

Rapid Residue Analysis of Chloramphenicol in Milk by Liquid Chromatography Coupled to Tandem Mass Spectrometry

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ABSTRACT

Chloramphenicol (CAP) is widely used in the growth of animals and marine culture as it is broad spectrum antibiotic. Due to the development of resistance of microorganisms against antibiotic many countries have completely banned the use of it. A Negative Electro spray ionization mode with liquid chromatography and mass spectrometry was used to monitor the contamination of CAP in milk. Milk samples were partially deproteinized and defatted by acetonitrile and then centrifugated for 10 min at 13000 rpm. Chromatographic separation was achieved in less than 20 min. Identification and quantification of the compound of interest was performed by using an electrospray ionization source in negative mode, applying multiple reaction monitoring of two diagnostic transition reactions for CAP (m/z 321→152 and m/z 321→121). Relative recoveries were calculated for raw milk (at 0.5, 1 and 1.5 times the MRPL) and ranged between 83% and 101%. Quality parameters of the method were established, and limit of detection of chloramphenicol antibiotic in raw milk was 0.1 μ g/ml. The validation of the method has been carried out according to the requirements set by the 2002/657/EC regulation. Excellent linear range was observed from the method quantitation limits to 1.5 ng/mL with correlation coefficients better than 0.99 for this compound. The method was accurate with recoveries ranging from 83% to 101%. Good intra-precision and intermediate precision were obtained with RSD better than 12%. The method is fairly robust with sample pH being the only critical control point. Finally, the applicability of the method was tested by analyzing 54 milk samples.

Key words: Chloramphenicol, LC-MS/MS, validation, EU commission.

Introduction

Chloramphenicol [D(-)-threo-2-dichloroacetamido-1-pnitrophenyl-1,3-propanediol] (CAP) is a broad-spectrum antibiotic, previously used in veterinary medicine (Woodward & Watson, 2004).

The adverse effects of this compound have led to restrict its use in both human and veterinary medicine. Among other applications, this drug has been used for the treatment of childhood meningitis. It is also a potent drug for typhoid fever. CAP has been used in veterinary practice for the prevention and treatment of many bacterial infections. However, toxic effects in humans such as aplastic anemia have been described. It has been banned for use in foodstuffs of animal origin in the European Union (Council, 1994; Epstein *et al.*, 1994).

The detection of CAP in animal food samples is highly important. Therefore, various methods such as liquid chromatography (Shen & Jiang, 2005; Vinas *et al.*, 2006), liquid chromatography–mass spectrometry (Bogusz *et al.*, 2004; Gantverg *et al.*, 2003; Mottier *et al.*, 2003; Neuhaus *et al.*, 2002; Pfenning *et al.*, 2002; Ramos *et al.*, 2003; Storey *et al.*, 2003), gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) (Impens *et al.*, 2003; Shen & Jiang, 2005), capillary zone electrophoresis (Wang, hang, & Fang, 1999), enzyme-linked immunosorbent assay (ELISA) (Scortichini *et al.*, 2005), spectrophotometry (Collado *et al.*, 2000) and chemiluminescence (Icardo *et al.*, 2003; Lindino & Bulhoes, 2004) have been applied for CAP determination in different samples. However, most of these methods need expensive apparatus and reagents and they are time-consuming. Therefore, a sensitive, rapid and cheap method for analysis is still needed.

In order to ensure human food safety, the European Union has set maximum residue permissible limits (MRPLs) in milk for CAP, such as 0.3 μ g/kg. It is indicated that they cannot be used in animals from which milk is produced for human consumption. This limit requires the development of sensitive and specific method to monitor and determine antibiotic residue in milk. Despite of the advances on the detection techniques, one of the main problems related to the determination of antibiotics in milk is the sample treatment, due to the high protein and fat content in this type of matrix, which can often interfere in analytical procedures. Most of the current extraction methods are long and tedious, involving liquid–liquid extraction (Ruyck *et al.*, 2002) or solid-phase extraction (SPE) (Bogialli *et al.*, 2005) which also include a previous step to precipitate the proteins.

