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# Chloramphenicol determination in milk by liquid chromatography – tandem mass spectrometry

**Vytautas Tamošiūnas[1],**

**Julijonas Petraitis[2] and**

**Audrius Padarauskas[1*]**

*[1] Department of Analytical and Environmental Chemistry, Vilnius University, Naugarduko 24, LT-03225 Vilnius, Lithuania*

*[2] Chemistry Department, National Veterinary Laboratory, Vilnius, Lithuania*

A high-performance liquid chromatography – tandem mass spectrometry (HPLC–MS–MS) method was developed for the determination of chloramphenicol (CAP) in milk products. Mass spectral acquisition was done using electrospray ionization in the negative ion mode applying multiple reaction monitoring of two diagnostic transition reactions for CAP (*m/z* 321→152 and *m/z* 321→257). Milk samples were extracted with ethyl acetate and evaporated to dryness, followed by a clean-up step using the liquid–liquid extraction with carbon tetrachloride / hexane (1:1 v/v) mixture. The calibration curve showed a good linearity in the concentration range from 0.02 to 1.0 μg/kg with the correlation coefficient above 0.995. The method gave a decision limit and a detection capability of 0.050 and 0.066 μg/kg, respectively. The mean recoveries of CAP from the milk samples spiked at 0.1–0.45 μg/kg were in the range of 86–92%. The applicability of this technique was demonstrated by analysis of milk products.

**Key words**: high-performance liquid chromatography, tandem mass spectrometry, chloramphenicol, milk products

## INTRODUCTION

Chloramphenicol (CAP) is an effective antibiotic that has widely been used since the 1950s to treat food-producing animals (Fig. 1). Because of the well-known risk of aplastic anemia and carcinogenic properties of CAP, its use in human and veterinary medicine is limited by its toxicity. Consequently, since 1994 the European Community has totally banned the use of CAP in food-producing animals [1]. Recently, the European Union (EU) has revised the technical criteria that must be applied in the screening and confirmatory analysis of veterinary drug residues in food of animal origin [2]. The minimum required performance limit (MRPL) for the detection of CAP residues in food of animal origin has been fixed at 0.3 μg/kg [3]. Thus, a sensitive and reliable method for the determination of CAP at residual levels is urgently needed.

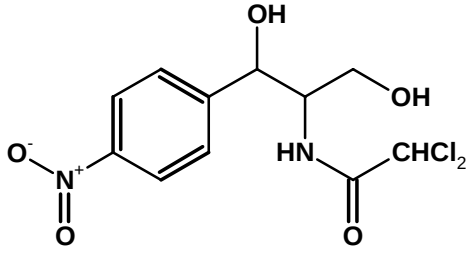


**Fig. 1.** Structural formula of chloramphenicol

In the past decade, several analytical methods have been developed for the screening and quantitation of CAP in foods and biological fluids. For screening purposes, immunochemical tests [4–6] and chromatographic techniques such as high-performance liquid chromatography (HPLC) with diode array detection and gas chromatography (GC) with electron capture detection have been used [7–9]. However, the confirmation of suspect-positive samples must be carried out by mass spectrometry (MS) coupled to an adequate chromatographic separation. For this purpose, GC–MS methods using either electron impact (EI) or chemical ionization (CI) have been reported [10–12], but these procedures still require a tedious derivatization step prior to final analysis. Liquid chromatography methods, however, do not require a derivatization step and HPLC–MS sensitivity approaches that of GC–MS.

Tandem mass spectrometry (MS–MS) is a more sophisticated technique allowing a very effective isolation of analyte ions from the noise-producing matrix. The potential of HPLC with tandem mass spectrometric detection (HPLC–MS–MS) has already been demonstrated for analysis of complex food matrices such as meat, seafood, eggs, honey, and particularly for analysis of antibiotic residues [13–18].

This paper focuses on the optimization of HPLC–MS–MS technique for the determination of CAP in milk products. Validation of the screening and confirmatory methods was performed according to EU Legislation.

* Corresponding author. E-mail: audrius.padarauskas@chf.vu.lt

## EXPERIMENTAL

HPLC–MS–MS analyses were performed using a LC Waters Alliance 2695 separations module (Waters, Milford, MA, USA) coupled via an electrospray interface (ESI) to a Quattro Ultima Pt Micromass mass spectrometer (Wythenshawe, UK). The instrument was operated in multiple reaction-monitoring (MRM) mode.

