Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic with historical veterinary uses in all major food-producing animals (see Figure 1 for structure). It has serious side effects on humans that may cause aplastic anemia, and the suspected carcinogen effect is also thought to be dose independent. Consequently, chloramphenicol has been banned for use in all food-producing animals by the European Union (EU), USA and Canada. A minimum required performance limit (MRPL) for chloramphenicol determination was recently set by the EU at 0.3 µg/kg (ppb) in all foods of animal origin, such as meat, seafood, egg, milk, honey, etc. However, residues of CAP at unacceptable levels continue to be found in food imports, as a result of illegal use in some countries to mask the poor hygiene conditions of animal-raising farm and to augment animal growth. The growing food safety concerns call for intensive surveillance of chloramphenicol in food products.

Analysis of residual of chloramphenicol in foodstuff is challenging because of the complicated sample matrices and stringent requirements of both low quantitation limit (<0.3 ppb) and method validation. The technique of liquid chromatography separation followed by tandem mass spectrometry detection, LC-MS/MS, is the technology of choice because of its sensitivity and specificity. A sample cleanup process is generally required to remove the sample matrix prior to the LC-MS/MS run. Typically, this involves the costly and labor-intensive solid phase extraction (SPE) and/or liquid-liquid extraction (LLE) procedures.

In this work, we report a simple sample preparation procedure involving only the acetonitrile protein precipitation and dilution to extract the CAP from milk, followed by a high-speed LC separation and detection by a triple quadrupole mass spectrometer operated in selected reaction monitoring (SRM) mode. The sample preparation is simple, fast, and inexpensive, and the method exceeds the sensitivity and specificity requirements for both screening and confirmatory assays. Validation according to the European Commission Decision 2002/657/EC has also been performed.

Goal

To develop a simple, rapid, and sensitive LC-MS/MS method for analyzing chloramphenicol in milk. The method should be suitable for both screening and confirmatory purposes.

Experimental Conditions

Sample Preparation

Standards and Regents: Chloramphenicol (98%) was purchased from Sigma-Aldrich (St. Louis, MO) and d5-chloramphenicol (100 µg/mL in acetonitrile) as internal standard from Cambridge Isotope Lab (Andover, MA). Regent grade water, acetonitrile and methanol were from Thermo Fisher Scientific (Pittsburgh, PA).

Procedures:

0.5 g Milk + d5–CAP (0.3 ppb) as IS
+ 0.75 mL CH3CN, vortex 1 min, Centrifuge @ 14000 rpm for 10 min
Take 0.7 mL Supernatant + 0.3 mL Water, store at 4°C for ≥ 1 hr
Pipette 0.8 mL upper solution for LC-MS/MS Analysis

Chromatography Conditions

HPLC Module: Accela High Speed LC System
Column: Hypersil GOLD™ 50 mm × 2.1 mm and 1.9 µm particle size (Thermo Scientific, Bellefonte, PA)
Column Temperature: Ambient
Mobile Phase: A: Methanol  B: Water
Gradient: Time (min)  A %
0.0-0.6 5%
2.3 100%
2.35-3.0 5%
Flow Rate: 500 µL/min
Injection Volume: 20 µL (with loop)
Table 2: Relative ion abundances at various CAP concentrations in milk and the associated tolerances required by Decision 2002/657/EC

<table>
<thead>
<tr>
<th>CAP Spiked Level (µg/kg)</th>
<th>Relative Ion Abundance of 257/152</th>
<th>Relative Ion Abundance of 194/152</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (n=6) %RSD (n=6) Tolerance by Decision 2002/657/EC</td>
<td>Mean (n=6) %RSD (n=6) Tolerance by Decision 2002/657/EC</td>
</tr>
<tr>
<td>0.05</td>
<td>96% 16% 20%</td>
<td>26% 21% 25%</td>
</tr>
<tr>
<td>0.15</td>
<td>92% 7.6% 20%</td>
<td>28% 25%</td>
</tr>
<tr>
<td>0.30</td>
<td>93% 15%</td>
<td>31% 15%</td>
</tr>
<tr>
<td>0.50</td>
<td>99% 3.4%</td>
<td>31% 17%</td>
</tr>
</tbody>
</table>

Note: Relative ion abundance values were calculated by relative peak area ratios

Table 1: SRM transitions for CAP and d5-CAP (IS)

<table>
<thead>
<tr>
<th>Mass Spectrometer Conditions</th>
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<tbody>
<tr>
<td>Mass Spectrometer: TSQ Quantum Access triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA)</td>
</tr>
</tbody>
</table>