Recently new procedures based on matrix solid phase dispersion with hot water extraction have been proposed in order to simplify the extraction step (Bogialli *et al.*, 2005). Most of these papers have determined

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single drug classes. Only few methods have focused on the determination of several types of antibiotics in milk (Koesukwiwat *et al.*, 2007) because the different physico-chemical properties of the analytes increase the complexity of the extraction process. In the last few years, a fast and inexpensive method for the analysis of pesticides in foods (including fat matrices), named QuEChERS (quick, easy, cheap, effective, rugged and safe) has been reported (Anastassiades *et al.*, 2003; Lehotay *et al.*, 2005). The method is based on an acetonitrile extraction/partitioning of the contaminants and water and proteins are removed from the sample by salting out with sodium chloride and magnesium sulphate, followed by dispersive-SPE clean up, which implies the addition of small amounts of bulk SPE packing sorbents to the extracts (Anastassiades *et al.*, 2003). After sample clean up, the mixture is centrifuged and the resulting supernatant can be analyzed directly. QuEChERS is a fast and inexpensive method which has been mainly applied for the extraction of different classes of pesticides (Plössl *et al.*, 2006; Garrido *et al.*, 2008) but only one paper has used this methodology for the determination of pharmaceuticals in blood sample (Paya *et al.*, 2007). In consequence its applicability could be extended to the extraction of antibiotics in complex matrices such as milk.

The aim of the work was to develop a method for the determination of selected veterinary antibiotic (CAP) in milk. The method involves a simple extraction technique based on QuEChERS procedure, which implies an extraction with acetonitrile without further clean up and analytical determination by liquid chromatography coupled with tandem mass spectrometry (MS/MS). The combination of LC-MS/MS and QuEChERS extraction provides a fast and simple method that can be applied by routine laboratories, which have to analyze large numbers of samples.

Objective

The aim of this study was to develop and validate a confirmatory method for determination of CAP in milk.

Experimental

Chemicals and materials

Methanol and acetonitrile were HPLC grade and were purchased from Merck (Darmstadt, Germany). Sodium hydroxide, citric acid monohydrate, ammonium hydroxide, formic acid and sodium chloride were purchased from Riedel-deHaen $\geq 99\%$. Ethylenediamine Tetra Acetic Acid disodium salt dihydrate (Na₂-EDTA) was purchased from Fluka $\geq 99\%$. Commercial antibiotic standard chloramphenicol (CAP) was supplied by Dr. Ehrenstorfer GmbH, Gogginger Str. 78 D- 8900 Augoburg.

Standard solutions

Stock standard solution of 1000 $\mu\text{g/ml}$ was prepared by dissolving 10 mg of CAP in 10 ml methanol in volumetric flask to obtain a final concentration and store at $-20\text{ }^\circ\text{C}$, it was stable for six month. The working mixed solution was prepared 40 MRPL reference standard solutions in one mixture by diluting appropriate volume of the stock solution with methanol and store at $-20\text{ }^\circ\text{C}$. Calibration mixture solutions were prepared by taken appropriate volume of the working solution mixture and complete with stock buffer to prepare standard solution at 10 MRPL. This standard was used to prepare the calibration mixtures at concentration levels 0.5, 1, 2 and 5 MRPL with dilution solvent. Calibration mixture solutions must be prepared daily with each set of samples. Standard in matrix was prepared one point standard in the targeted blank matrix daily (with each set of samples) at the level of interest (1MRPL) to compensate the matrix effect on the sample result. The sample result must be corrected against standard in matrix.

Buffer solution:

Stock buffer (50mM Ammonium formate solution): 1.73 ml formic acid was added to 900 ml water then the PH was adjusted at 2.78 ± 0.1 with ammonia solution 10% then 50 ml methanol was added and completed to 1L with deionised water.

Apparatus:

LC-MS/MS System, Agilent 1200 series liquid chromatography system equipped with Applied Biosystem (API 4000 Qtrape) tandem mass spectrometers with electrospray ionisation (ESI) interface. Separation was performed on a C18 column ZORBAX Eclipse XDBC18 4.6mm x 150 mm, 5 μm particle sizes. The injection volume was 25 μl . A gradient elution program was at 0.3ml/min flow rate, in which one reservoir contained 10 mM ammonium formate solution in MeOH: H₂O (1:9, v: v) and the other contained methanol. The ESI source was used in the negative mode, and Nitrogen was used as nebulizer gas, heater gas and collision gas according to manufacturer's settings; source temperature was 300°C , ion spray potential 5500V, decluster potential and collision energy were optimized using Harvard apparatus syringe pump. The

multiple Reaction Monitoring Mode (MRM) was used in which one MRM was used for quantification and other was used for confirmation.

