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Quantitative determination of chloramphenicol in milk powders by isotope dilution liquid chromatography coupled to tandem mass spectrometry

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Abstract

A method is described for the determination of residues of the illegal antibiotic chloramphenicol (CAP) in milk powders. The analyte is quantified by liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (LC–ESI-MS–MS) operating in negative ion multiple reaction monitoring mode (MRM) after a liquid–liquid extraction followed by a clean-up step on solid phase extraction (SPE) cartridge. Because of the presence of two chlorine atoms in the CAP molecule, four specific transition reactions of CAP were monitored by MS–MS in selecting *m/z* 321 → 257, 321 → 152 ($^{35}Cl_2$) and *m/z* 323 → 257, 323 → 152 ($^{37}Cl^{35}Cl$). Two calibration curves were constructed by plotting the area ratio of *m/z* 321 → 152 versus 326 → 157 and *m/z* 321 → 257 versus 326 → 262 against their corresponding amount ratio. Indeed, even if *m/z* 321 → 152 was found to give a higher MS–MS response (calibration curve used by default), an interfering chemical substance was sometimes observed for some milk extracts and not for the transition *m/z* 321 → 257. The quantitation method was validated according to the European Union (EU) criteria for the analysis of veterinary drug residues at 0.1, 0.2 and 0.5 μg/kg concentration levels using d_5-CAP as internal standard. The decision limit (CCα) and detection capability (CCβ) of CAP in milk were calculated for *m/z* 321 → 152 at 0.02 μg/kg and 0.03 μg/kg, respectively, and for *m/z* 321 → 257 at 0.02 μg/kg and 0.04 μg/kg, respectively. At the lowest fortification level (i.e. 0.1 μg/kg), repeatability and within-laboratory reproducibility were calculated for *m/z* 321 → 257 both at 0.02 μg/kg and for *m/z* 321 → 152 at 0.03 and 0.05 μg/kg, respectively. Moreover, the measurement of uncertainty of the analytical method was calculated at the same spiking levels and falls within the precision values of the within-laboratory reproducibility. This method can be applied to several types of milk powders (e.g. full cream, skim) and can serve as a monitoring tool to avoid that unacceptable levels of residues of CAP enter the food chain.

Keywords: Chloramphenicol; Milk powder; Measurement uncertainty

1. Introduction

Recently, considerable problems related to veterinary drug residues in the food chain have arisen, exemplified by the crises in 2001/2002 of chloramphenicol (CAP) and nitrofurans in animal-derived foods from South-East Asia and South America [1–3]. Such issues have repercussions on the global trade of food, resulting in rejection and potentially destruction of foods at the port of entry of receiving countries. Raw material suppliers and food manufacturers may also incur significant losses. In fact, the number of EU alert notifications related to veterinary drug residues have increased substantially over the past 5 years, i.e. 67 in 1997 to 434 in 2002. In 2002, 21% of the alert notifications were due to veterinary drugs, and together with the chemical notifications (30%) matched those recorded for microbial (pathogen) contamination (30%) [4]. The necessity of administration of veterinary drugs to combat diseases and enhance productivity is evident. However, legislation may differ considerably in different countries. For many food commodity-residue combinations, there are no set maximum residue limit (MRLs) or clear

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guidance on the level of residues permitted. This makes interpretation in certain commodities difficult. The global trade of food is continuously growing, and there are certain gaps in knowledge on the global accessibility and use of antimicrobial drugs. The recent incidents of CAP and nitrofurans in animal-derived foods originating from South-East Asia emphasize this deficiency. Too fast and extensive growth may be accompanied by lapses in food safety and quality systems, especially in countries where legislation is not well established and/or enforced.

CAP is a broad spectrum bacteriostatic antibiotic, obtained naturally (*Streptomyces venezuelae*) or by chemical synthesis [5]. Due to its potential side effects in certain individuals, the most serious being aplastic anaemia (a rare but fatal blood disorder), the drug is not recommended for the treatment of minor diseases, but is reserved for the therapy of serious infections (e.g. typhoid fever, meningitis) [6–7]. In veterinary medicine, CAP has been shown to be a highly effective and well-tolerated antibiotic since the potentially fatal side effects in humans have not been reported in animals. However, because of its toxicity in humans, the use of CAP is prohibited in food-producing animals within the EU since 1994 [8]. No MRL has been established for CAP in animal-derived foods, as its toxic effects are not dose-dependent. Thus, the EU has defined a minimum required performance limit (MRPL) for CAP in food of animal origin at a level of 0.3 μg/kg [9].

