Determination of the Chloramphenicol residues in milk and milk products using LC/MS/MS

**Purpose**

The European Union prohibits the use of chloramphenicol (CAP) as a veterinary drug for food producing animals. For this reason a rapid and sensitive confirmatory method for the identification and quantification of CAP in milk and milk products has been developed using the API 3000™ LC/MS/MS System. Although many analytical procedures are described in the literature, the presented method has the advantages of high recovery and short analysis time. The method was validated according to the EU Commission Decision 2002/657/EC.

**Overview**

Chloramphenicol (Figure 1) is a broad-spectrum antibiotic previously used in veterinary medicine because of its broad range of activity and its low cost. Chloramphenicol is primarily bacteriostatic. CAP binds to the 50S subunit of the ribosome and inhibits bacterial protein synthesis. It has a wide spectrum of activity against gram-positive and gram-negative cocci and bacilli, including anaerobes.

Because of the side effects in humans, particularly dose independent fatal aplastic anemia, the use of CAP for the treatment of food-producing animals is prohibited in several countries (e.g. European Union, Canada, United States, and most Asian counties). The Commission Decision 2002/657/EC necessitates control of CAP residues in edible tissues, milk, and milk products.

Methods to detect CAP residues in biological matrices, especially in milk, include immunoassays and mass spectrometry in combination with GC and HPLC. Immunological methods are suitable for screening purposes, whereas mass spectrometric methods are utilized for confirmation. Methods for the determination of CAP residues using GC/MS require additional chemistry steps, such as silylation of CAP before the samples can be analyzed. LC/MS, on the other hand, requires no derivatization and can be directly analyzed, minimizing possible derivatization problems, saving time, and preventing compound losses.

This paper describes a confirmatory research method based on HPLC and triple quadrupole mass spectrometry that is suitable for quantitative determination of CAP residues in milk and milk products in a low concentration range.
Table 1. Clean up for milk samples based on method by Hormazabal et al.\(^7\) as presented at the 16th International Mass Spectrometry Conference in Edinburgh.\(^8\)
**Key Features**

- Sensitive and selective Multiple Reaction Monitoring (MRM) scan function for linear quantitative analysis with wide dynamic range
- Patented LINAC® collision cell allows multi-component analysis at reduced MRM dwell times maintaining sensitivity and preventing crosstalk
- Rugged and reliable triple quadrupole MS system for maximum uptime
- Compatible with the broadest range of ionization sources for the analysis of a wide range of compounds

**Experimental Conditions**

Blank milk samples were fortified with CAP in the concentration range of 0.05 – 1.5 µg/L. The cleanup consisted of an acetonitrile extraction, followed by defatting with chloroform and then solid phase extraction on a styrene-divinyl benzene polymer cartridge (Table 1). The final extract was analyzed on an API 3000™ LC/MS/MS system using a TurboIonSpray® source in negative ion mode. Evaluation was performed by selecting the characteristic product ions for chloramphenicol and using Multiple Reaction Monitoring of four transitions m/z 321→152, 321→176, 321→194 and 321→257. For quantification, the transition m/z 321→152 was chosen. The method was validated according to the EU Commission Decision 2002/657/EC.

All solvents are HPLC-grade unless otherwise noted. Demineralized water was used in all experiments. All standard solutions of chloramphenicol (Sigma, Deisenhofen, Germany) were prepared with methanol and kept at +4°C. Bond Elute LMS SPE columns of crossed linked styrene divinyl benzene polymer (1 cc/25 mg) were used for solid phase extractions. After solid phase extraction and drying under nitrogen the sample was redisolved in water and then filtered on a 0.45 µm cellulose filter.

An Agilent 1100 Series HPLC equipped with an autosampler and degasser (Agilent, Palo Alto, CA, USA) was used for chromatographic analysis in this study. Eluent A consists of H₂O with 2 mM ammonium acetate and eluent B is methanol. A 50µL sample was injected onto a Luna column (50 mm x 2.0 mm, 3 µm particle size) (No. 008-4256-B0 Phenomenex, Torrance, CA, USA) equipped with a guard column (No. AJ0-4350, Phenomenex, Torrance, CA, USA). The sample was eluted with the following gradient at a flow rate of 350 µL/min:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>10.1</td>
<td>55</td>
<td>45</td>
</tr>
</tbody>
</table>

An API 3000™ LC/MS/MS system with a TurboIonSpray® source was operated in the negative ion mode using the following parameters:

- Nebulizer gas (air): 8
- Curtain gas (N₂): 10
- CAD gas (N₂): 7
- Dwell time: 250 ms/MRM transition
- Temperature: 400°C
- Ion spray voltage (IS): -3500
- Entrance potential (EP): 10
- Declustering potential (DP): -30
- Focusing potential (FP): -300
- Cell exit potential (CXP): -15