Extraction and clean up:

CAP was extracted from milk using an extraction procedure based on buffered QuEChERS methodology. Briefly the procedure was as follows: 2 g of sample was weighed in a polypropylene centrifuge tube (40 mL), 1 ml from both 1 M Sodium citrate buffer at PH 4 and 0.5 Na₂EDTA were added, 10mL of acetonitrile was added, and the mixture was vortex for 1 in. The mixture was centrifuged at 13000 rpm for 10 min at -4 °C for phase separation and the supernatant was decanted in 100 ml round- bottomed flask. The extraction step was repeated with another 10 ml acetonitrile, and then shaken for 5 min at 900 rpm followed by centrifugation again as previously described and combined the supernatant at the same 100 ml round-bottomed flask, then evaporated at 40 ±2 °C and 200 µl NaCl solution as soon as the aqueous bubbles appear to avoid the back-suction. Finally, 2mL of dilution solvent was added and 25µL were injected onto the HPLC–MS/MS system under the optimized conditions.

Method validation:

For the validation study, most of the performance characteristics were determined following the procedures described in 2002/657/EC Commission Decision. Blank milk was used as negative control milk. The procedure was validated using the blank samples fortified 6 replicate with the working mixed solution, at (0.15, 0.3 and 0.45µg kg⁻¹) for three days.

Results and Discussion

Complete Validation of CAP was done as per EU decision 2002/657 and all parameters were complied with EU requirement. Trueness, Coefficient variation (CV) and Recovery were calculated at three different concentrations as per EU decision i.e. at 1, 1.5 and 2 of MRPL. CC_α and CC_β were calculated at MRPL (0.3µg/kg).

CAP eluted from the LC at about 12.2 min. The mass spectrometer was operated in the negative ion mode using selected reaction monitoring, and the precursor ion at m/z = 321 yielded two main product ions of m/z = 152 and 121. Under our conditions, the m/z 152 product was the base peak, and it was used for quantitation as shown in figure (1).

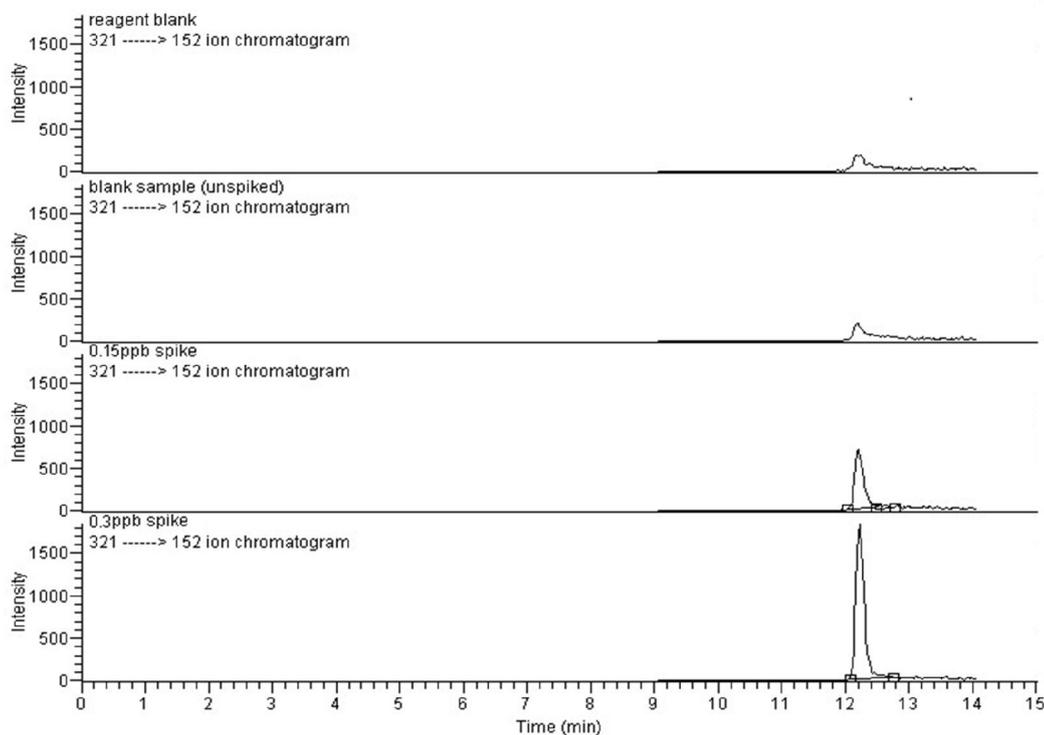


Fig. 1: m/z 152 Ion Chromatograms

The parameters taken into validation were: response linearity, decision limit ($CC\alpha$), detection capability ($CC\beta$), reliability and accuracy (repeatability and within-laboratory reproducibility).