Several analytical methods have been developed for the analysis of CAP in animal-derived foods and include rapid test kits (radio- and enzyme-immunoassays) [10–11], thin layer chromatography [12–13], and chromatographic techniques such as gas chromatography (GC) coupled to an electron capture detector [14], immunoaffinity chromatography [15], molecular imprinted polymers with voltammetric detection [16] or high performance liquid chromatography (HPLC) with ultraviolet detection [17–19]. However, none of the above-mentioned methods have the required specificity to unequivocally confirm a positive result. Indeed, only methods utilizing MS as the determinative step are considered by EU guidelines as unambiguous confirmatory techniques. Several authors have already described the analysis of CAP in liquid milk (raw and skim) and milk powders (whole and skim) using either GC coupled to mass spectrometry (MS) [20] or HPLC coupled with MS [21] or MS–MS [22–23]. However, none of the MS-based methods reported employs an isotope labelled surrogate to reliably quantify CAP in milk products. To highlight the utility of confirmatory methods, Gaudin et al. have reported the final results of a European inter-laboratory study for the screening of CAP in raw milk by ELISA test kits, showing a total false positive rate of 16.7% and a total false negative rate of 2.2% [24].

In this study, we describe a LC–MS–MS method for the quantitation and confirmation of residues of CAP that can be applied to a wide range of milk powders and liquid milk (raw and skim). The method has been validated according to the EU guidelines pertaining to the performance of analytical methods and the interpretation of results [25].

2. Experimental

2.1. Chemicals

Chloramphenicol (CAP) was supplied by Riedel-de-Haen (Seelze, Germany). Internal standard $^{2}[H_5]$-chloramphenicol (d_5-CAP, ring-d_4, benzyl-d_1; chemical purity >98%, isotopic purity 99.8%) was purchased from Cambridge Isotope Laboratory (Andover, MA, USA). Radiolabeled ^{14}C-CAP (specific activity 55 mCi/mmol; radiochemical purity 99.1%) was purchased from Moravek (Brea, CA, USA). Oasis HLB solid phase extraction (SPE) cartridges (500 mg, 12 cc) were from Waters AG (Rupperswill, CH). All other reagents and solvents were of analytical-reagent grade and supplied by Merck (Darmstadt, Germany). Deionised and bi-distilled water was obtained from a Milli-Q water purification apparatus (Millipore, Bedford, MA, USA).

2.2. Sample preparation

The milk powder samples of different manufacturers were used for this study and were purchased off-the-shelf from local supermarkets. A 5.00 ± 0.05 g test portion of milk powder was weighed into a 50 mL Falcon polypropylene tube (Becton Dickinson, Pont de Claix, France) and fortified with 0.5 μg/kg of d_5-CAP (250 μL of an aqueous 10 ng/mL solution). A solution (15 mL) of trichloroacetic acid (10% (v/v) in water) was added. The mixture was vortexed and then heated at 65 °C for 1 h in a thermostated water bath. After cooling down to room temperature the mixture was centrifuged at 13,000 rpm for 15 min (10 °C) and the supernatant filtered over glass wool and then rinsed with an additional water portion (10 mL). The pH of the filtrate was adjusted to 5.0 with a 0.1 M sodium acetate buffer solution. An Oasis HLB SPE cartridge (500 mg) was conditioned with successively methanol (6 mL), water and hydrochloric acid (10 mM), each 4 mL. The milk extract was loaded onto the SPE cartridge and penetrated at 1–2 drops/s by applying a slight vacuum. The column was rinsed with consecutively water (4 mL), water/methanol (95/5, v/v, 2 mL) and water/methanol (50/50, v/v, 2 mL). CAP and d_5-CAP were finally eluted with methanol (2 mL) and the extract dried under a stream of nitrogen at 60 °C. The dry residue was taken up in water (0.4 mL), the pH of the extract adjusted to 6.5 (with 1N hydrochloric acid) and transferred to a 1.5 mL Eppendorf tube. A liquid/liquid extraction was conducted by adding acetonitrile/dichloromethane (4/1, v/v, 0.6 mL). After thoroughly mixing, the solution was centrifuged at 7000 rpm for 5 min (Eppendorf centrifuge). The upper organic layer was transferred into a 2 mL reactivial (Pierce). The liquid–liquid extraction was repeated twice and the pooled organic fractions evaporated to dryness under a stream of nitrogen at 60 °C. The dry residue was taken up in water (200 μL) and filtered through a 0.2 μm nylon filter (Spartan 13/0.2 RC, Schleicher & Schuell) directly into a HPLC vial.

