Application Note: 407

Simple and Rapid Analysis of Chloramphenicol in Milk by LC-MS/MS

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Key Words

- TSQ Quantum Access™
- Accela™ High Speed LC System
- Antibiotic
- Food Residue Analysis
- SRM

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.

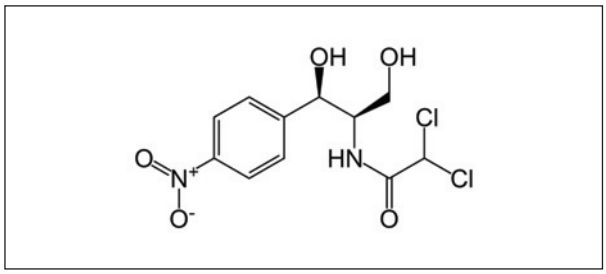


Figure 1: Structure of chloramphenicol

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

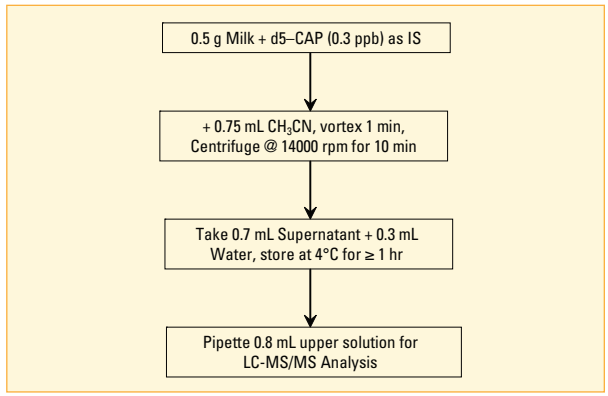
To develop a simple, rapid, and sensitive LC-MS/MS method for analyzing chloramphenicol in milk. The method should be suitable for both screening and confirmatory purposes.

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:



Chromatography Conditions

HPLC Module: Accela High Speed LC System (Thermo Scientific, San Jose, CA)
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)

Mass Spectrometer Conditions

Mass Spectrometer: TSQ Quantum Access triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA)
Source: ESI-, 3000 V
Sheath Gas: 45 unit
Auxiliary Gas: 10 unit
Capillary Temperature: 300 °C
Source CID: -7 V
Q1 and Q3 Peak Width (FWHM): 0.7 Da
Scan Time: 0.1 s
Collision Gas: Ar (1.5 mTorr)
SRM Transitions: 3 SRMs for CAP, 1 SRM for d5-CAP (see Table 1)

	Precursor Ion	Product Ion (Collision Energy)
CAP (M^-H^-)	320.93	152 (17)* 257 (15) 194 (16)
d_5-CAP (M^-H^-)	326.93	157 (17)*

* Product ion used for quantitation

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

Results and Discussion

Sample Preparation: A major goal for the method development in this study is to avoid using the labor intensive and time-consuming SPE or LLE procedures as in literatures. In curret work, the proteins from milk were removed with acetonitrile precipitation at ratio of 1.5:1 (v/v Acetonitrile:Milk), followed by dilution with water, which is necessary for gradient chromatographic separation. At such ratio, protein removal was not complete, trace amount of precipitates of proteins appeared after the sample was stored at 4 °C for some time. Thus, the supernatant was taken for LC-MS/MS analysis after the sample was stored at 4 °C for ≥ 1 hr.

Choice of Quantitation and Qualification Ions: Three product ions were chosen to give an Identification Points (IPs) of 5.5 to meet the requirement of ≥ 4.0 IPs by the Decision 2002/657/EC for confirmatory assay of the prohibited substances such as CAP. The *m/z* 152 was chosen as quantitation ion, the *m/z* 257 and 194 as confirmation ions, consisting with those reported in literatures.

The results of relative ion abundance measured at various concentrations are given Table 2. Both relative ion abundance ratios of 257/152 and 194/152 meet the requirements set by Decision 2002/657/EC.

Note that we found the 321>257 transition is more likely subjected to matrix interferences in many other cases of different matrices, thus if two SRM transitions need to be selected (4.0 IPs) for the method, 321>152 and 321>194 are preferred.

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 R^2= 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-

CAP Spiked Level (µg/kg)	Relative Ion Abundance of 257/152			Relative Ion Abundance of 194/152		
	Mean *n*=6	%RSD *n*=6	Tolerance by Decision 2002/657/EC	Mean *n*=6	%RSD *n*=6	Tolerance by Decision 2002/657/EC
0.05	96%	16%	20%	26%	21%	25%
0.15	92%	7.6%		28%	25%	
0.30	93%	15%		31%	15%	
0.50	90%	3.4%		31%	17%	

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

Table 2: Relative ion abundances at various CAP concentrations in milk and the associated tolerances required by Decision 2002/657/EC

bration data of (0.05-0.15-0.30 µg/kg) to obtain the Y-intercept and its standard deviation, $SD_{Y\text{-intercept}}$,

$$CC\alpha = Y\text{-intercept} + 2.33 * SD_{Y\text{-intercept}}$$

Similarly, the CCβ can be calculated from CCα and the standard deviation of 20 measurement of samples spiked at CCα level. Here the latter term is approximated with the within-laboratory reproducibility data of 0.15 µg/kg spiking level, thus,

$$CC\beta = CC\alpha + 1.64 * SD_{0.15\ \mu g/kg}$$

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 CCα and CCβ are 0.087 µg/kg and 0.12 µg/kg, respectively.