Source: ESI+, 3000 V
Sheath Gas: 45 unit
Auxiliary Gas: 10 unit
Capillary Temperature: 300°C
Source CID: -7 V
Collision Gas: Ar (1.5 mTorr)
Scan Time: 0.1 s
Q1 and Q3 Peak Width (FWHM): 0.7 Da
Capillary Temperature: 300°C
Auxiliary Gas: 10 unit
Sheath Gas: 45 unit
Source: ESI-, 3000 V

Method Performance: Figure 2 shows representative SRM chromatograms for a blank and 0.05 µg/kg spiked milk samples. As shown, with high-speed LC, each chromatographic run is only 3 min, allowing high throughput for screening assay. All three SRM traces for CAP at 0.05 µg/kg spiked samples can be well quantified. Note that the 0.05 µg/kg spiked in milk is equivalent to 0.46 pg injected on column by assuming a full recovery.

It should also be noted that with the high-speed LC separation of only 3 min for each chromatographic run, the CAP peak width (at 10% above baseline) is as narrow as 6 s. Under current MS acquisition conditions, there are 13-14 points across each peak, enough for maintaining a well-defined peak shape for accurate integration.

A representative calibration curve from standards prepared in milk is shown in Figure 3. Good linearity from 0.05 to 1.0 µg/kg with correlation coefficient of R²= 0.9954 (Weighting factor W = 1/X) was obtained.

Table 3 shows excellent recovery and within-laboratory reproducibility of the method (at four different days). Decision Limit (CCα) and Detection Capability (CCβ): According to Decision 2002/657/EC, the Decision Limit CCα is the minimum CAP concentration at which a sample is really non-compliant with an error probability of 1% (α=0.01), and the Detection Capability (CCβ) is the minimum amount of CAP that can be quantified and confirmed with an error probability of 5% (β=0.05).

Two methods can be used for calculating the CCα according to the Decision. One is to use the S/N ratio of 3:1 of blank samples, similar to those for estimation of limit of detection. The other is to use the intercept of calibration curve at low levels and the within-laboratory reproducibility. The former method does not work well for LC-MS/MS because the very low background (noise count ~0) of SRM chromatogram often yields unrealistically low values for CCα. Thus we use the latter approach by using cali-
bration data of (0.05-0.15-0.30 µg/kg) to obtain the Y-intercept and its standard deviation, SD_{Y-intercept}:

\[ CC_{\alpha} = Y\text{-intercept} + 2.33 \times SD_{Y\text{-intercept}} \]

Similarly, the \( CC_{\beta} \) can be calculated from \( CC_{\alpha} \) and the standard deviation of 20 measurement of samples spiked at \( CC_{\alpha} \) level. Here the latter term is approximated with the within-laboratory reproducibility data of 0.15 µg/kg spiking level, thus,

\[ CC_{\beta} = CC_{\alpha} + 1.64 \times SD_{0.15 \mu g/kg} \]

Where \( SD_{0.15 \mu g/kg} \) is the within-laboratory reproducibility (in standard deviation) of the 0.15 µg/kg in Table 3. The calculated values of \( CC_{\alpha} \) and \( CC_{\beta} \) are 0.087 µg/kg and 0.12 µg/kg, respectively.

<table>
<thead>
<tr>
<th>CAP Spiking Level</th>
<th>Within-laboratory Reproducibility (n = 20)</th>
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<tbody>
<tr>
<td>µg/kg</td>
<td>Mean (%)</td>
</tr>
<tr>
<td>0.05</td>
<td>97%</td>
</tr>
<tr>
<td>0.15</td>
<td>101%</td>
</tr>
<tr>
<td>0.30</td>
<td>104%</td>
</tr>
<tr>
<td>0.50</td>
<td>94%</td>
</tr>
</tbody>
</table>

Table 3: Recovery and Reproducibility Data
Conclusions
A simple, rapid and sensitive method for analysis of CAP in milk by LC-MS-MS has been developed and validated. The sample preparation by protein precipitation and dilution is very simple to perform and avoids the use of SPE or LLE. With the high-speed Accela LC coupled to a triple quadruple TSQ Quantum Access, each analytical run is as short as 3 min. The method can be used for the purposes of both high-throughput screening and rapid confirmatory assays.

For screening assay, the method can detect < 0.050 µg/kg CAP in milk. For confirmatory assay, the method validated according to Decision 2002/657/EC gives a CCα =0.087 µg/kg and CCβ = 0.12 µg/kg, both below the MRPL of 0.3 µg/kg.

References

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