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Article

The Validation of a Method for the Determination of Chloramphenicol in Milk

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Abstract: The liquid chromatography tandem mass spectrometry (LC-MS/MS) method with the liquid-liquid extraction and SPE (Waters OASIS HLB column) cleaning process was validated for the determination of chloramphenicol (CAP) residues in milk. The optimisation of the mass spectrometry parameters was performed by injecting the CAP standard solutions into the electrospray ion (ESI) source. The mass spectrometer was operated in the negative ESI mode. The validation data fulfilled the requirements established in the Regulations Decisions 2020/657/EC. During the validation process the chloramphenicol-D5 as an internal standard was used. The obtained results indicated the good linearity ($R^2 > 0.99$) within the range of 0.1–2.0 μg/kg. The mean recovery for spiking levels was 96.5 ±10.59%. The limit of quantification (LOQ) was 0.1 μg /kg. The repeatability was 10.6%. The method fulfilled all the 2020/657/EC guidelines and thus can be extended for the routine analysis of CAP residues in milk.

Keywords: validation**;** chloramphenicol; LC-MS/MS; milk.

1. Introduction

The milk and dairy products are considered to be the essential components of a balanced human diet as they have tremendous nutritional values regarding a good proportion of proteins, fats and important minerals [1]. According to Puvača et al. [2], significant health benefits of milk and its products are related to the proteins, especially their nutritive value and biological properties.

In the veterinary practice the usage of antibiotics is extensive - from the treating of diseases and microbial infections to the dietary supplements [3]. Chloramphenicol (CAP), or the 2,2-dichloro-*N*-[(1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide, is a broad-spectrum antibiotic, isolated from *Streptomyces venezuelae*, which belongs to the amphenicol drug family and has been used through the history as a cure for various infections in the veterinary medicine. Its chemical structure comprises both the lipophilic and hydrophilic groups, as well as the substituents (Figure 1). Since the polar and nonpolar groups are present, it is soluble in organic solvents (acetone, ethanol, ethyl acetate and methanol) and slightly soluble in water [4].

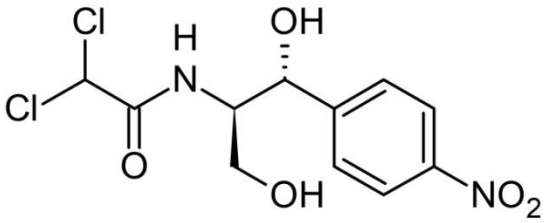

Figure 1. Structural formula of chloramphenicol

The first usage of the CAP was in the late 1940s and it considered the scrub typhus (Asia) and epidemic typhus (South America) treatment [5]. Considering the fact that it is affordable, available and effective in suppressing various microorganisms, since the 1950s it has been used all over the planet in veterinary treatments, including the food-producing animals [4]. However, it can negatively affect the humans, leading to the aplastic anemia, including the suspected dose-independent carcinogenicity. Its genotoxic effects led to its restrictions in many countries and the banning for use in food-producing animals within the European Union (EU) and the United States (US) [5]. After that, the minimum required performance limit (MRPL) was set at 0.3 μg/kg for the aquaculture products, eggs, honey, meat, milk and urine, according to the Council Directive 96/23/EC [6]. Even so, the unacceptable levels of the CAP residues are still found in imported food due to the illegal usage in order to boost the animal growth and cover up the inappropriate hygiene of the animal-raising farms [10]. During the last 12 years, the Rapid Alert System for Food and Feed (RASFF) reported 456 cases of the CAP incidents in numerous matrices, out of which 49 were the dairy products [7].

Since the safety concerns are rising, the intensive monitoring of the CAP in food products is necessary. The examination of the CAP residues is difficult due to the complex sample matrices, as well as the requirements considering the low limit of quantification and method validation [10]. The uncontrolled and unthoughtful antibiotics usage may lead to the drug residues detections in milk, which opposes a risk for human health due to their ability to induce allergic reactions in hypersensitive individuals, or to cause the development of drug-resistant bacteria. This speaks in favor of the importance of the antibiotic residues' analysis which must guarantee food safety [3].

Several techniques are used for the determination of the CAP residues in different matrices, including milk; the methods like ELISA [7], GC-MSD and GC-ECD [5], HPLC-DAD [8] and LC/MS/MS [9, 11]. For the CAP determination by the gas chromatography the derivatization of this antibiotic is a necessary procedure. This step is not required for the CAP determination by the liquid chromatography.