**Figure 2.** LC/MS/MS chromatogram of the CAP standard (50 pg in 50 µL injection volume). Ion traces of all four transitions are presented.
The collision energy (CE) was optimized for each of the 4 MRM transitions monitored throughout the HPLC run. The collision energy for each MRM transition is listed below:

<table>
<thead>
<tr>
<th>Precursor ion (Q1)</th>
<th>Product ion (Q3)</th>
<th>Collision energy (CE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>321.0</td>
<td>152.0</td>
<td>-24</td>
</tr>
<tr>
<td>321.0</td>
<td>257.0</td>
<td>-12</td>
</tr>
<tr>
<td>321.0</td>
<td>194.0</td>
<td>-16</td>
</tr>
<tr>
<td>321.0</td>
<td>176.0</td>
<td>-20</td>
</tr>
</tbody>
</table>

Under these conditions, the m/z 152 product ion was the base peak (Figure 3).

**Results and Discussion**

Commission Decision 2002/657/EC set the performance criteria for mass spectrometric detection as a confirmatory method following either an on-line or an off-line chromatographic separation. When mass fragments are measured a system of identification points shall be used according to this definition. For the confirmation of substances listed in Group A of Annex I of Directive 96/23/EC (e.g. chloramphenicol) a minimum of 4 identification points is required.

According to this definition, four identification points are obtained using LC/MS/MS with 1 precursor and 2 product ions. The presented research method detects 4 product ions, thus the performance criteria for confirmation is fulfilled.

Figure 3 shows the 4 MRM chromatograms in spiked milk samples. Due to the sample matrix peak detected in MRM chromatogram of the product ion m/z 257 the product ion m/z 152 was chosen for quantification purposes. The MRM chromatogram of m/z 321→152 is without matrix peak in the front of the chromatograms (Figure 4).

The validation was made according to the criteria of Commission Decision 2002/657/EC. The limit of decision (ccα) was determined using 20 blank milk samples with 3 parallel analysis and calculated using the lowest diagnostic ion signal/noise ratio as described in the EU regulation.

The calculated ccα was 0.03 µg/L. The limit of detection (detection capability, ccß) was determined using 20 blank milk samples, spiked with 0.1 µg/L CAP (duplicate samples). The calculated ccß was 0.1 µg/L.
The recovery of the assay was tested using spiked blank milk samples in from 0.1 µg/L to 0.5 µg/L CAP concentration range (n=12). The calculated recovery was about 70% with a coefficient of variation of 5%.

The precision was determined using calibration curves, prepared with spiked blank milk samples from 0.05 to 1.5 µg/L concentration range (n=7). The number of replicates of each concentration was 10. Table 2 shows the coefficient of correlation for all 4 MRM transitions. The coefficients of correlation of the calibration curves calculated for all 4 MRM transitions were > 0.99.

The “intra-day” reproducibility was determined at 0.1 µg/L concentration (n=6x3) and it was < 9%. The interferences in the chromatogram from different milk samples were investigated and eliminated using an appropriate HPLC column with guard column and using appropriate product ions. The product ions used are listed in Table 2.

For the chromatographic separation, the deviation of the retention time between standard and samples were inside of 2.5% — the average retention time of the standard was 2.54 minutes and 2.56 minutes for the milk samples.

For the relative ion intensities of mass spectrometric detection the deviation of relative ion intensity in spiked milk samples vs. standard was lower than 10%.

**Conclusion**

The presented method is able to detect and to quantify chloramphenicol residues in milk and milk products in the lower ppb concentration range. With the excellent sensitivity of the API 3000™ system, the limit of detection (ccα) and the limit of decision (ccβ) were calculated to be 0.03 µg/L and 0.1 µg/L respectively. This method along with the proven reliability and stability of the API 3000 system provides for a dependable and consistent determination of CAP as demonstrated by the precision of the correlation coefficients (r²) at > 0.99 for the 4 calibration curves and the < 9% variability in the “intra-day” reproducibility calculations. Due to its high specificity, sensitivity, and stability this LC/MS/MS assay on the API 3000 system fulfills the criteria of the EU regulation 2002/657/EC.

**References**


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<table>
<thead>
<tr>
<th>Precursor ion (Q1)</th>
<th>Product ion (Q3)</th>
<th>Coefficient of Correlation (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 321</td>
<td>152</td>
<td>0.9993</td>
</tr>
<tr>
<td>m/z 321</td>
<td>257</td>
<td>0.9994</td>
</tr>
<tr>
<td>m/z 321</td>
<td>176</td>
<td>0.992</td>
</tr>
<tr>
<td>m/z 321</td>
<td>194</td>
<td>0.998</td>
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Table 2. Coefficient of correlation (r²) of the calibration curves of all four transitions.
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Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: 650.638.5800
Toll Free: 800.345.5224
Fax: 650.638.5884

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