HPLC separations were performed on a $C_{18}$ SymmetryShield column (10 cm × 2.1 mm I.D., 3.5 μm particle size) fitted with a SymmetryShield $C_{18}$ precolumn (1 cm × 2.1 mm I.D., 3.5 μm particle size) (Waters). The mobile phase was a water–acetonitrile gradient. The $CH_3CN$ content was increased from 20 to 60% in 4 min. The mobile phase flow-rate was set at 0.2 ml/min, and 20 μl of the extract was injected into the HPLC–MS–MS system.

Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Acetonitrile, diethyl ether, ethyl acetate, hexane, carbon tetrachloride, formic acid were HPLC grade and used as received (Merck, Darmstadt, Germany). CAP (purity >99%) standard was purchased from Fluka (Buchs, Switzerland), while its deuterated internal standard with five deuteriums ($d_5$-CAP, purity >98%) was obtained from Cambridge Isotope Laboratory (Andover, MA, USA).

Stock solutions of CAP and $d_5$-CAP at the concentration of 0.1 mg/ml were prepared in methanol and stored at 4 °C, protected from light. Working solutions used for spiking blank samples were obtained by appropriate dilution.

## RESULTS AND DISCUSSION

### MS–MS detection

CAP and its deuterated internal standard $d_5$-CAP were first analyzed in negative ESI–MS mode to select characteristic ions as the precursors (Fig. 2). The full scan mass spectra of CAP and $d_5$-CAP displayed several intense ions, and the most abundant were *m/z* 321 and *m/z* 326 for CAP and for $d_5$-CAP, respectively, which correspond to the deprotonated molecular ion $(M–H)^-$.

According to Commission Decision 2002/657/EC [2], for banned substances, at least one parent ion and two transitions with two different product ions are required to confirm the presence of the analyte studied. Both CAP and $d_5$-CAP were then analyzed by HPLC–ESI–MS–MS in a negative ionization product ion scan mode by selecting *m/z* 321 and *m/z* 326 ions as the precursor ion, respectively. The full scan mass spectra of these ions are compared in Fig. 3. As one can be see, two main fragment ions were obtained from the collision-induced dissociation (CID) experiments of these ions, giving rise to respectively *m/z* 257 and *m/z* 152, *m/z* 262