The accurate, sensitive and robust analytical methods are needed for monitoring and controlling the compliance of the zero tolerance level of CAP [12]. So, the aim of this study was to develop a sensitive validated method for determining CAP residues in milk samples according to the Commission Decision No. 2002/657/EC requirements [13].

2. Materials and Methods

2.1 Chemicals and reagents

Chloramphenicol, analytical standard purity >99% (Sigma-Aldrich), analytical standard of chloramphenicol-D5 (Sigma-Aldrich) used as an internal standard (IS), acetonitrile and methanol HPLC grade (J.T. Baker), formic acid analytical grade (J.T. Baker), ammonia 35% (J.T. Baker), ethyl acetate LC grade (J.T. Baker). NaOH, hexan, acetone, trifluoroacetic acid, di-natriumphosphat and citric acid monohydrate, all p.a (Merck).

CAP basic standard (1mg/mL): Measure 10 mg (±0,1mg) of CAP and dilute in 10 mL of methanol.

Intermediate CAP solution (10 μg/mL): 100 μL of basic CAP solution and add 10 mL of methanol.

Working CAP standard WS1 (50 ng/mL): 50 μL of intermediate CAP solution and add 10 mL of methanol.

Working CAP standard WS2 (5 ng/mL): 500 μL of WS1 and add 5 mL of methanol.

2.2 Validation parameters

The limit of quantification (LOQ) was set in accordance with the Council Directive 96/23/EC and MRPL of 0.3 μg/kg. The LOQ was 0.1 μg/kg.

The calibration was done at seven calibration levels (0.1, 0.15, 0.2; 0.3, 0.5, 1.0 and 2.0 μg/kg) (Table 1).

Table 1. Spiking sample procedure.

C ($\mu g/kg$)	**Sample (g)**	**V (μL) of WS2**	**V(μL) of WS 1**
0.1	2	40	
0.15	2	60	
0.2	2	80	
0.3	2	120	
0.5	2		20
1.0	2		40
2.0	2		80

The accuracy of the method was tested as a recovery; by spiking a blank sample in which no analyte of interest was found at four levels (0.1, 0.3, 0.5 and 1.0 μg/kg).

The precision of the method was examined as a relative standard deviation (RSD, %). The eligibility criteria are defined in the Regulations Decisions 2020/657/EC and 98/179/EC. The intralaboratory reproducibility (%RSD_R) was tested under repeatability conditions (%RSDr) with the same method, on the same samples, by two analysts in three days.

2.3 Sample extraction

Centrifugate the milk sample for 15 min at 6000 rpm/ 4 °C before the analysis. Pipette 2 mL of skim milk into a 50 mL PP cuvette, add 20 μl of IS and 100 μL 20% TCA and mix for 30s. Add 10 mL of McIlVaine buffer and mix for 10 min and centrifuge for 10 min at 6000 rpm/4 °C. After that, clean the sample by SPE columns (Waters OASIS HLB column). Condition the column with 6 mL of methanol and 6 mL of deionized water. Add 2 mL of filtered sample on the column. Wash the column with 6 mL of deionized water. Elute CAP with 6 mL of methanol. Evaporate the eluate under a stream of nitrogen at 40 ° C. Dissolve the residue in 500 μL of 15% methanol in water using vortex (10 min). The final extract was centrifuged for 10 min at 13,000 rpm/4 °C. Filter the supernatant into the autosampler vial.

2.4 Chromatohraphic conditions

An HP Agilent Technologies 1290 Infinity LC system equipped with an autosampler and coupled with an Agilent 6470B triple-quadrupole mass spectrometer (Agilent Technologies, US) with the Agilent Jet Stream ion source was used. The system was controlled by the MassHunter Workstation V.10.0. software from Agilent Technologies Inc 2006-2018. The liquid chromatography

separation was performed on a Zorbax Eclipse XDB-C18 column (10mmx2.1 mm, 1.8 μm) from Agilent Technologies.

