HPLC-MS Analysis of Chloramphenicol Residues in Milk and Powdered Milk Products+

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Chloramphenicol (CAP) is a broad-spectrum antibiotic with bacteriostatic action but also has toxic properties, which is why its presence in food and feed is prohibited in Croatia and the European Union.

In the aim of consumer protection it is essential to develop a sensitive analytical method for detection of CAP fractions lower than $w = 0.3$ μg kg^{-1}. For the efficient control and monitoring of CAP, a rapid, sensitive, and selective method for its identification and quantification, using high performance liquid chromatography in combination with mass spectrometry LC-MS, has been developed.

The cleaning procedure was based on the AOAC official method 993.32. HPLC-MS analysis used the ODS Hypersile column and the water/acetonitrile gradient. Electrospray negative ionization (neg ESI) was used before single ion monitoring (SIM) detection of three *m/z* 321, 323 and 325. As additional criteria, the ratio between these masses in real and spiked milk samples was also investigated in accordance with theoretical values of the isotope pattern for 2 chlorine atoms present in the analyte.

The detection limit of 0.1 μg kg^{-1} was achieved. The mean value of recovery was 94 %, the correlation coefficient of the calibration curves calculated for 2 *m/z* values was higher than 0.99.

Fourty samples of milk and milk products were tested with the HPLC-MS method, and obtained results showed that samples had CAP 0.37, 0.29, 0.39 μg kg^{-1}, respectively. All the other analysed samples contained CAP concentrations below the detection limit.

Key words: *Chloramphenicol, liquid chromatography, mass spectrometry, milk*

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic with bacteriostatic action. It acts by binding to the 50S subunit in the prokaryotic cell, inhibiting protein synthesis. Due to its low cost, optimal activity in the range between pH 7.4–8.0, and long half-life in solution, it was widely used for years in veterinary medicine. Recently, has been discovered that it could cause neoplastic anemia[1], especially in children, as well as hypersensitivity.

Since March 2003, the presence of chloramphenicol in food and feed is prohibited in Croatia[2] as it was earlier prohibited in the European Union[3-4].

In the aim of consumer protection it was essential to develop a sensitive analytical method for the detection of CAP fractions lower than $w = 0.3$ μg kg^{-1}.

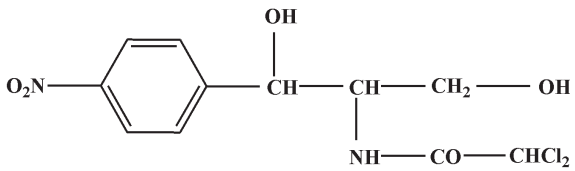

Fig. 1 – *Structure of chloramphenicol (CAP)*
Slika 1 – *Struktura kloramfenikola (CAP)*

Methods for detecting CAP residue in milk and other biological materials described in literature, apart from several advantages, also have some disadvantages, which will be discussed later. The immunoassay[5-7] technique, which is very useful for screening purposes due to its simplicity, often gives false positive results. Gas chromatography with electron capture detector[8-9] (ECD) is sensitive enough, but needs derivatization and is not confirmatory. Some research describes the use of easy and low time-consuming capillary electrophoresis (CE)[10], however this also is a non-confirmatory method. Some voltammetric[11] methods are also present, but the detection limit is too high. On the other hand, high performance liquid chromatography (HPLC)[12-13] with conventional detectors is a non-confirma-

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tory method (CAP presence cannot be confirmed in four control points as prescribed by the EU Directive 96/23/EC[4]). Therefore, a rapid sensitive and selective method for CAP identification and quantification using LC-MS has been developed. There are a few LC-MS methods also described in literature, however these instruments are too expensive for rutine analyses[14-18].

Experimental

Reagents

The chloramphenicol (CAP) standard was 99.9 % purity, supplied by WHO Centre for Chemical Reference Substances, Stockholm, Sweden. All other chemicals were at least HPLC Grade and supplied by *J. T. Baker*. The water was purifed with a Milli-Q water purification system from Millipore (Bedford, MA, USA)

Apparatus

The HPLC system, equipped with auto sampler SIL-10 -Advp, thermostat column oven and degasser, Shimadzu Corp. The MS system – LCMS-2010, single quadrupole mass spectrometer with electrospray probe (ESI) Shimadzu Corp. (Kyoto, Japan).

