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Determination of chloramphenicol residues in milk powder by mass spectrometric method

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ABSTRACT

Chloramphenicol (CAP) is no longer a first-line agent for any indication because of resistance and safety concerns; it has been banned in several countries, including the European Union (EU), for treatment of food-producing animals. But CAP is still illegally used in animal farming because of its easy access and low cost. Therefore, effective detection techniques are required for a strict control of this compounds entry in food chain. So aim of the present study was to optimize and validate the analytical parameters for analysis of chloramphenicol residues by liquid chromatography tandem mass spectrometry (LC-ESI-MS-MS) in milk powder. Sample preparation performed by extraction of milk samples with ethyl acetate and evaporated to dryness, followed by a clean-up step using the liquid liquid extraction with isooctane. Multiple reactions monitoring (MRM) acquisition method in negative ionization mode with the transitions of m/z 320.54→151.94, 320.54→ 256.9, 325.7→157.1 (IS) used. For the quantification transition m/z 320.54 → 151.94 was chosen by matrix-matched calibration curves, ranging from 0.15 to 0.75 μg kg^{-1}, with regression coefficients of 0.99. The recovery, repeatability, reproducibility and CCalpha and CCbeta were evaluated. The limit of decision (CCα) and detection capability (CCβ) for milk powder was 0.085 and 0.109 μg kg^{-1}. The repeatability (RSD %) was lower than 12.5 % and reproducibility (RSD %) was less than 12.43 %. On the basis of these analytical parameters and performances we have placed optimization step in a method as a monitoring tool for unacceptable levels of residues of CAP in milk powder.

KEYWORDS

Milk;
Chloramphenicol residues;
Chromatography;
Mass spectrometry;
Veterinary drug residues;
Food analysis;
LC-MS/MS.

INTRODUCTION

Chloramphenicol (CAP) is a bacteriostatic antimicrobial. It is considered a prototypical broad-spectrum antibiotic. It is effective against a wide variety of Gram-positive and Gram negative bacteria, including most anaerobic organisms. Due to resistance and safety concerns, it is no longer a first-line agent for any indication. The most serious adverse effect associated with CAP treatment is bone marrow toxicity, which may occur in two distinct forms: bone marrow suppression, which is a direct toxic effect of the drug and is usually reversible, and aplastic anemia, which is idiosyncratic (rare, unpredictable, and unrelated to dose) and generally fa-

tal[1]. In spite of its potential toxicity, CAP is sometimes used at therapeutic doses for treatment of serious infections in humans; however, it has not been possible to identify a safe level of human exposure to CAP because of the unpredictable effects of dose on different patient population. Due to these reasons CAP for humans and food-producing animals have been banned in several countries, including the European Union (EU). CAP is still illegally used in animal farming because of its easy access and low cost. Therefore, effective detection techniques are required for a strict control of this compound. As a consequence, the use of CAP in food-producing animals has been forbidden within the EU since 1994[2], and no maximum residue limit (MRL) has been established in animal-derived foods. In March 2003 Commission Decision 2003/181/EC set an Minimum required performance limit (MRPL) of 0.3μg kg^{-1} for chloramphenicol in meat, milk, eggs, aquaculture products and honey. Which means all methods should be able to detect and confirm to this level. With growing concerns over food safety and the need to increase sample-throughput in analytical testing laboratories, there is a constant requirement for accurate, simpler, faster and improved analytical methods. The complexity of food matrices and the presence of much potential interference, require specific and selective methods of analysis. Various analytical methods have been reported for the determination of CAP in food product. But in last few years HPLC-UV, ELISA, GC-ECD, HPTLC like technique are rarely used because such detector is not enabling adequate quantification and confirmation of this analyte at trace levels, in agreement with the EU criteria[3]. As per criteria mass spectrometric methods must be utilized for confirmation. GCMS methods can provide definitive confirmative and quantitative results but require a derivatization step. The combination of LC-MS offers a rapid, simplified, specific and sensitive alternative to GC-MS methods and removing the need for derivatization reactions. Most suitable technique Liquid chromatography coupled with mass spectroscopic detection was used to determine CAP residues in food matrixes, enabling adequate confirmation and quantification of this analyte at trace levels, in agreement with the EU criteria. During literature review we found that recent reported paper are basically based on honey and animal product (fish, egg and prawns etc.) but in case milk specially milk powder few studies were dedicated, this work totally based on milk powder analysis for CAP for day today analysis. 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[4,5,19], chloroform–acetone[6], or acetonitrile[7-10,20], followed by washes with, hexane[6,8,19] or chloroform[7,8], carbon tetrachloride / hexane[4] or solid-phase extraction[10-12,18], and others are molecular imprinted polymers (MIPS)[13-15], dispersive liquid– liquid microextraction (DLLME)[16], C18-dispersive solid extraction[5], matrix solid-phase dispersion (MSPD)[17]. The scope of the present study was to develop liquid chromatography coupling to tandem mass spectrometry method for the detection of CAP in milk powder, using simple and economic liquid–liquid extraction for sample extraction and cleanup. The developed methodology gave satisfactory recoveries and clean final extracts. Applicability of the proposed method was tested by participating Food Analysis Performance Assessment Scheme (FAPAS) Proficiency testing and analyzing the real milks samples. As a whole, the method proved to be simple, economic, and reached the required sensitivity. Hence, it provides a suitable means for routine analysis of CAP residue in real milk samples.