The column temperature was maintained at 35 °C. The LC runs were performed with mobile phase A: methanol, and mobile phase B: water. The gradient used, ranged from 30 to 80% of mobile phase B in 4 min. Then the 80% of B mobile phase was kept during 4 min. Finally, the mobile phase was backed to the initial condition (30% of B) in 2 min, which was kept for 2 min, to condition the column for the next injection. The total chromatographic run time (with post-time) was thus 12 min. The injection volume was 10 μL and the operational flow rate was 0.3 mL min−1. The ionisation source settings were: gas temperature, 250 °C; gas flow, 13 Lmin-1, nebuliser pressure, 40 psi, sheath gas temperature 350 °C, sheath gas flow 12 Lmin-1, capillary voltage, (+) 4000 V and (-) 3000V. Nitrogen was used as the nebuliser gas, and helium for the collision gas. The LC-MS/MS system was operated in the MRM (multiple reaction monitoring) mode, with a unit mass resolution set for Q1 and Q3. Energy of fragmentor (Frg), collision energy (CE) and cell accelerator voltage (CAV), were optimised using MassHunter Optimizer software. Optimal MS/MS parameters values are shown in Table 2. Identification was based on the retention time tolerance of ± 0.1 min, acquisition of at least two selected reaction monitoring transitions, and multiple reaction monitoring (MRM) ratio of product ions response (q/Q) with a tolerance of ± 30%, taking the retention time and response ratio of the standard in solvent as the reference value.

Table 2. MS/MS parameters.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Frag (V)	Collision energy (V)	CAV	Polarity
CAP	321	152	100	17	3	Negative
		257		10	3	
CAP-D5	326	157	100	20	3	Negative
		262		10	3	

3. Results

3.1. Validation parameters

Linearity

It was examined by the use of seven spiking levels. The obtained correlation coefficient was R^2 0.9991 with the y = 0.539560x + 0.025389. The calibration curve is shown on the Figure 2.

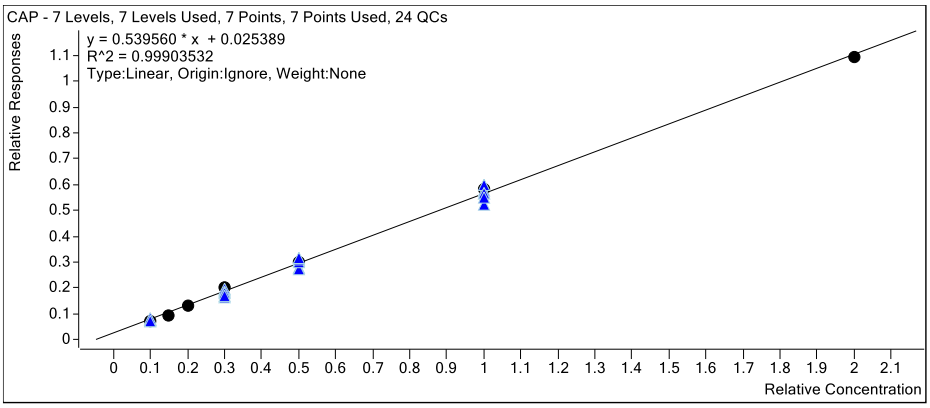

Figure 2. CAP calibration curve.

Recovery

All the values obtained during the validation process including the recovery (for spiking levels 0.1, 0.3, 0.5 and 1.0 µg/kg), the RSD, % and RSDr) were shown in the table 3.

Tabele 3. Obtained data during the validation study.

Analyt	Spiking level (µg/kg)	Average recovery (µg/kg)	SD	RSD (%)	% Rec.	RSDr (%)	% Rec. average
CAP	0.1	0.090	0.005	5.40	89.66	13.58	92.9
CAP		0.096	0.009	9.55	95.83		
CAP		0.093	0.007	8.00	93.30		
CAP	0.3	0.280	0.015	5.27	93.23	12.12	94.3
CAP		0.279	0.018	6.41	93.14		
CAP		0.290	0.026	8.83	96.59		
CAP	0.5	0.504	0.035	7.03	100.71	10.67	98.7
CAP		0.475	0.020	4.29	94.95		
CAP		0.502	0.034	6.78	100.40		
CAP	1.0	1.035	0.027	2.58	103.52	6.00	101.4
CAP		1.011	0.026	2.61	101.14		
CAP		0.994	0.047	4.75	99.44		

The obtained average recovery was 96.5 ±10.59%.