LC column – Thermo ODS Hypersil-Keystone Narrow-Bore 2.1 · 100mm · 3 μm, Agilent Technologies Bellefonte, PA 16823, USA.

HPLC-MS analysis used the ODS Hypersile column and the water/acetonitrile high pressure gradient with a volume flow rate of $Q = 0.2$ mL min^{-1}. The gradient was as follows: 20 % B increased to 100 % B in 10 min, and hold at 95 % B for 2 min. Eight minutes were enough for post time reequilibration of the column. Injection volume of 10 μL for standards and samples was used, and column temperature was maintained at $T = 30$ °C.

Electrospray negative ionisation was used prior to single-ion monitoring MS detection of three *m/z* 321, 323 and 325.

The interface quantities were: The temperature of negative ion electrospray probe (ESI(–)) was $T = 250$ °C. Curved desolvation line (CDL) temperature or temperature of the heated capillary was $T = 230$ °C. The nebulizer drying gas was (N_2) with flow rate $Q = 3.0$ L min^{-1}, and block temperature was set at $T = 230$ °C.

Mass spectra were acquired in selective ion monitoring mode (SIM) with $t = 0.25$ s of scanning interval. Detector gain was set $U = 2.5$ kV, probe high voltage at $U = 4.5$ kV, CDL voltage at $U = 25.0$ kV and Q-array voltage on gain for scanning.

Sample Preparation

The cleaning and detection used the modified AOAC Official method 993.32[4,6] for multiple sulfonamide residue in raw bovine milk. 10 mL or 10 g of homogenized milk sample was weighed and extracted with 50 mL chloroform and 25 mL acetone $\psi = 2 : 1$, respectively. The milk and solvents were shaken vigorously twice for a 1 min and vented. Phase separation lasted 1 min, and the extract was shaken by hand vigorously for 1 more min then vented. Phase separation lasted 5 min. Both extraction solutions were drawn off into a 100 ml round-bottom flask and filtered through prewashed filter paper. The extract was evaporated to dryness at 30 °C. The residue was dissolved in 1 ml $c = 0.02$ mol L^{-1} sodium acetate buffer at pH 4.8, vortexed for 1 min. After that, 5 ml of hexane was added and vortexed for 1 min. The extract was transferred to a conical tube and phase separation lasted 2 min. Vortexed again for 1 min and let phase to separate 15 min. The aqueous layer was filtered through an 0.45 μm, 13-mm modified hydrophilic PTFE filter and analyzed using LC-MS.

This method was also applied to the milk powder samples and dry baby food, previously prepared for consumption.

Results

In the aim to validate this method for CAP identification and quantification in milk and milk products, the linearity of standards and spiked samples (5 calibration points) were investigated in the range of $\gamma = 1$–24 pg μL^{-1}, 0.1–2.4 μg kg^{-1} respectively. Fig. 2 shows the results.

Chloramphenicol is a forbidden substance therefore in accordance with the EU Directive 96/23/EC for confirmation techniques, one more confirmation point was necessary for positive results. This was the isotope pattern between real samples and standards as shown in Tables 1 and 2.

The relation between these three masses (*m/z* 321, 323 and 325) and the match with theoretical values when two atoms of chlorine were present in the isotope pattern were determined.

The intensity ratio for the isotope pattern for standards and samples, as shown in Tables 1 and 2, was satisfactory due to the good correlation between the isotope pattern for standards and samples. The higher the CAP concentration in both standards and samples, the better the ratio between the theoretical and obtained isotope patterns. This is more obvious in *m/z* 325 because of the small areas.

