DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Chloramphenicol in Bovine Milk by Liquid Chromatography/Tandem Mass Spectrometry

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A rapid confirmatory liquid chromatographic/tandem mass spectrometic method was developed for determination of chloramphenicol in bovine milk. Chloramphenicol was extracted directly from milk by solid-phase extraction on a C_{18} cartridge. The extract was further cleaned up on neutral aluminium oxide. Three transition products were monitored in negative ion mode after atmospheric pressure chemical ionization. The detection capability related to the transition product of lowest abundance was 0.03 μg/kg. The mean recovery was 90% at levels of 0.1–0.2 μg/kg. The relative repeatability standard deviations were 4.3, 3.8, and 2.8% at levels of 0.1, 0.2, and 1.0 μg/kg, respectively.

Chloramphenicol (CAP) is a broad-spectrum antibiotic active against Gram-positive and -negative bacteria. The compound has been banned in several countries, including the European Union (EU), for treatment of food-producing animals. Chromatographic methods for determination of CAP in milk include methods based on either gas chromatography (GC) with electron capture detection (1), GC coupled with mass spectrometry (GC/MS; 2), liquid chromatography (LC) with UV detection (3–9), LC with electrochemical detection (10), and LC coupled with single quadrupole MS (LC/MS; 11) or tandem MS (LC/MS/MS; 12). Extraction and cleanup of CAP residues in milk for LC analysis have mainly been based on liquid–liquid extraction with solvents such as ethyl acetate (3, 5, 12), acetone (12), chloroform–acetone (9), or acetonitrile (4, 11), followed by washes with dichlormethane (4), isooctane (3), hexane (4, 9, 12), or chloroform (3, 11), or solid-phase extraction (SPE; 11). Although liquid–liquid extractions may give clean extracts, they are generally not suitable for high throughput or automated analysis. In all cases, the reported limits of detection were 1 μg/kg or above.

The objective of the present study was to develop a simple and sensitive LC/MS/MS confirmatory method for determination of CAP residues in milk, reducing sample pretreatment to a minimum.

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Experimental

Materials

(**a**) *Stock solution of CAP (1000 μg/kg).*—CAP (Cat. No. 85,744-0, Aldrich, Steinheim, Germany) dissolved in methanol.

(**b**) *Standard solutions of CAP (2, 20, 40, 60 μg/L).*—Prepared by diluting stock solution in 25% acetonitrile.

(**c**) *Standard solution of CAP for spiking of samples (100 μg/L).*—Prepared by diluting stock solution with water.

(**d**) *Solvents.*—Methanol and acetonitrile, chromatography grade.

(**e**) *Purified water.*—From Milli-Q Plus apparatus (Millipore, Bedford, MA).

(**f**) *LC mobile phase.*—25% acetonitrile in 5mM ammonium acetate adjusted to pH 4.0 with acetic acid.

(**g**) *Commodities.*—Raw milk was obtained from individual farmers in all regions of Denmark. Nonhomogenized and standardized bulk milk (3.5% fat) pasteurized to inactivation of the alkaline phosphatase enzyme (LP milk), homogenized and standardized bulk milk (3.5% fat) pasteurized to inactivation of the alkaline phosphatase enzyme (hLP milk), and bulk skim milk pasteurized to inactivation of the peroxidase enzyme (HP skim milk) were purchased from local grocery stores. Inactivation of the alkaline phosphatase enzyme is typically performed by heating the milk to 72°C for 15 s in a plate heat exchanger unit. Inactivation of the peroxidase enzyme is typically performed by heating the milk to 87°C for 15 s. Bulk milk treated at ultra high temperature (UHT milk), whole milk powder, and skim milk powder were obtained from production plants in Denmark. None of the milk samples were frozen before analysis.

Apparatus

(**a**) *Liquid chromatograph.*—Agilent 1100 Series system consisting of binary pump, solvent degasser, autosampler, and column oven (Agilent Technologies, Waldbronn, Germany).

(**b**) *Analytical column.*—NovaPak C_{18}, (3.5 μm, 150 × 4.6 mm id; Waters Corp., Milford, MA).

(**c**) *Mass spectrometer.*—Sciex API 2000 triple quadrupole instrument equipped with heated nebulizer for atmospheric pressure chemical ionization (APCI; Applied Biosystems, Foster City, CA).

(**d**) *Centrifuge.*—Sigma Model 4K15 (Osterode, Germany).

(**e**) *Vortex mixer.*—VF2 (IKA, Staufen, Germany).

(**f**) *pH meter.*—PHM 93 (Radiometer, Copenhagen, Denmark).

