# Rapid Determination of Chloramphenicol Residues in Honey by Liquid Chromatography Tandem Mass Spectrometry and the Validation of Method Based on 2002/657/EC

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## Abstract

A simple and rapid liquid chromatography tandem mass spectrometry (LC-MS/MS) method of analysis for the chloramphenicol (CAP) in honey was developed. Samples were cleaned-up with hexane and followed extraction with acetonitrile. LC separation was achieved by using a Phenomenex Luna C-18 column and acetonitrile – water as a mobile phase. The mass spectrometer was carried out in a multiple reaction monitoring mode with negative electrospray interface. Validation of the method was done according to criteria of Decision Commission No 2002/657 EC. Samples were fortified at CAP levels between 0.15 and 0.45 μg/kg with CAP-5d as internal standard. The precision within-day was lower than 9% and recovery was in the range of 91-104%. The limit of decision (CCα) and detection capability (CCβ) were 0.11μg/kg and 0.14 μg/kg, respectively. The validation method was successfully applied for determination of CAP in honey.

**Key words***: honey, chloramphenicol, residue, LC-MS/MS.*

## Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic previously used in veterinary medicine. Recently some honeys on the international market have been found contaminated with CAP residues. The EU prohibits the use of CAP as a veterinary drug for food producing animals. For CAP maximum residue limit (MRL) could not be set. Therefore a minimum required performance level (MRPL) was set at 0.3 μg/kg [3]. Various analytical methods have been reported for the determination of CAP in honey and other biological materials. These included the rapid enzyme test kits linking immunosorbent assay (ELISA) and mass spectrometric in combination with gas chromatography (GC-MS) or liquid chromatography (LC-MS) [1-9]. The ELISA method is suitable for screening purposes whereas mass spectrometric methods are utilized for confirmation. GC-MS methods can provide definitive qualitative and quantitative results but require a derivatization step. The combination of LC-MS offers a rapid, simplified, specific and sensitive alternative to GC-MS methods and removing the need for derivatization reactions.

The present work describes a rapid method for the determination and confirmation of CAP in honey, based on liquid chromatography with tandem mass

spectrometric (LC-MS/MS) in electrospray negative ion mode. The method was validated according to Commission Decision 2002/657//EC.

## Material and Methods

Reagents. Analytical standard chlorampenicol from Sigma-Aldrich, chloramphenicol D5 (100 µg/ml in acetonitrile) was used as an internal standard from Cambridge Isotope Laboratories (FSD-117-100, 98%), ethyl acetate LC grade and acetonitrile LC-MS grade were from Baker.

Standard solution. A CAP standard stock solution of 1,0 mg/ml was prepared by dissolving 100 mg CAP in 100 ml of acetonitrile and this solution was diluted 50 times in acetonitrile obtaining an intermediate standard solution of 20 ug/ml. A CAP working solution of 50 ng/ml was made by diluting a stock solution with acetonitrile. An internal standard of CAP-d5 was prepared by dissolving an ampoule with 100 ug/ml in acetonitrile, which was adequately diluted till a working solution of 1,5 ng/ml was obtained. All standard solutions were kept at $-20C^0$ and protected from light for no longer than 3 months.

Liquid chromatography - conditions. LC analyses were performed on a C18 Luna column (150 x 2 mm i.d., 5 µm) (Phenomenex, Torrance, USA) using an Agilent 1100 series liquid chromatograph equipped with a binary pump and an autosampler. The column was thermostated at 40 $^0$C. Mobile phase was water and acetonitrile (80:20; v/v). The flow rate was set at 200 µl/min and the injection volume was 20 µl. The linear gradient program was shown table I.

Table I. Program of gradient mobile phase

| Time (min) | Water (%) | Acetonitrile (%) |
|---|---|---|
| 0.0–0.1 | 80 | 20 |
| 0.1–7.0 | 0.0 | 100 |
| 7.0–7.3 | 80 | 20 |
| 7.3–20 | 80 | 20 |

Using these conditions, retention time of CAP and CAP-d5 was observed at 6.8 min.

Mass spectrometry - conditions. MS analyses were performed on an API 3000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbo-ionspray interface. The source block temperature was 400 $^0$C and the electrospray capillary voltage was –3500V. Nitrogen was used as collision gas. MS detection was performed in negative mode using Multiple Reaction Monitoring (MRM).

The four transitions were monitored m/z 321→257, 321→ 194, 321→ 152, 326→ 157(IS) and for quantification, the transition m/z 321→257 was chosen. The dwell

time for each transition reaction was 500 ms and the declustering potential was 91V. The MRM transition and their collision energies are shown in table II.