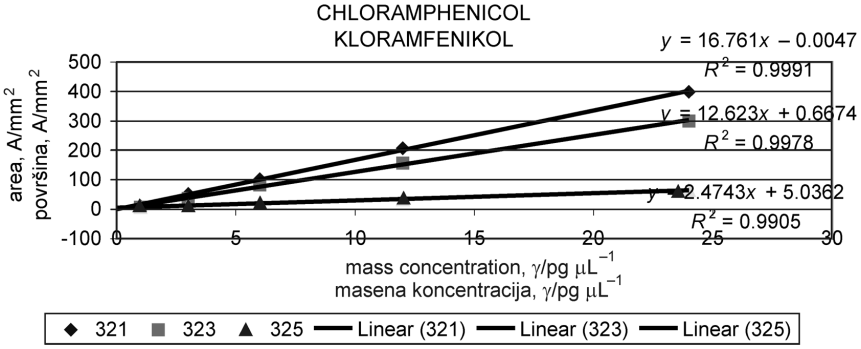


Fig. 2 – *Linearity in concentration range (1–24 pg μL^{-1}) and coefficients of correlation* R *for three* m/z

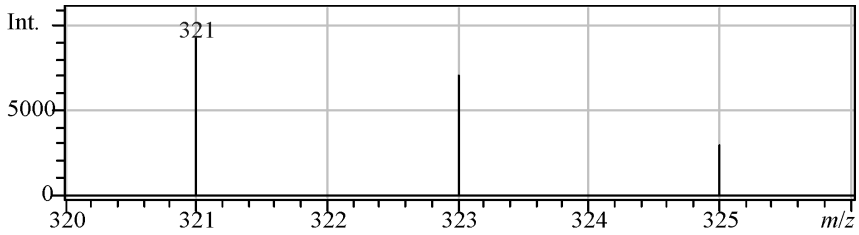
Slika 2 – *Linearnost u kalibracijskom području (1–24 pg μL^{-1}) i koeficijent korelacije* R *za tri karakteristične mase*

T a b l e 1 – *Intensity ratio – isotope pattern for real samples*
T a b l i c a 1 – *Omjer intenziteta izotopa klora u realnim uzorcima*

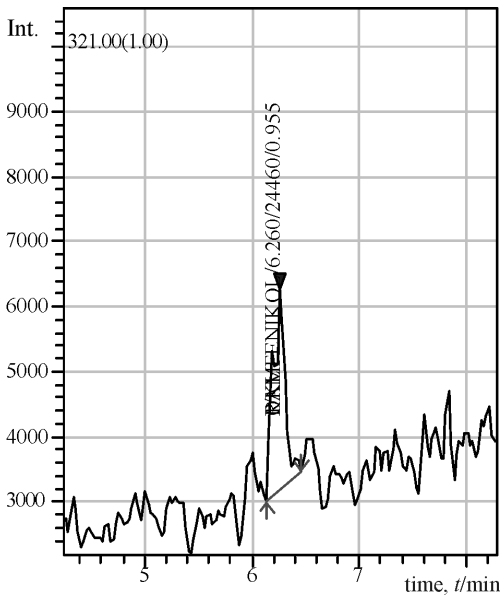
Replicates Replike	321	323	325
1	100	87	18
2	100	78	48
3	100	89	48
4	100	81	37
5	100	87	36
6	100	60	42
Average value Srednja vrijednost	100	80.33	38.16
CV koeficijent varijacije	0	10.80	11.14

T a b l e 2 – *Intensity ratio – isotope pattern for chloramphenicol standards at different mass concentration*
T a b l i c a 2 – *Omjer intenziteta izotopa klora za CAP-standarde pri različitim masenim koncentracijama*

γ_{CAP}/pg μL^{-1}	321	323	325
1	100	87	66
3	100	73	52
6	100	86	41
12	100	94	29
24	100	72	27
240	100	70	13
Average value Srednja vrijednost	100	80.33	38
CV koeficijent varijacije	0	9.93	19.05



F i g. 3 – *Isotope pattern for both standards and samples when two chlorine atoms were present in the molecule*
S l i k a 3 – *Omjer izotopa klora u standardima i u uzorcima kada su u molekuli vezana dva atoma klora*



F i g. 4 – *LC-MS chromatogram of milk sample spiked with 0.1 μg kg^{-1} chloramphenicol*
S l i k a 4 – *LC-MS-kromatogram uzorka mlijeka obogaćenog s 0,1 μg kg^{-1} kloramfenikola*

The mean value of recovery was 94 % for six replicates with rsd 7.50 % calculated on *m/z* 321, rsd 7.06 % calculated on *m/z* 323 and 10.38 % calculated on *m/z* 325, respectively.