2.3. LC–ESI-MS–MS conditions

The LC–MS–MS conditions are identical to those described for the determination of CAP in meat-based samples and honey [26–27]. HPLC analyses were performed on a C18 reversed-phase (RP) SymmetryShield HPLC column (150 mm × 2.1 mm i.d., 3.5 μm) fitted with a SymmetryShield RP C18 precolumn (10 mm × 2.1 mm i.d., 3.5 μm) (Waters, Milford, MA) using a Perkin-Elmer HPLC 200 pump series system (Norwalk, CT). The mobile phase was constituted for solvent A: water and solvent B: acetonitrile. The linear gradient program was: 0–3 min 0% B; 3–10 min 100% B; held for 5 min at 100% B before coming back at 0% B in 1 min and followed by a re-equilibration time of 4 min (constant flow rate of 300 μl/min). Using these conditions, the retention time of CAP was observed at 8.2 min. The injection volume was 15 μl and the entire HPLC flow was directed into the MS detector between 6 and 12 min using a VICI diverter (Valco Instruments, Houston, TX).

MS detection was done on a Sciex API 3000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ionization source (resolution 0.7 amu, full width half mass). Nitrogen was used for the nebulizer and collision gases at pressures of 0.87 bar and 5 mTorr, respectively; for the TurboIonSpray and curtain gases and flow-rates of 7.5 l/min and 13 ml/min, respectively. The source block temperature was set at 350 °C and the electrospray capillary voltage to 3.5 kV. The declustering potential and the dwell time for each transition reaction were set at 65 V and 100 ms. Data acquisition was performed using the Sciex Analyst software in negative multiple reaction monitoring (MRM) alternating eight transition reactions (*m/z* 321 → 152, 321 → 257, 323 → 152 and 323 → 257 for CAP and *m/z* 326 → 157, 326 → 262, 328 → 157 and 328 → 262 for d_5-CAP).

2.4. Determination of extraction recovery using radio-labelled standard

A solution of ^{14}C-CAP in ethanol/water (2/98, v/v; 100 μCi/mL) was diluted 4000-fold in water. Milk powder samples were spiked with ^{14}C-CAP at a level of 0.7 μg/kg (25 μL of a 147 ng/mL solution, 25 nCi/mL) in quadruplet experiments. Extraction was then performed as described above. Radioactivity was measured by liquid scintillation counting with an LKB-Wallac 1219 Rackbeta counter (Perkin-Elmer Life Sciences, Regensdorf, Switzerland). For this purpose, the final extract (600 μL) was thoroughly mixed with Ultima Flo M scintillator (10 mL) (Packard, Meriden, USA) prior to counting.

2.5. Quantitation

CAP was quantified by means of two external calibration curves (response ratio versus amount ratio) constructed from six calibration levels ranging from 0 to 2 μg/kg (0.5 μg/kg d_5-CAP). One calibration curve was representative of *m/z* 321 → 257 versus *m/z* 326 → 262 and the second curve representative of *m/z* 326 → 157 versus *m/z* 323 → 157. Calibration standards (aliquoted and stored at −20 °C until use) were injected before and after analyses of the samples to confirm their stability as well as that of the instrument and both data sets were used to establish the calibration curves. Uncertainty measurements were calculated from the cause and effect diagrams linked to the main relationship and to the determination of standard concentration according to specific guidelines [28–30].

3. Results and discussion

3.1. Sample extraction and clean-up

Preliminary studies were first conducted using radiolabeled ^{14}C-CAP to optimise solvents and SPE conditions to enhance the recovery of CAP from milk powders. The sample preparation of CAP in milk was similar to the one developed in honey [27] but the test portion was higher and a protein precipitation step was added before SPE. Using the described methodology, the overall recovery of ^{14}C-CAP spiked at a concentration of 0.7 μg/kg into a blank milk powder was calculated at 30 ± 4% (n = 4).