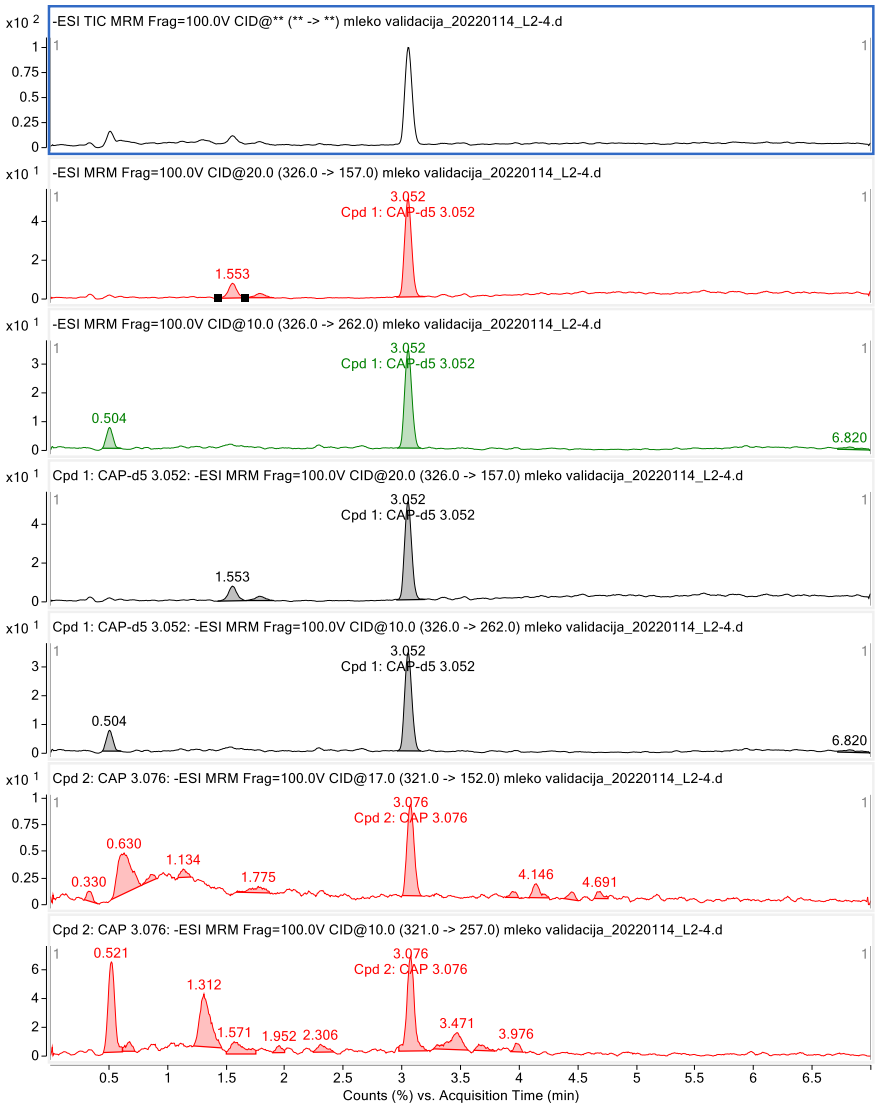

Figure 3. LC-MS/MS chromatographic peaks of the CAP (0.3 µg/ kg).

4. Discussion

The optimization of the mass spectrometry parameters was performed by injecting the CAP standard solutions into the electrospray ion (ESI) source. The mass spectrometer was operated in the negative ESI mode. The validation data fulfilled the requirements established in the Regulations Decisions 2020/657/EC. During the validation process the chloramphenicol-D5 as an internal standard was used. The obtained results indicated the good linearity ($R^2 > 0.99$) within the range of 0.1–2.0 µg/kg. The mean recovery for the spiking levels was 96.5 ± 10.59%. The limit of quantification (LOQ) was 0.1 µg /kg. The repeatability was 10.6%. The method fulfilled all the 2020/657/EC guidelines and thus can be extended for the routine analysis of CAP residues in milk.

5. Conclusions

In this study, the liquid-liquid extraction and SPE (Waters OASIS HLB column) cleaning process followed by LC-MS/MS in negative ESI mode, for the detection and quantification of chloramphenicol in milk has been developed. The method demonstrated acceptable inter- and intra-assay recovery at LOQ, good repeatability and within-lab reproducibility, and met all the Regulations Decisions 2020/657/EC guidelines of the method validation. The validated method is

simple, less expensive, takes less time for analysis, and uses minimal solvents, chemicals, and lab-wares. The method is sensitive and can be applied for the routine analysis of chloramphenicol contaminants in milk at or below the MRPL.

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Conflicts of Interest: The authors declare no conflict of interest

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