CAP Spiking Level (µg/kg)	Within-laboratory Reproducibility (n = 20)		
	Mean (%)	SD (µg/kg)	%RSD
0.05	97%	0.0065	14%
0.15	101%	0.020	13%
0.30	104%	0.037	11%
0.50	94%	0.042	8.0%

Table 3: Recovery and Reproducibility Data

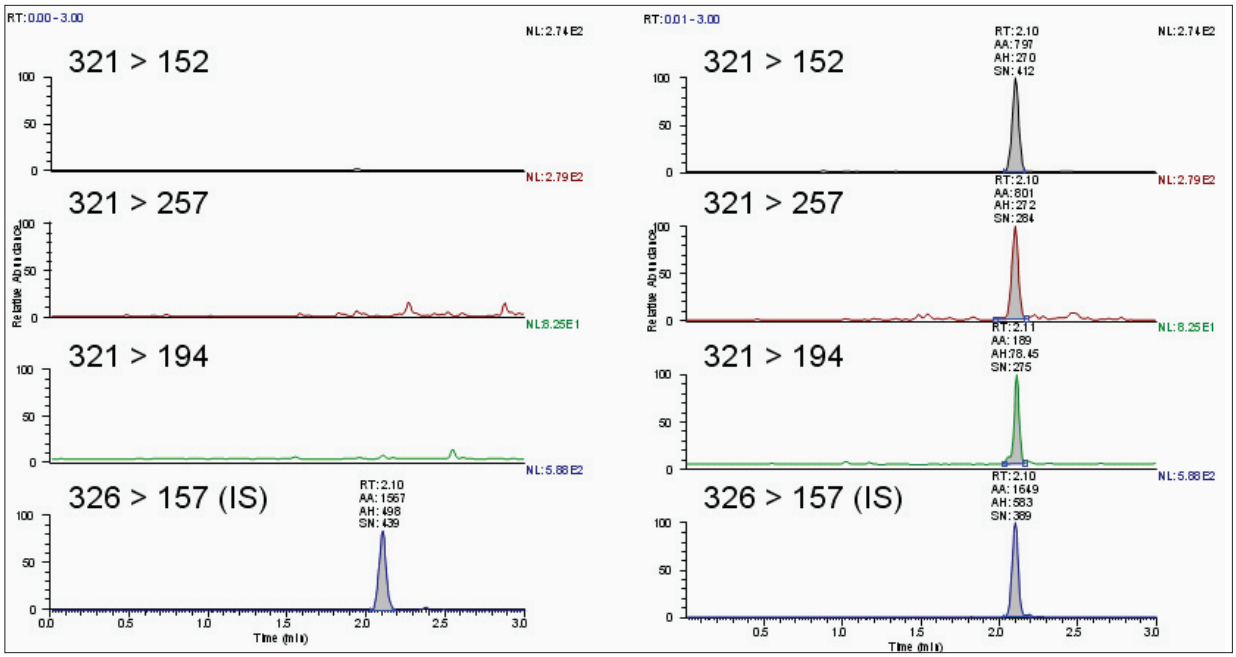


Figure 2: SRM chromatograms for milk blank and 0.050 µg/kg spiked milk samples

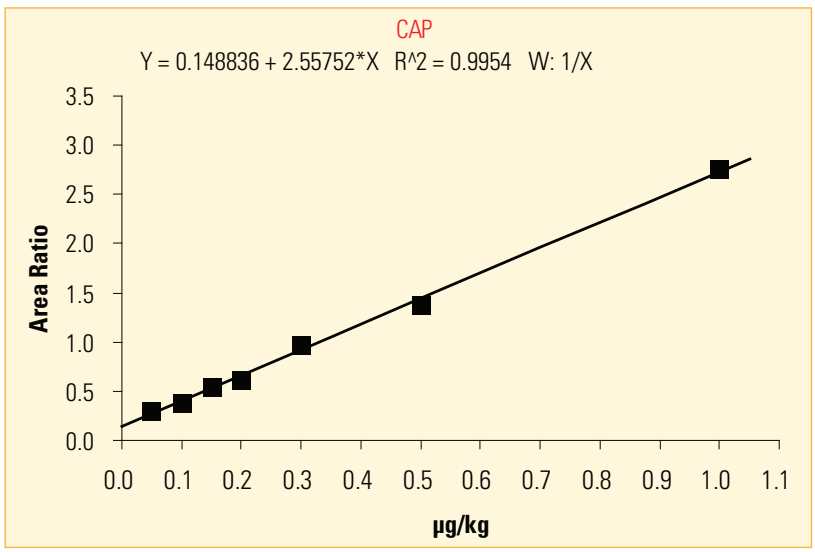


Figure 3: Calibration of CAP in milk

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.

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