Table 1. Factors and factor levels used for testing ruggedness of procedure for determination of CAP in raw milk and pasteurized and homogenized milk

Factor	Factor level 1	Factor level 2
C_{18} brand	IST	Varian
Volume of wash water used to rinse C_{18} cartridge after sample application, mL	3.0	5.0
Vacuum drying time before elution of CAP from C_{18} cartridge, s	0	10
Volume of acetonitrile used for elution of CAP from C_{18} cartridge, mL	2.5	3.5
Aluminium oxide brand	IST	Varian
Volume of 90% acetonitrile used for washing aluminium oxide cartridge after sample application, mL	1.0	3.0

(**g**) *Evaporator.*—Temperature-controlled heating block with manifold for nitrogen flow (Mikrolab Aarhus, Aarhus, Denmark).

(**h**) *Vacuum manifold.*—For SPE cartridges (Waters Corp.).

(**i**) *SPE columns.*—C_{18} cartridges, 500 mg, 3 mL [(Cat. No. 220-0050-B, International Sorbent Technology (IST), Hengoed, UK; or Cat. No. 1210-2028, Bond Elut from Varian, Harbor City, CA)]; neutral aluminium oxide cartridges, 1000 mg (Cat. No. 714-0100-C, IST; or Cat. No. 12166045B, Varian).

(**j**) *Filters.*—Acrodisc 13 PVDF, 13 mm × 0.45 μm, disposable (Gelman Sciences, Ann Arbor, MI).

(**k**) *Containers.*—Polypropylene centrifuge tubes with screw cap, 15 and 50 mL (Sarstedt, Nümbrecht, Germany).

Sample Preparation

A volume of ca 20 mL milk or reconstituted milk powder (2 g in 18 mL water) was centrifuged at 3000 *g* for 5 min at 20–25°C. A 10 mL volume of the noncream layer was heated to 38–40°C and pulled through a 500 mg C_{18} cartridge (flow rate ca 2 mL/min), which had previously been conditioned with 5 mL methanol followed by 3 mL water. The cartridge was washed with 4 mL water, dried by vacuum (20 inches Hg) for 5 s, and eluted with 3 mL acetonitrile. The eluate was mixed with 150 μL water and percolated through a 1000 mg neutral aluminium oxide cartridge, which had previously been washed with 5 mL 90% acetonitrile. The cartridge was rinsed with 2 mL 90% acetonitrile, and the combined effluent was evaporated to bare dryness at 50–55°C. The residue was redissolved in 500 μL 25% acetonitrile and filtered. The procedure, thus, resulted in a 20-fold concentration of CAP residues.

Preparation of Matrix Calibration Standards

Preparation of matrix calibration standards for determination of true recovery.—Samples of LP milk were extracted according to the procedures. The final residue was redissolved in 500 μL standard solution containing 2, 20, 40, and 60 μg/L CAP, *Materials* (**b**).

Preparation of method matrix calibration standards.—Samples of LP milk were spiked with CAP standard, *Materials* (**c**), to levels of 0.1, 1, 2, and 3 μg/kg before centrifugation and were extracted according to the procedures.

LC–MS/MS Analysis

A volume of 100 μL extract was injected into the LC system for 15 min. The LC flow rate was 0.6 mL/min, and column temperature was kept at 25 ± 1°C. The eluent was diverted to waste for the first 5 min after injection by post-column switch. The APCI probe temperature was kept at 500°C, and the nebulizer current was set at –2 μA. Selected reaction monitoring (SRM) was used for the transitions *m/z* 321→152, 321→194, and 321→257. The potentials were –23 V between orifice and skimmer (declustering potential), –350 V between focusing ring and skimmer (focusing potential), and –10 V between skimmer and entrance quadrupole (entrance potential). The potential differences between en-

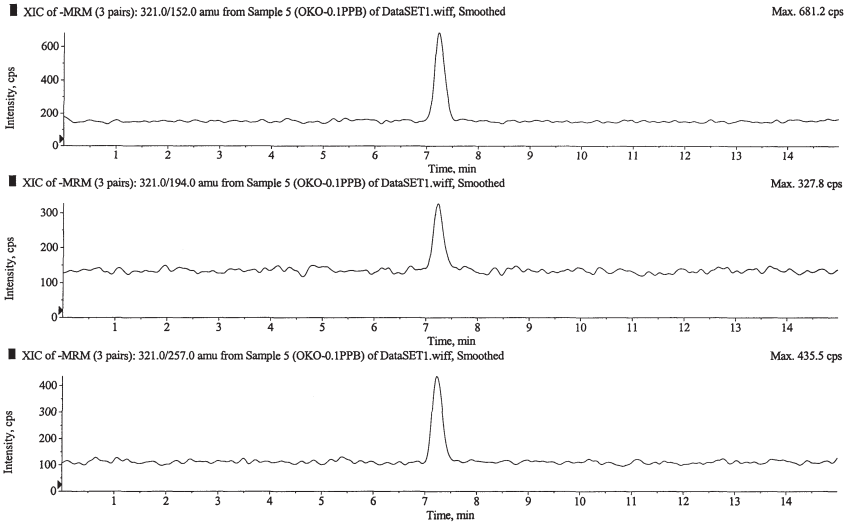

Figure 1. LC–APCI–MS/MS chromatograms of milk fortified with 0.1 μg/kg CAP.