3.2. LC–ESI-MS–MS

CAP and its internal standard were first analysed and optimised in negative ESI-MS and ESI-MS–MS. The full mass spectra of CAP and its deuterated internal standard display several intense ions at *m/z* 321.1 and 323.1, and at *m/z* 326.1 and 328.1, respectively, which correspond to the characteristic isotopic cluster of the two chlorine atoms. Two main fragment ions were obtained from the collision induced dissociation (CID) experiments of *m/z* 321, 323, 326 and 328, giving rise to respectively *m/z* 257 and 152, *m/z* 257 and 152, *m/z* 262 and 157, *m/z* 262 and 157 (Fig. 1). Their respective fragmentation patterns have been reported previously [26]. Therefore, the peak areas of the transition reactions *m/z* 321 → 152 and *m/z* 321 → 257 for CAP (*m/z* 326 → 157 and *m/z* 326 → 262 for d_5-CAP) were monitored for quantitation, with the second transition showing more intense signals by a factor of approximately 1.5. Moreover, as CAP contains two chlorine atoms, two additional transition reactions *m/z* 323 → 152 and 323 → 257 (*m/z* 328 → 157 and 328 → 262 for d_5-CAP) were also recorded for additional analyte certainty. Figs. 2 and 3 depict the LC–MS–MS chromatograms of blank and spiked (at a concentration level of 0.1 μg/kg) milk powder extracts. The typical LC retention time of CAP was 8.2 min.

3.3. Method performance

A good linearity was obtained for calibration curves in solvent with a slope and a correlation coefficient for *m/z* 321

