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DETERMINATION OF AMOXICILLIN AND SULFADIMIDIN RESIDUES IN MILK WITH LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS) USING LIQUID-LIQUID EXTRACTION (LLE)

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Abstract

Antibiotics and sulphonamides are widely used in animal production to prevent (prophylactic use) and treat (therapeutic use) diseases as well as to promote growth. However, all drugs that are administered to food-producing animals may lead to residues in the edible tissues, milk, or eggs. The presence of these residues may pose potential health risks, including allergic reactions, direct toxic effects, carcinogenic effects and a change in the resistance of bacteria exposed to antibiotics.

Due to harmful effects of residues, legislation regarding the control and to monitor these residues in food of animal products are given in EU Council Regulation 37/2010/EU. The aim of this study is to optimize and validate the LC-MS / MS method for residues analysis of Amoxicillin and Sulfadimidin in milk using Liquid-Liquid Extraction.

Milk samples, were collected during 2016. The extraction method was done according to the method of Liquid-Liquid Extraction. The optimization of chromatographic conditions and MS / MS detectors is achieved by a mix of standards. The chromatographic separation was achieved on a Kinetex® C18 column (1.7 μM 100 A, LC Column 50 x 2.1 mm) column followed by tandem mass spectrometry using an electro spray ionization source in positive mode. Validation of the method was performed according to Decision 2002/657/EC.

Optimization and validation process was carried out according to Commission Decision 2002/657/EC criteria. In validation process, decision limit (CCα), detection capability (CCβ), and linearity. The linear regression analysis showed good correlation with R^2=0.994 and R^2=0.996. The method of Liquid-Liquid Extraction was inappropriate because it was not possible to detected the residues of Amoxicillin. Method successful validation according to the European Union requirements and its application to samples demonstrated its efficiency for veterinary control of drugs in milk.

Keywords: Amoxicillin, Sulfadimidin, milk, optimization, validation, LC-MS / MS.

Introduction

Human health is related directly to the environment, and in particular the nature and quality of the food.

Antibiotics and sulphonamides drugs are chemical substances that suppress the growth of microbes and may eventually destroy them. The mechanism of action of antibiotics falls into following categories:

- Inhibitor of cell wall synthesis – bacitracin, cephalosporin, penicillin, vancomycin
- Inhibitor of cell membrane function – polymyxins
- Inhibitor of protein synthesis – aminoglycosides, chloramphenicol, lincosamides, macrolides, tetracyclines

- Inhibitor of nucleic acid synthesis– fluoroquinolones, metronidazole,rifampicin, sulfonamides, trimethoprim
- Inhibitor of enzymes involved in the synthesis of folic acid- sulfonamides, trimethoprim (H. Richard Adams Wiley 2001)

Antibacterial agents, are widely used in animal production to prevent (prophylactic use) and treat (therapeutic use) diseases as well as to promote growth (M. Khaskheli *et al* 2008)

Beta-lactam antibiotics are most commonly used for the treatmentof gram positive and gram negative bacterial infections. They have abroad spectrum of bactericidal action hampering the synthesis ofbacterial cell-wall.

Sulphonamides are among the oldest groups of antibiotic/antimicrobial agents. They are widely used in the treatment of bacterialdiseases of dairy cattle. They have a wide spectrum of bacteriostaticaction, affecting gram positive and gram negative organisms (Sulejmani Z. et al 2012)

However, all drugs that are administered to food-producing animals may lead to residues in the edible tissues, milk, or eggs. The presence of these residues may pose potential health risks, including allergic reactions, direct toxic effects, carcinogenic effects and a change in the resistance of bacteria exposed to antibiotics (Iran Mohammad Hosein Movassagh et al 2010, Seyda Ergin Kaya et al 2009).

Due to harmful effects of residues, legislation regarding the control and to monitor these residues in food of animal products. The European Union has established safe maximum residue limits (MRLs) for these drugs (Dimitrieska-Stojkovic Elizabeta et al 2011).

In the EU, the maximum residue limit (MRL) for antibiotics isestablished according to (EU) 37/2010. Decision 2002/657/EC also establishes the method for determination of these compounds in milk and animal tissues (Commission Regulation (EU) 37/2010, Commission decision: 2002/657/EC).