Table II. MRM transitions monitored for CAP and internal standard CAP-d5

| Compound | Precursor m/z | Product m/z | Collision energy (eV) |
|---|---|---|---|
| CAP | 321 | 152 | 18 |
| CAP | 321 | 194 | 14 |
| CAP | 321 | 257 | 14 |
| CAP-d5 (IS) | 326 | 157 | 23 |

Sample preparation. The honey samples (1.0 ±0.01g) were weighed in 25 ml a centrifuge tubes and fortified with 100 µl of working internal standard CAP-d5 and diluted with 2.0 ml water. To the sample 2.0 ml hexane was added, well mixed, centrifuged and the upper layer was discarded. Four ml of acetonitrile was added to the aqueous phase, mixed, centrifuged and acetonitrile was evaporated until being dry under a stream of nitrogen using a heating block at 45 $^0$C. The dry residue was redissolved in 0.5 ml mobile phase acetonitrile: water (50:50, v/v) and was filtered through a 0.45 µm disposable filter. Twenty µl were injected into LC-MS/MS.

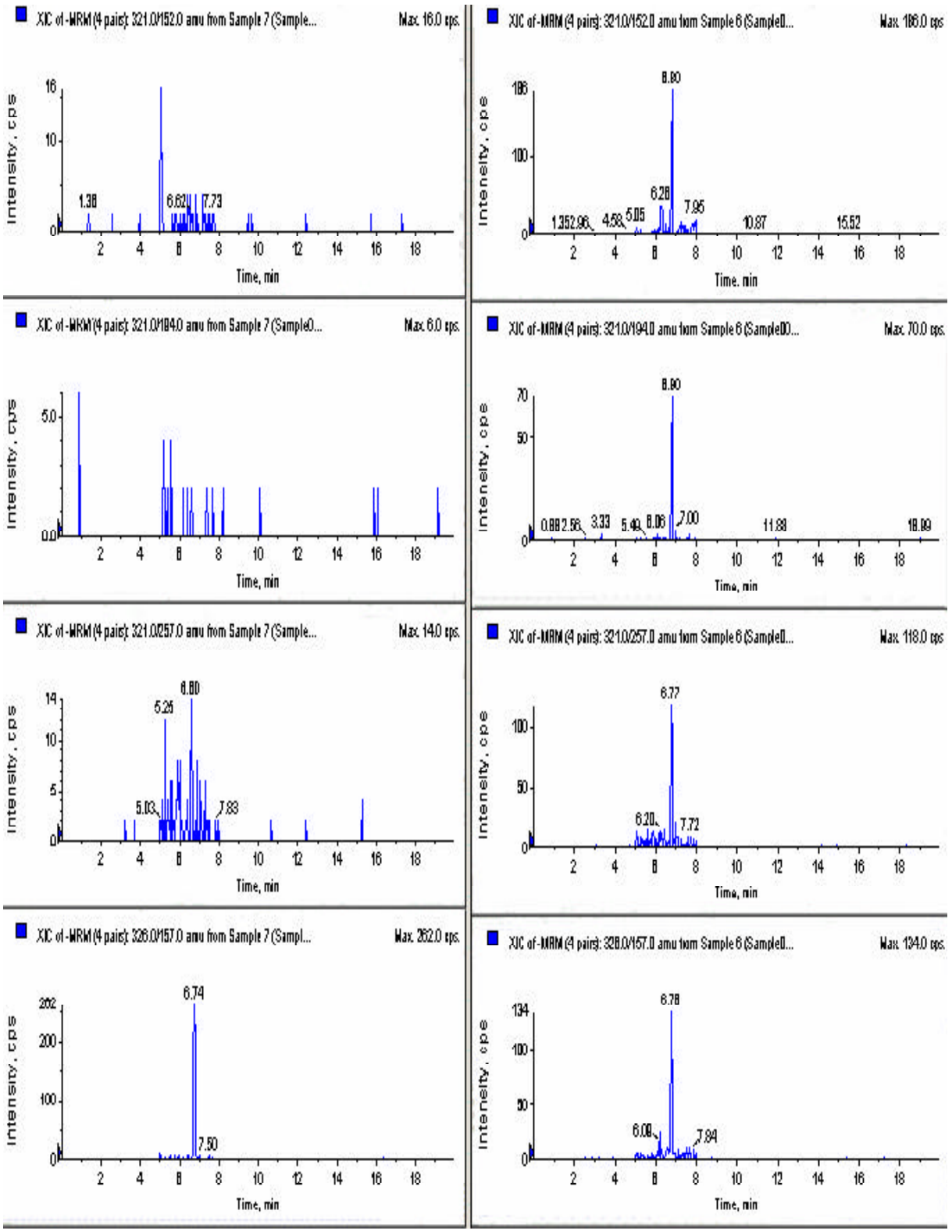
Calibration curves at five concentrations levels were prepared by spiking blank honey samples with CAP at the following concentrations: 0.0 (blank samples), 0.1, 0.15, 0.30 and 0.45 µg/kg. A fixed amount of an internal standard CAP-d5 was added to all the samples. The calibration curves were obtained relating to a ratio of CAP area (m/z 257)/ CAP-d5 area (m/z 157) with CAP concentration in µg/kg. A calibration curve with matrix was made every day.