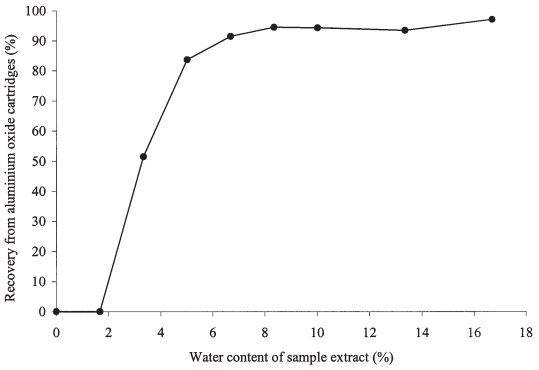

Figure 2. Effect of water concentration of sample extract on recovery of CAP from aluminium oxide cartridges.

trance quadrupole and collision cell quadrupole (collision energy) were –20 V for the *m/z* 152 transition and –14 V for the *m/z* 194 and 257 transitions. Nitrogen was used as collision gas. Data acquisition and processing were performed with Analyst Software 1.1 (Applied Biosystems).

Detection Capability

The detection capability (CCβ; 13) was determined on 35 different milk samples, which included 15 raw milk samples from Reed Danish Breed and Danish Holstein herds and 5 raw milk samples from Jersey herds (3.5–6.1% fat), 5 samples of LP milk, 5 samples of hLP milk, and 5 samples of HP skim milk. The samples were spiked before centrifugation and extraction with CAP to a level 3 times the signal-to-noise ratio for the 321→194 transition. The samples were, thus, spiked with CAP to a level of 0.015 μg/kg. The spiked samples were mixed and stored for 1 h at 5–7°C before extraction. The CCβ was determined as the spike level plus 1.64 times the standard deviation (SD) of the 35 measurements.

Precision and Recovery

The repeatability standard deviation (i.e., the variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample and in a short interval of time), the intralaboratory reproducibility standard deviation (i.e., the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators under different experimental conditions), and the recovery were determined on samples of LP and hLP milk. The samples were spiked to levels of 0.1, 0.2, and 1 μg/kg with CAP and were analyzed in duplicate on each of 8 days. Repeatability was calculated in accordance with ISO standard 5725-2, 1994 (14). The intralaboratory reproducibility was calculated by the same principle used for determination of reproducibility (14). The recovery was also determined on 20 different raw milk samples, 5 HP skim milk samples, 5 samples of reconstituted whole milk powder (2 g in 18 mL water), 5 samples of reconstituted skim milk powder (2 g in 18 mL water), and 5 samples of UHT milk. All samples were spiked to a level of 0.2 μg/kg, mixed, and stored for 1 h at 5–7°C before centrifugation and extraction.

Ruggedness

The method was tested for matrix-induced effects on signal intensity. Standards and final sample extracts of raw and hLP milk spiked with 4 ng CAP were analyzed in attenuated order. The test was performed on the same samples as used for CCβ determination.

The influence of small variations in the analytical procedure was tested by using a fractional factorial IV design with 3 center points on raw and hLP milk spiked with CAP to a level of 0.2 μg/kg. The varied factors are listed in Table 1. Peak area response was monitored. Creation of the experimental design and data processing were performed with Modde version 4.0 (Umetri, Umeå, Sweden).

The stability at 5–7°C of calibration standard solutions and final extracts of milk spiked with CAP to a level of 0.1 μg/kg was tested over a period of 8 days.

Results and Discussion

Because of the liquid nature of milk, it was possible to perform a simple direct extraction of CAP by SPE. The only pretreatment was centrifugation for removal of fat globules. The residual fat content in the milk phase was about 0.06% for raw and LP milk, and about 2.2% for hLP milk. The residual fat globules did not pose a problem for extraction. The acetonitrile eluate from the C_{18} cartridge was percolated through a neutral aluminium oxide cartridge to obtain a purified colorless extract. When cleanup on aluminium oxide was omitted, a significant amount of matrix components was visible in the final dry extract.

