg/ L. Results were very satisfactory for cephalosporin antibiotics which the CCβ was assessed to be above the MRL for the chosen transitions. Maximum residue level (MRLs) is the maximum concentration of residue resulting from the use of medicinal product that may be legally permitted or recognized as acceptable in or on a food, allocated to individual food commodities.

The recovery values ranged from 84.53 to 95.70 % with relative standard deviations (RSD%) no larger than 17.25 %. The obtained recovery values in our study were very satisfactory for all substances according to Commission Decision 2002/657/ EC (Commission Decision 2002/657/EC). The absence of any interfering

peaks around the analytes and retention times demonstrated the selectivity of the clean up procedure. No results were obtained above 21%, which represents a significantly lower value when compared with the criteria value accepted by the Horwitz equation (Commission Decision 2002/657/EC).
In the study of Magda *et al*., 2016, EDTA buffer was used to extract residues where the recovery values were obtained from 62% to 108%. Extraction with Na_2EDTA-McIlvaine buffer was described by Barbara *et al*., 2018, were providing recovery rate up to 200% and high standard deviations (up to 60%).
Amatya (2010) was determine simultaneously 19 drugs of three classes (seven Penicillins and Cephalosporins, and five Quinolones) in cow milk using LC-MS/MS. NaH_2PO_4 was used to extract residues. SPE clean up and concentration was done using OASIS HLB cartridge, where the recovery values were obtained from 38% to 117%. The CCα ranged from 4.6 $\mu g/kg^{-1}$to 112 $\mu g/kg^{-1}$, and the CCβ ranged from 5,3-124 µg/kg-1.
The results obtained for validation parameters for the method in our study corresponded to the literature data by Bohm, *et al*. 2009, Ortelli, *et al.* 2009, Karageorgou, *et al.* 2018, Zhang, *et al*. 2015; Junza, *et al*. 2014.

5. Conclusion

In conclusion, the LC-MS/MS method was sensitive and suitable for qualitative and quantitative analysis of cephalosporin antibiotics residues in milk. Sample preparation techniques and a mass spectrometry method were optimized for confirmatory purposes. The method was successfully validated according to the European Union requirements, and will be used for routine control of antibiotic drugs in milk. Monitor of antibiotics residues is necessary to ensure food safety and to prevent exposure of the consumers.

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