According with 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 Journal of RM 80/2011).

The aim of this study is to optimize and validate the LC-MS / MS method for residues analysis of Amoxicillin and Sulfadimidin in milk using Liquid-Liquid Extraction (LLE).

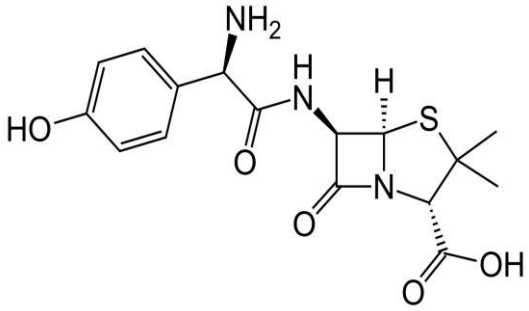
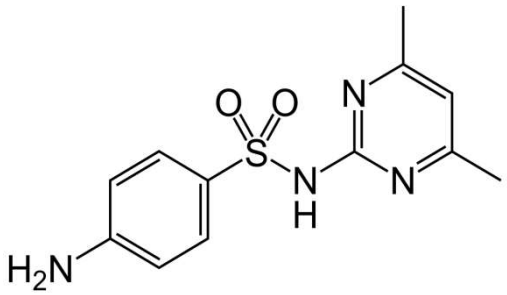
Materials and methods

2.1. Chemicals and reagents:

Methanol (LC-MS purity), acetonitrile (LC-MS purity), water (LC-MS purity), formic acid (0.5% HCOOH), dimethyl sulfoxide (DMSO), ammonium hydroxide (2M NH4OH), sodium chloride (NaCl)

2.2. Reference standards:

- Amoxicillin (A8523) – Sigma - 98%
- Sulfadimidin (46802) – Fluka – 99.8%

Amoxicillin (A8523) – Sigma - 98%	Sulfadimidin (46802) – Fluka – 99.8%
	

2.3. LS-MS / MS column

The chromatographic separation was achieved on a Kinetex®C18 (1.7µM100A, LC Column 50x2.1 mm) column followed by tandem mass spectrometry using an electro spray ionization source in positive mode.

2.4.Confirmatory LC-MS/MS method

LC-MS/MSis one of the most promising techniques for the analysis of antimicrobials in animal tissues, because it allows drug quantification and confirmation at trace levels.

2.5. Extraction of analytes by liquid-liquid extraction:

a) Transfer 2 ml of milk in 15 ml of PP tube
b) Add 5 ml of acetonitrile ACN
c) Vortex 1 min
d) Centrifuge for 5 minutes at 3000 rpm, at + 20ºC
e) Add 0.25 g NaCl
f) Ultrasonic bath for 5 minutes, vortex for 1 minute
g) Centrifuge for 5 minutes at 3000 rpm at + 20ºC
h) Take 100 µl, dry in N_2 flow and dissolve the residue with 500 µl mobile phase.
i) LC-MS/MS (Helio A. Martins-Júnior*et al 2007*)

2.6. Chromatographic conditions

The mobile phase A: aqueous solution containing 0.5% of formic acid (0.5% HCOOH / H2O), and mobile phase B was acetonitrile (CH3CN)). The best results were observed at 25°C, 0.4 mL/min as the flow rate, 10 µL volume of injection and the auto sampler temperature was 10°C (22). To achieve good partitioning of the analyzed components, the method was optimized under laboratory conditions.
Phase and flow ratios are given in Table 1.

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	60.0	40.0
11.0	0,4	0.00	100.0
13.0	0,4	0.00	100.0
13.1	0.4	98.0	2.0
17.0	0.4	98,0	2.0

Table 1. Chromatographic conditions

2.7. Optimization of the MS / MS method

In order to determine the retention time and the mass spectrum of antibiotics involved in this study, individual standards of these analytes with a concentration of 10 µg / mL were directly injected into the mass detector, whereby the mass spectrum of these analytes in the ESI + (positive ionic spectrum) and were determined main precursor ions, product ions (daughter ions) (R.W. Han et al 2015, Andreia Freitas et al 2012, Solutions Guide LC/MS

Applications for Drug Residues in Foods, Ramon Companyó Glenn Kennedyet al 1998, C. Nebot et al 2012, A. Iglesias Murielle Gaugain-Juhel).
The MS / MS conditions are given in Table 2.