The MS/MS conditions were optimized by infusion of 500 μg/L CAP standard using a TurboIonSpray interface (Sciex). The flow rate was 3 μL/min. The optimization was first performed in the MS mode and then in the SRM mode. Optimization of compound- and instrument-dependent parameters was performed manually by ramping the individual parameters. The APCI interface parameters were then optimized by flow injection of 10 μL volumes of a 50 μg/L standard solution of CAP into mobile phase (flow 0.6 mL/min).

Table 2. Relative repeatability standard deviation (RSD_r) and intralaboratory reproducibility standard deviation ($RSD_{R,intra}$) determined on spiked samples of LP and hLP milk

Fortification level, μg/kg	Measured mean concentration, μg/kg	RSD_r,[a] %	$RSD_{R,intra}$,[a] %
0.1	0.10	4.6	5.0
0.2	0.20	3.9	4.9
1.0	1.01	3.4	4.4

[a] One duplicate analysis of each sample matrix at each level was conducted on each of 8 days.

Table 3. True recovery determined on spiked samples.

Sample matrix	*N*	Concn, μg/kg	Recovery mean ± SD, %[a]
hLP milk	8	0.1	89 ± 3.7
	8	0.2	90 ± 3.5
	8	1.0	91 ± 2.8
LP milk	8	0.1	93 ± 3.6
	8	0.2	90 ± 3.5
	8	1.0	92 ± 3.0
Raw milk	20	0.2	92 ± 3.2
HP skim milk	5	0.2	91 ± 3.0
Reconstituted skim milk powder	5	0.2	91 ± 4.1
Reconstituted whole milk powder	5	0.2	87 ± 2.7
UHT milk	5	0.2	90 ± 3.8

[a] SD = Standard deviation of single determinations.

The dominant precursor ion obtained from APCI was *m/z* 321 ($[M–H]^-$). This ion was fragmented in the collision cell, which produced several product ions. The most sensitive transitions were *m/z* 152, 257, and 194 with average relative abundances of 100:65:35 at a level of 0.1 μg/kg (Figure 1). The average relative abundances at a level of 1 μg/kg (100:64:35) were not significantly different. The use of 3 transitions gave 5.5 identification points exceeding the EU requirement of 4.0 for confirmation of banned substances (13). The relative abundances of the 3 transition ions measured in pure standard solutions were 100:64:36, which were not significantly different from those obtained in matrix samples. The transition product *m/z* 152 was used for quantitative measurements.

The method was developed and validated to meet a minimum required performance limit (MRPL) of 0.1 μg/kg. However, it was observed that at least 50 mL milk fortified with CAP to levels of 0.1 and 20 μg/kg could be applied to the extraction and cleanup procedure without significant loss of recovery. In some cases, it was necessary to filter reconstituted milk powder through a glass fiber filter before SPE of large volumes.

The residual matrix components in the sample extract enhanced the signal response by 14% on average. The enhancement was stable within analytical series, and no significant differences between the matrix groups were observed. Therefore, we decided to use matrix calibration standards rather than an internal isotope standard, which increased the accessibility of the method.

The recovery of CAP from aluminium oxide cartridges was dependent on the water concentration of the acetonitrile extract (Figure 2). The residual amount of water on the C_{18} cartridge after vacuum suction for 5 s was about 150 mg (determined by weighing cartridges used for method blanks). To obtain stable recovery, 150 μL water was added to the 3 mL acetonitrile phase obtained from elution of the C_{18} cartridge before cleanup on the aluminium oxide cartridge.

The ruggedness test of the method did not show any significant effect of the factor variations listed in Table 1.

Calibration curves were created using nonweighted linear regression analysis with lines forced through the origin. The coefficients of determination (R^2) for the *m/z* 152 transition were 0.9988–0.9999 in the precision study. The corresponding slope converting concentration (μg/kg original sample) to peak area (counts) was 73 000 ± 2000 (mean ± SD). The mean standard error of slope estimates was 650 counts × kg/μg.

The CCβ related to the less sensitive ion transition was determined as 0.03 μg/kg from a mean result of 0.017 μg/kg and an SD of 0.007 μg/kg obtained on blank control samples spiked to a level of 0.015 μg/kg. The relative repeatability standard deviation (RSD_r) and relative intralaboratory reproducibility standard deviation ($RSD_{R,intra}$) were <5% at a level of 0.1 μg/kg (Table 2). The mean recovery was 90% (Table 3). Standard solutions containing 2 μg/kg CAP and final sample extracts of milk spiked to a level of 0.1 μg/kg were stable for at least 8 days when stored at 5–7°C.

Using the procedure described, it was possible to run more than 1100 matrix samples in subsequent series without significant decrease in sensitivity induced by contamination of the curtain plate or the orifice/skimmer region.

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