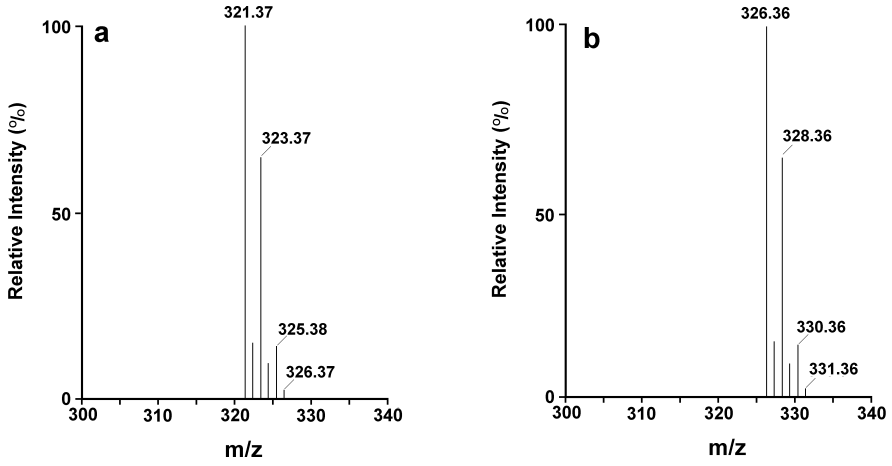


**Fig. 2**. Full scan mass spectra of (*a*) CAP and (*b*) $d_5$-CAP

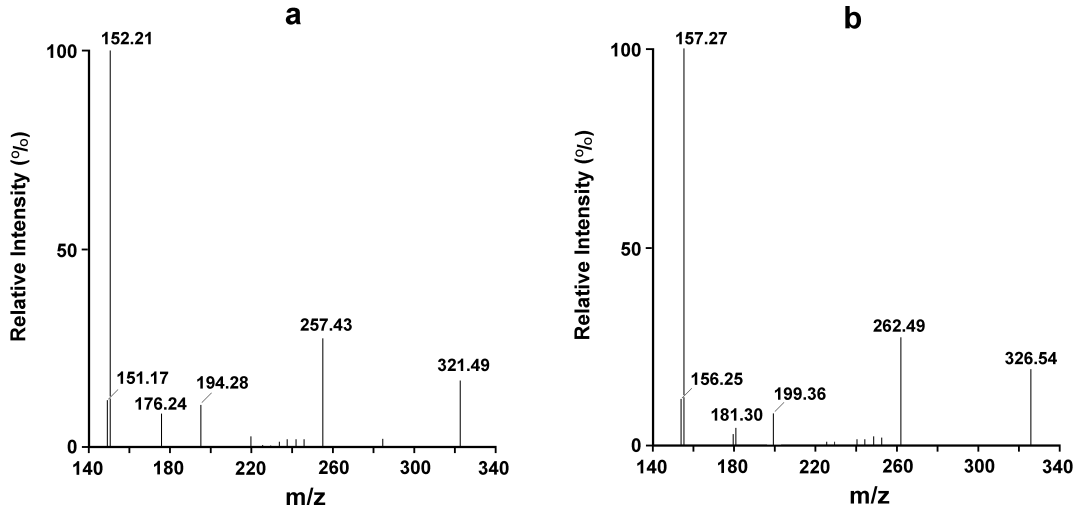


**Fig. 3**. Collision-induced dissociation mass spectra of (*a*) CAP *m/z* 321 and (*b*) $d_5$-CAP *m/z* 326

and *m/z* 157. Their respective fragmentation patterns are in good agreement with previous findings [19].

The selected transitions for CAP and the internal standard and the optimal MS–MS conditions are given in Table 1. Concentrations were calculated by comparing the ratio of *m/z* 321→152 response CAP with the ratio of *m/z* 326→157 response $d_5$-CAP.

Table 1. **MS-MS transitions monitored for CAP determination and optimal MS–MS conditions**

| Compound | Precursor ion (*m/z*) | Ion transitions (*m/z*) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| CAP | 321 | 321→152 (more intense) | 52 | 15 |
| | | 321→257 (less intense) | 52 | 10 |
| $d_5$-CAP | 326 | 326→157 (more intense) | 54 | 15 |
| | | 326→262 (less intense) | 54 | 10 |

**Sample extraction and clean-up**

Several sample preparation procedures for the determination of CAP in various matrices have been published [20]. All of the methods included extraction and a sample clean-up process that allowed trace levels of the analyte to be estimated in biological samples. The much superior selectivity and sensitivity of the tandem mass spectrometer lead to a much simpler and faster sample preparation. Thus, preliminary studies were conducted to optimize solvents and extraction conditions. The extraction efficiency of CAP from spiked blank samples was studied using two relatively polar extractants: diethyl ether and ethyl acetate. The recoveries were determined by comparing the peak area obtained from spiked blank samples with those obtained from aqueous standard solutions. The obtained results showed that ethyl acetate provided higher (≥60%) recoveries of CAP from spiked blanks. In addition, to further improve the extraction efficiency, we attempted to increase the extraction time from 15 min to 45 min. This resulted in an increase in the recoveries of CAP up to 85–90%. Based on these results, ethyl acetate was selected as the extractant for further studies.

For the clean-up of extracted CAP, various solid-phase extraction (SPE) procedures using silica, cation-exchange or octadecyl columns were proposed [8, 11]. A potential drawback of SPE procedure is that it requires a large number of individual steps (column preconditioning, sample loading, washing and elution) and thus significantly prolongs the analysis time. In order to shorten the sample clean-up procedure and to allow a higher sample throughput, conventional liquid–liquid extraction was adopted. The following three non-polar extractant systems were studied: carbon tetrachloride, hexane and a carbon tetrachloride / hexane (1:1 v/v) mixture. Sample clean-up performance was evaluated by performing a standard addition of CAP to the blank samples just before the clean-up procedure in order to avoid the loss of analyte during the first extraction step. The obtained results showed that all three solvents provided adequate recoveries (≥90%), but using carbon tetrachloride and hexane slightly higher amounts of interfering compounds remained in the aqueous phase and did not allow CAP to be screened at low levels.