Type of ionization	ES+	Desolvation gas flow (L/Hr)	500
Capillary (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

Table 2. MS/MS conditions

2.8.Validation procedure
The method was validated using the regulatory guidelines from the Commission Decision 2002/657/EC, concerning the performance of analytical methods.
In validation process, decision limit (CCα), detection capability (CCβ), linearity of the method were studied.
The linearity of the method was determined by the coefficient of correlation (R^2) from the calibration curves for each components.
For determination of CCα and CCβ the milk was enriched with the standards below the MRL value, and during this were prepared 18 replicates.
Antibiotics and sulphonamides were divided into two groups, and the concentration of the standards was determined depending on the MRL value. The calibration curves have six points and the concentrations were as follows: for the first group of 2-50 ng / ml, and for the second group of 50-200 ng / ml.

Results and discussion

3.1. Optimization of MS/MS method
Optimization of the MS / MS method and the standards injection into the MS detector was achieved ionic scanning and analyzed parameters were determined such as: precursor ions, daughter ions and retention time) that are presented in Table 3.

Compound	Formula/Mass		Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode	Retention time
Amoxicillin	365.4+H^+=366.4	1 2	367.07 367.07	28 28	159.96 90.89	16 40	ES+ ES+	5.06
Sulfadimidin-Sulfametazin	278.3+H^+=279.3	1 2	278.95 278.95	34 34	185.93 91.93	18 36	ES+ ES+	2.70

Table 3. Retention time, precursor and daughters ions

The results obtained for precursor ions, daughter ions, retention time correspond to the literature data by Andreia Freitas et al., Susan B. Clark et al., YanyanFangi et al. al., Rameshwari Amatya et al. (Susan B. Clark1 e al 2012, R.W. Han et al 2015, Andreia Freitas et al 2012, Yanyan Fang, Rameshwari Amatya et al 2010)

3.2. Linearity

The linear regression analysis showed good correlation with R^2 from 0.9941 for Amoxicillin and 0.9964 for sulfadimidin.

Linearity of the method was performed according to Decision 2002/657/EC

Compound	R^2
Amoxicillin	0,9941
Sulfadimidin	0,9964

Table 4. Linearity of the method

3.3.Determination of CCαand CCβ

Compound	Level of Spike ng / ml	The value obtained ng / ml	CCα (ng/ml)	CCβ (ng/ml)
Amoxicillin	2	Not detected	Not detected	Not detected
Sulfadimidin	50	42,38	47,02	49,46

Table 4. CCα ,CCβ

From the results shown in Table 5 it can be concluded the obtained value for CCα and CCβ was less than MRLs (Maximum Residue Limits).

The method of Liquid-Liquid Extraction (LLE) was inappropriate because it was not possible to detected the residues of Amoxicillin. In the future, the research will be continue using of the solid phase extraction method (SPE), which increases the sensitivity and selectivity for drug residue discovery and will be determined other validation parameters.

In Turkey, by Kaja and Filazi (2010) from all analyzed samples only 1.25% were found to have been contaminated with beta-lactam residues.

In raw milk samples from Germany by Kress 2007 have been reports on presence ofsulfonamides whereas the prevalence for sulfonamideswas 1,1 and 1,6 %.

Conclusion

Monitor of antibiotics and sulphonamides residues is necessary to ensure food safety and to prevent exposure of the consumers.

- Optimization and validation process of the LC-MS / MS method according to the European Union requirements to determination of antibiotic and sulfonamide residues in milk
- The linearity of the method was carried out according to Commission Decision 2002/657/EC criteria
- The obtained value for CCα and CCβ was less than MRLs (Maximum Residue Limits).
- The method of Liquid-Liquid Extraction was inappropriate because it was not possible to detected the residues of Amoxicillin
- In the future, the research will continue using of the solid phase extraction method and will be determined other validation parameters.
- Milk in Macedonia in average, contains low levels of antibacterial residues and it could be considered as safe for human consumption.

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