EXPERIMENTAL

Apparatus

HPLC–MS–MS analyses were performed using a LC Waters Alliance 2695 separations module (Waters, Milford, MA, USA) coupled via an electrospray interface (ESI) to a Quattro Micro mass spectrometer (Waters, Milford, MA, USA). The instrument was operated in multiple reaction-monitoring (MRM) mode with Masslink software packages (Waters, Milford, MA, USA), for spectral and quantification data processing. LC separations were performed on a Zorbax Eclipse XDB-C18 column (150 × 2.1 mm, 5 μm particle size) (Agilent Technology).

Standard solutions

Stock solutions (1 mg mL^{-1}) of CAP and CAP -*d5* were prepared in acetonitrile and working standards were prepared by diluting the stock solution with water. All standard solutions were kept at –20 ^{0}C and

protected from light for no longer than 3 months.

Equipment parameters

The chromatographic separation was achieved using gradient mode water (mobile phase A) and acetonitrile (mobile phase B). Gradient is as follow; 95 % A for first 1.5 min then this change to 5 % A from 1.5 to 3.0 min. and maintain for 3 min. The conditions than return to the initial 95 % A in 1.0 min. and remain the same till the end of run. The mobile phase flow-rate was set at 0.3 ml/min, and 25 µl of the extract was injected into the HPLC–MS–MS system.

Sample extraction and clean-up

Extraction and clean-up procedure optimized for analysis of CAP residue in milk products (Full and skimmed milk powder) was as follows. Two grams of milk sample was weighed in a polypropylene centrifuge tube (50 ml capacity) and spiked with 200 µL of CAP -*d5* (5 µg L^{-1} Stock standard), 8 mL water was added. The sample was vortexed and allowed to stand for 10 min. 8 mL ethyl acetate were added and homogenisation for 5 min at 8000 rpm and then centrifuged at 9800g for 5 min. After centrifuge 4 ml of upper organic layer is transfer to a glass tube and evaporate it at 45^{0}C under nitrogen. The residue was dissolved in 0.5 ml of Isooctane and then extracted with 0.5 ml of water. After centrifugation for 5 min at 22000g, aqueous phase was transfer to LCMSMS vial and the aliquot of 25 µl was injected on the HPLC column.

Matrix-matched calibration

Matrix-matched calibration intended to compensate for matrix effects and acceptable interference. The matrix blank (a sample known not to contain detectable levels of the Analyte) should be prepared as for analysis of samples and analyte is added to a blank extract of a matrix. To test the linearity of the calibration curve, five blank milk matrixes are prepared as for analysis of samples and CAP and CAP -*d5* are added to a blank extract of a matrix at 0.15, 0.30, 0.45, 0.60 and 0.75 µg kg^{-1} levels for a calibration range of 0.15 to 0.75 µg kg^{-1} with the correlation coefficient above 0.99. Constructed a calibration curve using all standards using the ratio of [(CAP 151.94)/ (CAP -*d5* 157.1)] responses vs. CAP concentration in µg kg^{-1}.