Linearity and sensitivity

Usually, the quantification of drug residues is performed by using a matrix-matched calibration curve made from fortified blank samples prepared in the same matrix as the real samples. To test the linearity of the calibration curve, five standards of CAP in the blank milk matrix were analyzed. The calibration curve showed a good linearity in the concentration range from 0.075 to 1.5 $\mu\text{g}/\text{kg}$ with the correlation coefficient above 0.995. A similar slope and correlation coefficients were also observed for the calibration curve based on pure standard solutions prepared in water, indicating that there was no signal contribution and ion suppression from the matrix.

Recovery

The recoveries were obtained by spiking blank pool samples at three different levels (0.15, 0.3 and 0.45 $\mu\text{g}/\text{kg}$). Recoveries for fortified samples are reported in Table 1. Overall recoveries at 0.3 $\mu\text{g}/\text{kg}$ considering repeatability data ($n = 18$) was 101% for CAP, meeting Commission of the European Guidelines for RSD % (10% when the target level is ≥ 1000) and Commission of the European Guidelines for accuracy (80–110% for concentrations $>10 \mu\text{g}/\text{kg}$) for CAP.

Table 1: Mean and overall recoveries of CAP from milk samples fortified at 0.15, 0.3 and 0.45 $\mu\text{g}/\text{kg}$ ($n = 18$)

Fortification level($\mu\text{g}/\text{kg}$)	Parameter	Day 1	Day 2	Day 3	Overall
0.15 (0.5 MRL)	Recovery %	80.9	85.8	78.4	81.7
	RSD %	13.0	12.2	11.1	12.1
0.3 (1 MRL)	Recovery %	106.7	89.2	106.1	100.7
	RSD %	10.7	5.3	7.3	7.8
0.45 (1.5 MRL)	Recovery %	89.9	78.1	90.7	86.2
	RSD %	4.0	12.6	5.2	7.3

Repeatability and Reproducibility:

Eighteen samples of the blank pool were fortified with the analytes to yield concentrations of 0.15, 0.3 and 0.45 $\mu\text{g}/\text{kg}$ ($n = 6$, each level). This procedure was repeated on two other days with the same environmental conditions. Overall interlaboratory relative repeatability (RSDr) and overall within-laboratory reproducibility (RSDR) for all residues at 0.15, 0.3 and 0.45 $\mu\text{g}/\text{kg}$ were found below 13% (Table 2), meeting Codex Alimentarius and Commission of the European Communities guidelines for repeatability (20 and 15%, respectively, when the target level is $>10 \mu\text{g}/\text{kg}$).

Table 2: Repeatability and Reproducibility for the determination of CAP in spiked milk samples

0.5 times MRL			0.15 $\mu\text{g}/\text{kg}$			1.0 times MRL			0.3 $\mu\text{g}/\text{kg}$			1.5 times MRL			0.45 $\mu\text{g}/\text{kg}$				
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Replicate 1	0.099	0.117	0.101	Replicate 1	0.32	0.25	0.29	Replicate 1	0.41	0.37	0.39	Replicate 1	0.41	0.37	0.39	Replicate 1	0.41	0.37	0.39
Replicate 2	0.122	0.153	0.110	Replicate 2	0.32	0.26	0.32	Replicate 2	0.39	0.28	0.40	Replicate 2	0.39	0.28	0.40	Replicate 2	0.39	0.28	0.40
Replicate 3	0.106	0.140	0.120	Replicate 3	0.28	0.27	0.30	Replicate 3	0.40	0.35	0.43	Replicate 3	0.40	0.35	0.43	Replicate 3	0.40	0.35	0.43
Replicate 4	0.135	0.122	0.130	Replicate 4	0.29	0.28	0.34	Replicate 4	0.39	0.42	0.39	Replicate 4	0.39	0.42	0.39	Replicate 4	0.39	0.42	0.39
Replicate 5	0.129	0.129	0.110	Replicate 5	0.34	0.29	0.35	Replicate 5	0.40	0.34	0.44	Replicate 5	0.40	0.34	0.44	Replicate 5	0.40	0.34	0.44
Replicate 6	0.138	0.111	0.135	Replicate 6	0.37	0.26	0.31	Replicate 6	0.43	0.36	0.40	Replicate 6	0.43	0.36	0.40	Replicate 6	0.43	0.36	0.40
Mean	0.121	0.129	0.118	Mean	0.32	0.27	0.32	Mean	0.40	0.35	0.41	Mean	0.40	0.35	0.41	Mean	0.40	0.35	0.41
S.D.	0.016	0.016	0.013	S.D.	0.03	0.01	0.02	S.D.	0.02	0.04	0.02	S.D.	0.02	0.04	0.02	S.D.	0.02	0.04	0.02
C.V.	13.10	12.206	11.103	C.V.	10.71	5.29	7.28	C.V.	3.97	12.55	5.23	C.V.	3.97	12.55	5.23	C.V.	3.97	12.55	5.23
Recovery	80.93	85.820	78.444	Recovery	106.74	89.19	106.11	Recovery	89.87	78.09	90.74	Recovery	89.87	78.09	90.74	Recovery	89.87	78.09	90.74
Overall Mean	0.12			Overall Mean	0.30			Overall Mean	0.39			Overall Mean	0.39						
Overall SD	0.01			Overall SD	0.03			Overall SD	0.04			Overall SD	0.04						
Overall CV	12.09			Overall CV	11.43			Overall CV	9.97			Overall CV	9.97						