The correlation coefficient of the calibration curves calculated for the three most abundant m/z values was higher than 0.99. The detection limit of 0.1 μg kg^{-1} was achieved with the signal to noise ratio 10.87 for milk sample spiked with 0.1 μg kg^{-1} CAP.

There was an excellent correlation between standards and samples isotope pattern with a deviation of relative intensity lower than 10 %.

The deviation on retention times between standards and samples was 1 % (the average retention time for spiked samples 6.28 min (n = 6, rsd 0.029) and for the standards 6.25 min (n = 5, rsd 0.010)

Fourty samples of milk and milk products were tested with the LC-MS method, and the obtained results revealed that 3 samples had CAP 0.37, 0.29, 0.39 μg kg^{-1}, respectively. All the others showed CAP concentrations below the detection limit of 0.1 μg kg^{-1}.

Conclusions

The method presented enabled detection, quantification and confirmation of CAP residues in milk and milk products in the lower 10^{-9} (ppb) mass fraction range. In order to protect consumers from milk and milk products contaminated with CAP, the LC-MS method has been proven as rapid and sufficiently sensitive.

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List of symbols
Popis simbola

A	– area – površina
c	– (amount) concentration, mol L^{-1} – (množinska) koncentracija, mol L^{-1}
m/z	– relation mass and charge number of ion – odnos mase i naboja iona
Q	– volume flow rate, L min^{-1} – obujmni protok, L min^{-1}
R	– coefficient of correlation – koeficijent korelacije
T	– temperature, °C – temperatura, °C
t	– time, min – vrijeme, min
w	– mass fraction, μg kg^{-1} – maseni udjel, μg kg^{-1}
γ	– mass concentration, pg μL^{-1} – masena koncentracija, pg μL^{-1}
ψ	– volume ratio, V_{ch}/V_{ac} – obujmni omjer, V_{ch}/V_{ac}

SAŽETAK

HPLC-MS-analiza rezidua kloramfenikola u mlijeku i proizvodima od mlijeka u prahu

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Kloramfenikol je jedan od antibiotika sa širokim spektrom djelovanja. Zbog toksičnih svojstava zabranjena je njegova primjena u Hrvatskoj i u Europskoj uniji u prehrambenim namirnicama.

Ekstrakcija uzorka i čišćenje temeljeni su na modificiranoj AOAC-ovoj metodi 993.32. Tijekom HPLC-MS-analize uporabljena je kolona ODS Hypersile s gradijentnim programom kombinacije acetonitrila u vodi. Primijenjena je negativna ionizacija elektroraspršivanjem (neg ESI) molekula kloramfenikola i nakon toga praćenje tri odabrane karakteristične mase iona *m/z* 321, 323 i 325 radi detekcije, identifikacije i kvantifikacija analita.

Također, kao dodatni kriterij ispitan je i uspoređen odnos intenziteta te tri odabrane veličine u realnim uzorcima i u uzorku mlijeka nacijepljenog s kloramfenikolom prema teorijskoj vrijednosti karakterističnoj za prirodnu raspodjelu izotopa kada su dva atoma klora adicijski vezani u molekuli analita.

Postignuta je granica detekcije 0,1 μg kg^{-1} kloramfenikola u mlijeku. Srednja vrijednost iskorištenja bila je 94 %, a koeficijent korelacije kalibracijskih krivulja pri dvije *m/z* bio je veći od 0,99.

Ovom metodom analizirano je oko 40 uzoraka mlijeka i mliječnih proizvoda pri čemu su samo tri uzorka bila pozitivna s vrijednostima kloramfenikola 0,37, 0,29, 0,39 μg kg^{-1} mlijeka, dok su u ostalim uzorcima količine kloramfenikola bile ispod granice detekcije.

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