The complete sample extraction and clean-up procedure optimized for analysis of CAP residue in milk products was as follows. A 10 ml volume of Milli-Q water was added to 10 g aliquot of milk sample and the sample was defatted by centrifugation for 20 min (3500×*g* at –4 °C). A 3.0 ml volume of the defatted sample was transferred to a glass tube, 6 ml of ethyl acetate and 1 ml of 0.6 ng/ml $d_5$-CAP standard were added, and the mixture was agitated on a minishaker for 45 min. After phase separation, 4 ml of the upper layer (ethyl acetate) was evaporated to dryness at 50 °C under nitrogen stream. The residue was dissolved in 0.5 ml of water and then extracted with 0.5 ml of carbon tetrachloride/hexane (1:1 v/v) for 5 min. After centrifugation for 5 min at 25000 rpm, the aqueous phase was removed and the aliquot of 20 µl was injected on the HPLC column.

**Analytical performance**

Initially, two acetonitrile-water mobile phases containing formic acid (0.10 mol/l) and ammonium acetate (0.025 mol/l) buffers were tested for their ability to separate CAP from the sample matrix compounds. Unfortunately, both mobile phase systems gave an analyte carry-over effect. This problem was completely avoided with pure acetonitrile-water mobile phase using a linear gradient from 20% to 60% $CH_3CN$ in 4 min.

Method validation was carried out according to criteria described in [2]. The parameters taken into account were: response linearity, decision limit (CCα), detection capability (CCβ), reliability and accuracy (repeatability and within-laboratory reproducibility).

Usually, the quantification of drug residues is performed by using a matrix-matched calibration curve made from fortified blank samples prepared in the same matrix as the real samples. To test the linearity of the calibration curve, five standards of CAP in the blank milk matrix were analyzed. The calibration curve showed a good linearity in the concentration range from 0.02 to 1.0 µg/kg with the correlation coefficient above 0.995. A similar slope and correlation coefficients were also observed for the calibration curve based on pure standard solutions prepared in water, indicating that there was no signal contribution and ion suppression from the matrix. Consequently, CAP quantification in milk samples was performed using calibration curves obtained from pure standards.

The EU decision [2] introduces the concepts of decision limit (CCα) and detection capability (CCβ) for a

chemical analytical method. These parameters are to be used instead of the more familiar limit of detection and limit of quantification. The definition of the CCα for a forbidden compound is: "The limit at and above which it can be concluded with an error probability of 1% that a sample is noncompliant". The definition of the CCβ for a forbidden compound is: "The lowest concentration at which a method is able to detect truly contaminated samples with an error probability of 5%". This means that a signal responding to CCα or lower will be regarded as the background noise, while a signal corresponding to CCβ or higher will be regarded as originating from a forbidden compound. The CCα and CCβ were obtained using the calibration graph approach [2]. Blank material was fortified at five different concentrations (n = 20) and the standard error of the *y* intercept was calculated. The decision limit (CCα = 2.33 × standard error of the *y* intercept) and the detection capability (CCβ = CCα + (1.64 × standard deviation of 20 spikes at CCα)) for CAP were 0.050 μg/kg and 0.066 μg/kg, respectively. These data demonstrate that both values are significantly below the MRPL of 0.3 μg/kg.

The reliability and accuracy of the method were determined by spiking blank milk samples with CAP, resulting in three analytical series, each with three concentration levels (0.10, 0.30 and 0.45 μg/kg) and six samples per concentration level. The trueness was expressed in terms of recovery rates and the precision as relative standard deviation (RSD). The results are presented in Table 2. The performance characteristics of the method presented in this paper indicate that it may be used in food control.