Decision limits ($CC\alpha$) and Detection capabilities ($CC\beta$):

The EU decision introduces the concepts of decision limit ($CC\alpha$) and detection capability ($CC\beta$) for a chemical analytical method. These parameters are to be used instead of the more familiar limit of detection and limit of quantification. The definition of the $CC\alpha$ for a forbidden compound is: "The limit at and above which it can be concluded with an error probability of 1% that a sample is noncompliant". The definition of the $CC\beta$ for a forbidden compound is: "The lowest concentration at which a method is able to detect truly contaminated samples with an error probability of 5%". $CC\alpha$ and $CC\beta$ were calculated at MRPL (0.3 $\mu\text{g}/\text{kg}$) in matrix milk. For $CC\alpha$ and β calculation following equations was used.

$$CC\alpha = \text{MRL} + 1.64 * \text{SD of 20 fortified blanks at MRL}$$

$$CC\beta = CC\alpha + 1.64 * \text{SD of 20 fortified blanks at } CC\alpha$$

Table 3: Calculation of CC α and CC β of CAP by fortification at the MRL (0.3 $\mu\text{g}/\text{kg}$)

Replicate	Concentration	Replicate	Concentration
1	0.25	1	0.37
2	0.27	2	0.33
3	0.32	3	0.43
4	0.23	4	0.36
5	0.31	5	0.29
6	0.28	6	0.40
7	0.28	7	0.42
8	0.29	8	0.41
9	0.28	9	0.43
10	0.21	10	0.36
11	0.35	11	0.39
12	0.30	12	0.38
13	0.31	13	0.52
14	0.30	14	0.45
15	0.28	15	0.48
16	0.31	16	0.47
17	0.26	17	0.43
18	0.30	18	0.47
19	0.23	19	0.40
20	0.27	20	0.47
Mean	0.28	Mean	0.41
S.D.	0.03	S.D.	0.06
MRL	0.30	CC α	0.36
CC α	0.36	CC β	0.45

Limit of detection (LD)

The analysis of the 21 blank samples, allowed to calculate the limit of detection, determined as the average plus three times the standard deviation of the noise amplitude in windows defined 0.05 min of retention times observed in bracketing standards ($LD = \bar{y}_i + 3s_{y_i}$). Using this approach, the limit of detection obtained was 0.15 $\mu\text{g}/\text{kg}$.

Limit of quantification (LQ)

The limit of quantification (LQ) was defined as the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated conditions of the test. So, the LQ were established as the lowest concentration levels for which the method was validated with an accuracy and precision that felt within the ranges recommended by the European Communities Guidelines: 0.3 $\mu\text{g}/\text{kg}$.

The performance characteristics of the method presented in this paper indicate that it may be used in food control.

Table 4: Performance data of the HPLC–MS–MS method for the analysis of CAP in spiked milk samples

Parameter	Fortification levels $\mu\text{g}/\text{kg}$		
	0.15	0.3	0.45
Overall mean \pm SD (%)	82%	101%	86%
Trueness \pm SD (%)	18%	1%	14%
Precision (RSD%)	12%	7.8%	7.3%

Conclusion

From validation it can be concluded that the following method is very simple, cost effective and selective to analyze CAP in Milk. It require less run time and extraction time this method can be used commercially for the analysis of large quantity of samples. This method uses very sensitive and robust instrument LCMSMS for the analysis of samples so that it can work up to PPT level with high precision and accuracy i.e. fulfill the requirement of EU commission.

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