RESULTS AND DISCUSSION

MS–MS detection

CAP and its deuterated internal standard (IS) CAP -*d5* were first analyzed in Negative mode electrospray ionization and selected ion recording (SIR) were used. According to Commission Decision 2002/657/EC, for banned substances, at least one parent ion with two different product ions are required to confirm the presence of the analyte studied. Both CAP and CAP -d5 were then analyzed selection of product ion scan mode by *m/z* 320.54 and *m/z* 325.7 ions as the precursor ion, respectively three main fragment ions were obtained from the collision induced dissociation (CID) experiments of these ions, giving rise to respectively *m/z* 256.9 and *m/z* 151.94 *m/z* and *m/z* 157.1. In order to obtain maximum sensitivity the source temperature was set at 120 °C and desolvation temperature was 450 ^{0}C. Cone and desolvation gas flow were 70,700 L/hr. Nitrogen gas used as desolvation and cone gas and argon used as collision gas other analyte-specific parameters (Cone voltage (V), Collision energy (eV)) are shown in TABLE 1. The transitions monitored for quantification of CAP in samples was done using the calibration curves from *m/z* 320.54→151.94 (Figure 1). The *m/z* 320.54→256.9 transition was used for confirmation of results from the *m/z* 320.54→151.94 transition. For the internal standard CAP -*d5* (I.S.) *m/z* 325.7→157.1 was monitored. Furthermore as chloramphenicol contains two chlorine atoms, additional transition reactions *m/z* 322.7 → 151.94 was also recorded. Figure 2 shows HPLC– MS–MS chromatograms of matrix match standard with 0.300 µg kg^{-1} CAP. The reproducibility of ratios used for confirmatory purposes was studied in sample matrixes. All of them presented good RSD over three CAP-spiked concentration levels: mean ion ratio 22% for *m/z* 151.94 /157.1 with RSD 16.8 %, in the, 0.3 µg kg^{-1} CAP-spiked milk powder.

Sample preparation optimization

The optimal condition for sample preparations is challenging job because any designs and compromises must be affected on simplicity and speed of the procedure with broad applicability, high recovery and adequate selectivity. These analytical step can be affected by various factors like sample composition, type of

TABLE 1 : MS–MS transitions and conditions for chloramphenicol

Compound	Precursor Ion (m/z)	Transitions ion (m/z)	Cone voltage (V)	Collision energy (eV)
Chloramphenicol	320.54	320.54→151.94	30	23
		320.54→256.9	30	15
Isotopic ion	322.7	322.7 → 151.94	31	19
d5-CAP	325.7	325.7→157.1	30	17

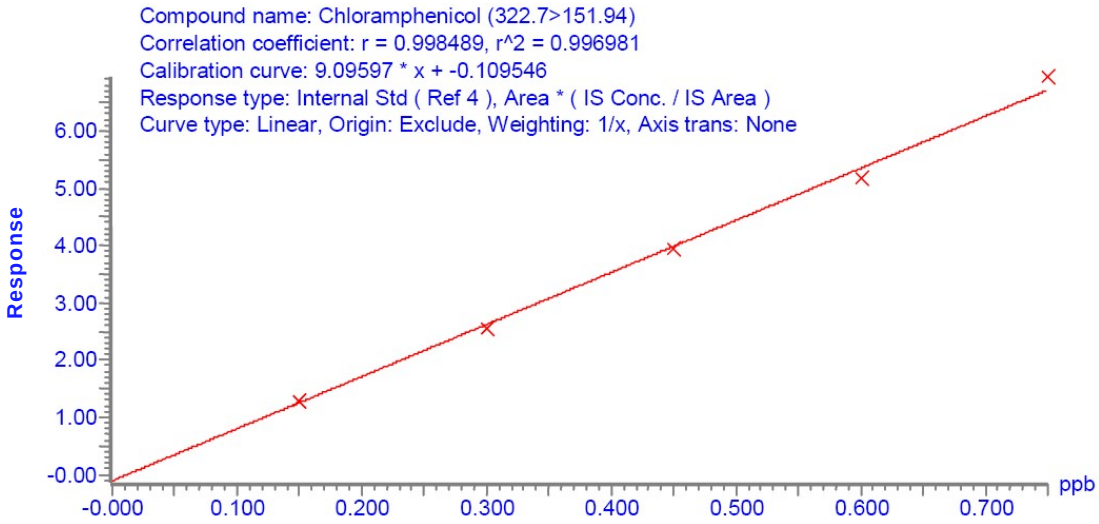

Figure 1 : Chloramphenicol calibration curves (m/z 320.54→151.94) from 0.15 to 0.75 µg kg^{-1}