Table 2. **Performance data of the HPLC–MS–MS method for the analysis of CAP in spiked milk samples**

| Parameter | Fortification level, μg/kg | | |
|---|---|---|---|
| | 0.10 | 0.30 | 0.45 |
| Overall mean ± SD | 0.09 ± 0.02 | 0.28 ± 0.02 | 0.44 ± 0.04 |
| Trueness ± SD (%) | 86.4 ± 16 | 92.0 ± 11 | 89.1 ± 15 |
| Precision (RSD%) | 18.8 | 12.6 | 15.4 |

Finally, the HPLC–MS–MS method was applied to milk samples collected in Lithuania during 2005. Figure 4 shows HPLC–MS–MS chromatograms of a milk sample and a milk sample spiked with 40 ng/kg CAP. Among the 75 samples analyzed, 11 samples (~15%) contained CAP residues with concentration levels that varied between a minimum of 0.07 μg/kg and a maximum of 0.2 μg/kg.

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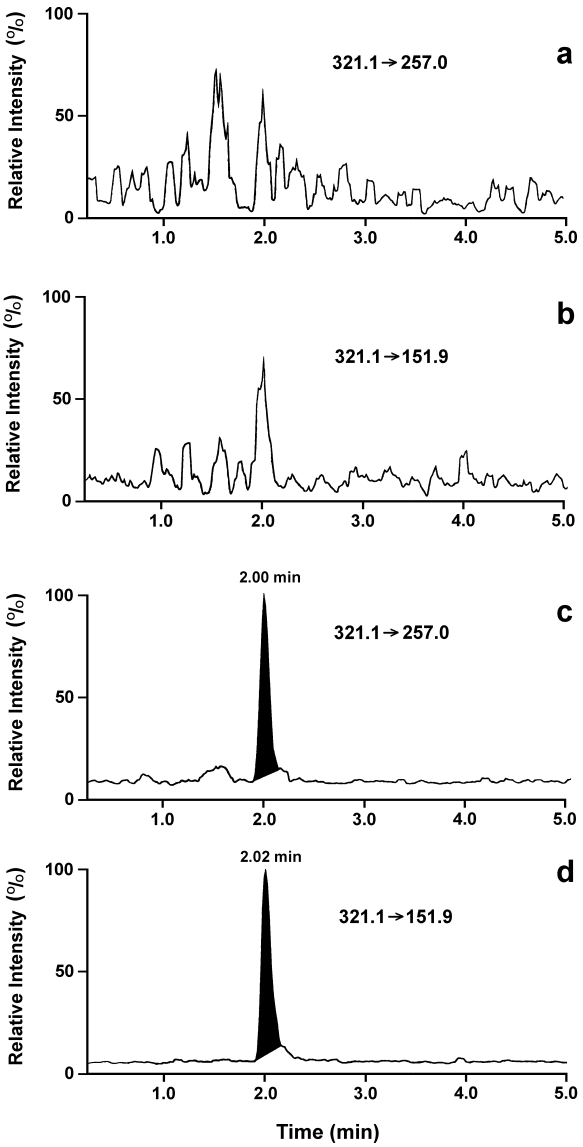


**Fig. 4**. HPLC–MS–MS chromatograms of blank milk sample (*a*, *b*) and milk sample spiked with 40 ng/kg CAP (*c*, *d*)

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**Vytautas Tamošiūnas, Julijonas Petraitis, Audrius Padarauskas**

**CHLORAMFENIKOLIO NUSTATYMAS PIENE SKYSČIŲ CHROMATOGRAFIJOS–DVIGUBOS MASIŲ SPEKTROMETRIJOS METODU**

Santrauka

Optimizuotas efektyviosios skysčių chromatografijos–dvigubos masių spektrometrijos metodas chloramfenikoliui piene nustatyti. Chloramfenikolis iš pieno mėginių ekstrahuojamas etilo acetatu, ekstraktas išgarinamas, skiedžiamas vandeniu, papildomai išvalomas ekstrahuojant anglies tetrachlorido/heksano mišiniu (1:1) ir analizuojamas.

Išmatuotos pagrindinės analizinės charakteristikos: kalibracinė kreivė yra tiesinė chloramfenikolio koncentracijų intervale 0.02–1.0 μg/kg; koreliacijos koeficientas – 0,995; sprendimo riba – 0,050 μg/kg; aptikimo geba – 0,066 μg/kg. Vidutinės chloramfenikolio standartinių priedų (0,1–0,45 μg/kg) piene išgavos siekia 86–92%. Metodas pritaikytas chloramfenikolio likučiams piene nustatyti.