## Results and Discussion

A gradient LC-ESI/MS/MS method was developed to separate and quantify with an internal standard, and confirm of CAP in honey. A MRM procedure was applied. The three transitions were monitored m/z 321→257, 321→ 194 and 321→152. According to Commission Decision 2002/657/EC[2] for the confirmation of banned substances a minimum of four identification points were required. The four identification points were obtained using LC-MS/MS with one precursor and two product ions. The presented research method detected 3 product ions. Thus the performance criteria for conformation was fulfilled. The method was validated according to the criteria of Commission Decision 2002/657/E [2]. According to these criteria, validation included the determination of specification, linearity, precision (within- and between-day), trueness, decision limit (CCα), and detection capability (CCβ).

Specificity of the method was checked by the preparation and analysis of blank and spiked honey samples from different origins to verify the absence of potential interfering compounds at CAP retention time. No interference was observed

around CAP retention times in honey samples. Figure 1 shows MRM chromatograms of blank and spiked CAP in honey at 0.3 µg/kg, respectively.




a b

Figure 1. LC-MS/MS chromatogram of honey extract, MRM mode, blank (a) and spiked matrix CAP 0.3µg/kg (b).

Response linearity was evaluated by calibration curves at five concentration levels. Calibration curves were built by using samples spiked in the 0.0–0.9 µg/kg and were repeated on three different days. The linear correlation coefficient was higher than 0.990 for the curves. Precision (within-day) and accuracy (recovery) were calculated from the analysis of blank honey spiked at three levels 0.5, 1.0, and 1.5 MPRL (0.15, 0.30, 0.45 µg/kg, respectively) of CAP. Six replicates were obtained for each concentration. Precisions (within-day) were found that satisfied the three levels studied and variation coefficients (CV%) were 5.5 – 8.3 %. The recovery (trueness) was calculated by comparing the measured concentration to the spiked concentrations. The average recovery was in the range of 91–104% for all levels. Precision (between-day) was calculated in spiked samples at 0.3 µg/kg on three different days (3x6). The coefficient of variation was lower than 12%. According to decision 2002/657/EC the coefficient of variation from the method was not the value calculated by the Horwitz. The coefficient of variation should be as low as possible in all cases. The precision and accuracy are presented in Table III.

Table III. Precision and accuracy for CAP determination in spiked honey samples

| Fortification levels (µg/kg) | 0.150 | 0.300 | 0.450 |
|---|---|---|---|
| Average (µg/kg) (n=6) | 0.156 | 0.274 | 0.435 |
| Within-day precision (%) | 8.3 | 6.6 | 5.5 |
| Recovery (%) | 104 | 91 | 97 |
| Between-day precision (%) (n= 3x6) | | 11.8 | |

The revised criteria also introduces the decision limit (CC$\alpha$) and detection capability (CC$\beta$) to replace the limit detection and quantification, respectively. CC$\alpha$ is defined as lowest concentration level that the identified substance can be declared as present with a statistical certainty of 1-$\alpha$ ($\alpha$=1%). CC$\beta$ is the smallest concentration of the analyte that can be detected, identified and quantified in a sample with an error probability of $\beta$ ($\beta \leq 5\%$). These limits were determined using six curves obtained at four levels 0.00, 0.15, 0.30 and 0.45 µg/kg. Six matrix calibration curves were prepared. The value CC$\alpha$ and CC$\beta$ were 0.11 and 0.14 µg/kg, thus below the MRPL set at 0.3 µg/kg by the EU.

This presented LC-MS/MS method is fast compared to the other presented methods which are very time consuming processes as derivatization procedure for GC-MS. The sample preparation is simple and has good precision and recoveries. The validation results are in accordance with the performance method of the European Commission Decision 2002/657/EC.

The method was used for routine analysis of CAP in honey samples. The commercial samples were collected in the eastern part of Poland in 2005-2006. CAP residues were not detected above the decision limit (CC$\alpha$ = 0.11 µg/kg) using this procedure.

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