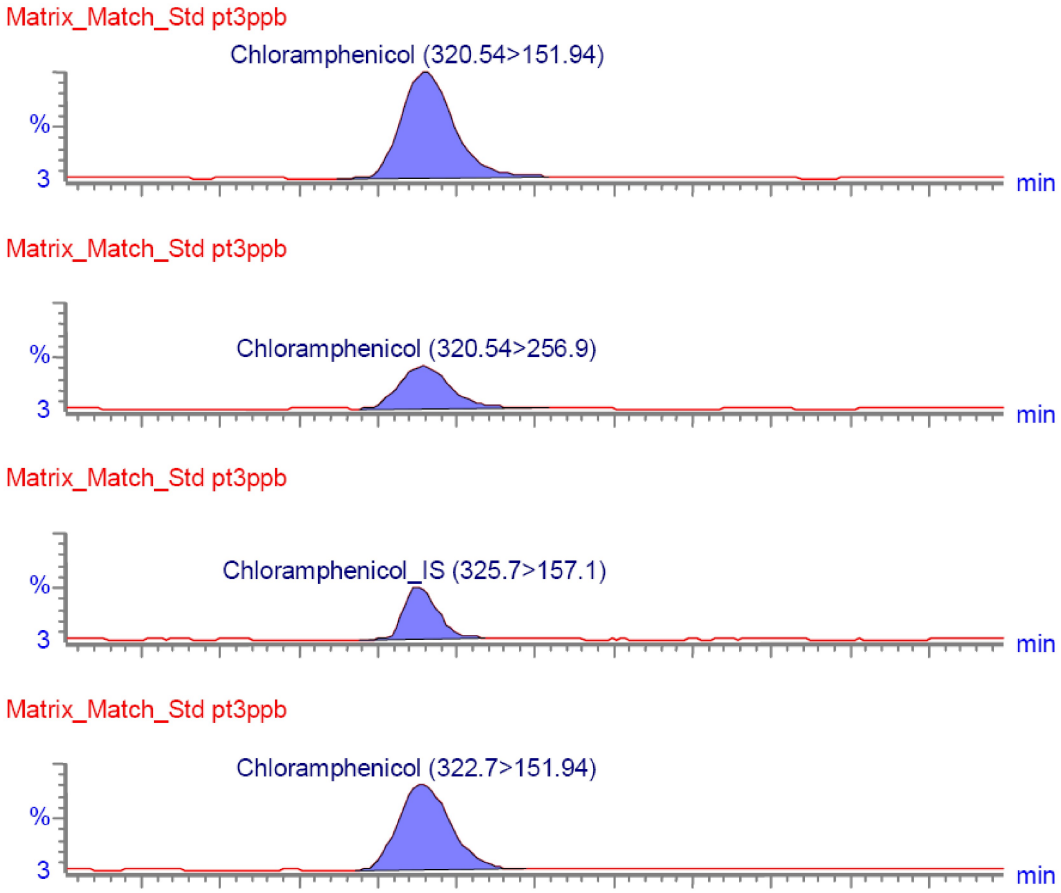

Figure 2 : Chloramphenicol chromatograms of matrix match std at 0.300 µg kg^{-1}

solvent used for extraction, ratio of solvent sample combination, extraction procedure, time spend for extraction etc. we sought to systemically compare and measure the effect of each of these adjustable parameters by using multiple tools of objective measurement.

Selection of extraction solvent

The choice of the solvent (s) for sample preparation is one of the most crucial decisions to be made when developing any method. In this context three organic solvents acetonitrile (MeCN) and hexane, ethyl acetate (EtAc) were compared in a series of experiments in which the only parameter changed was the type of solvent. The recoveries were determined by comparing the peak area obtained from spiked blank samples with those obtained from aqueous standard solutions. The obtained results showed that ethyl acetate provided higher (≥70%) recovery comparative acetonitrile (≥55%) and hexane (≥43%) of CAP from spiked sample. Based on these results, ethyl acetate was selected as the extractant for further studies.

Comparison of shaking versus blending of an incurred sample

We assessed the extractability of CAP residue and compared shaking versus blending as the approach for initial extraction step. Comparisons show 68 % recovery for blending (homogenisation for 2 min at 8000 rpm and stand for 10 min.) and 54 % recovery for shaking (Mechanical shaking for 30 min.) In addition, to further improve the extraction efficiency, we attempted to increase the extraction time (homogenisation 2 to 5 min). This resulted in an increase in the recoveries of CAP up to 85–90%.

Liquid–liquid extraction cleanup

In order to shorten the sample clean-up procedure and to allow a higher sample throughput, conventional liquid–liquid extraction was adopted. The following three non-polar extractant systems were studied: Chloroform, hexane and Isooctane. Isooctane not reported earlier for milk sample cleanup. it's selected due to it better defatting capacity. Sample clean-up performance was evaluated by performing a standard addition of CAP to the blank samples just before the clean-up procedure in order to avoid the loss of analyte during the first extraction step. The obtained results showed that isooctane provided better clean-up & recoveries (≥96%) compare to chloroform (82%), hexane (89%).

The complete sample extraction and clean-up procedure optimized for analysis of CAP residue in milk products was as describe in sample preparation above.

Validation

Method validation was carried out as described in the 2002/657/EU document. The parameters taken into account were: response linearity, specificity, Confirmation Criteria, decision limits (CCα), detection capability (CCβ), and recovery, repeatability and within-laboratory reproducibility. Usually to test the linearity of the calibration curve, four standards of CAP in the blank milk matrix were analyzed. Specificity of the method was checked by the preparation and analysis of blank and spiked milk samples to verify the absence of potential interfering compounds at CAP retention time. The CCα and CCβ were obtained using the calibration graph approach[3]. Blank material was fortified at five different concentrations and the standard error of the *y* intercept was calculated. The decision limit and the detection capability for CAP were 0.085 μg kg^{-1} and 0.109 μg kg^{-1} respectively. These data demonstrate that both values are significantly below the MRPL of 0.3 μg kg^{-1}. The reliability and accuracy of the method were determined by spiking blank milk samples with CAP, resulting in three analytical series, each with three concentration levels and six samples per concentration level. Individual 2 g samples were fortified to contain 0.20, 0.30, and 0.40 μg kg^{-1}, using 40, 60 and 80 μL of spiking Solution. Spiking Solution was prepared at a concentration of 10 μg kg^{-1}. The recovery was expressed in terms of percentage recovery and repeatability, reproducibility as relative standard deviation (RSD). The results are presented in TABLE 2.

Application

To determine the applicability of method we had participated in FAPAS® Proficiency Test 02151 in milk powder organised by FAPAS® (Food Analysis Performance Assessment Scheme**)**. Assigned value of chloramphenicol was 0.376 μg kg^{-1} and our reported result was 0.426 μg kg^{-1} with 0.6 Z-score. These results confirm the ability of the method to extract CAP from samples and determination in sample matrices without a loss of method performance.

TABLE 2 : Performance data of the HPLC–MS–MS method for the analysis of CAP in spiked milk samples

Parameter			
Decision limit (CCα) μg kg^{-1}		0.085	
detection capability (CCβ) μg kg^{-1}		0.109	
Fortification level, μg kg-1	0.200	0.300	0.400
Mean ± SD	0.187±0.025	0.279±0.032	0.391±0.046
Recovery (%)	93.15	93.96	97.97
Repeatability (RSD %)	12.50	9.64	11.51
Reproducibility (RSD %)	12.43	11.06	11.20
Average % ion Ratio ±SD (m/z 151.94 /157.1)	22±6.5	21.8±3.6	21±3.2

CONCLUSION

The method can be considered as rapid, as it utilises an efficient extraction protocol without use of SPE. The obtained data fulfils the requirements laid down in Commission Decision 2002/657/EC and allows the calculation of all relevant performance characteristics. It's reduced time and sample preparation steps show it's applicability for day today analysis in testing laboratory

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