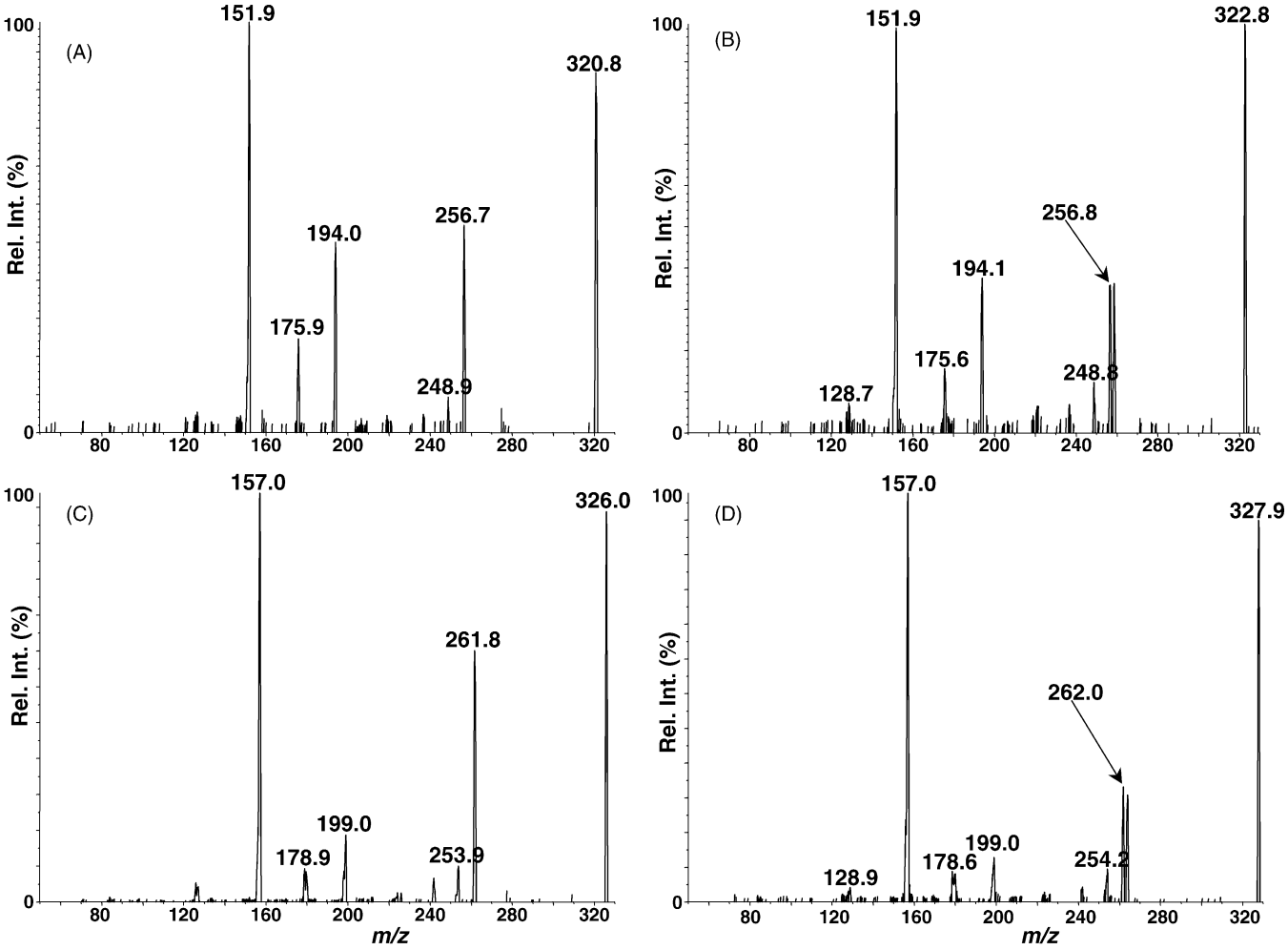

Fig. 1. CID mass spectra of CAP (A) *m/z* 321 ($^{35}Cl_2$), (B) *m/z* 323 ($^{37}Cl^{35}Cl$), and of d_5-CAP (C) *m/z* 326 ($^{35}Cl_2$) and (D) *m/z* 328 ($^{37}Cl^{35}Cl$). Nitrogen was used as collision gas with pressure and collision energy set at 5 mTorr and 20 eV, respectively.

→ 152 of 0.981 and 0.998, and for *m/z* 321 → 257 of 1.012 and 0.999, respectively. Similar slopes and correlation coefficients were also observed for matrix-matched calibration curves for *m/z* 321 → 152 of 1.142 and 0.998 and for *m/z* 321 → 257 of 1.035 and 0.988, respectively. Thus, quantification of CAP in milk samples was done using calibration curves obtained from solvent for practical reasons. By default, CAP was quantified using the calibration curve of *m/z* 321 → 152 (most intense MS–MS response), however, the second calibration curve was also used (*m/z* 321 → 257) in some milk extract samples due to the presence of an interfering co-eluting peak for the former transition reaction. Linearity was checked by calculating the standard deviation of the average of response factors (peak area ratios divided by the corresponding analyte concentration ratios of all standards), which should be below 15% to assume a linear response [31].

Residues of CAP in a sample were considered confirmed once all of the following method performance criteria were met: (a) the ratio of the retention time of the analyte to that of d_5-CAP shall be the same as that of the calibration standard in the milk matrix within a margin of ± 2.5%; (b) the presence of signal at both the four transition reactions for CAP and d_5-CAP was visible and similar results were obtained for the two transition reactions used for quantitation and (c) $^{37}Cl^{35}Cl$ ratios for *m/z* 323 → 257 versus *m/z* 321 → 257 and *m/z* 323 → 152 versus *m/z* 321 → 152 were within 0.33 ± 20% and 0.65 ± 15%, respectively. According to the EU criteria, a system of identification points (IPs) is used to define the number of ions and their corresponding ratios that must be measured when using MS techniques [25]. For the LC–MS–MS analysis of CAP, which belongs to Group A substances (with no MRL), a minimum of four IPs are required. In this case, measurement of two precursor ions (i.e. 321 and 323) earns 2IPs plus the four transition reactions (*m/z* 321 → 152, 321 → 257, 323 → 152 and 323 → 257; giving 6IPs, i.e. 1.5IPs each) leading to a total of 8 IPs. As a positive unambiguous confirmation of the presence of CAP in the extracts, the chlorine ratios ($^{37}Cl^{35}Cl$) of *m/z* 323 → 257 versus 321 → 257 and *m/z* 323 → 152 versus 321 → 152 were calculated from the analysis of standard solutions

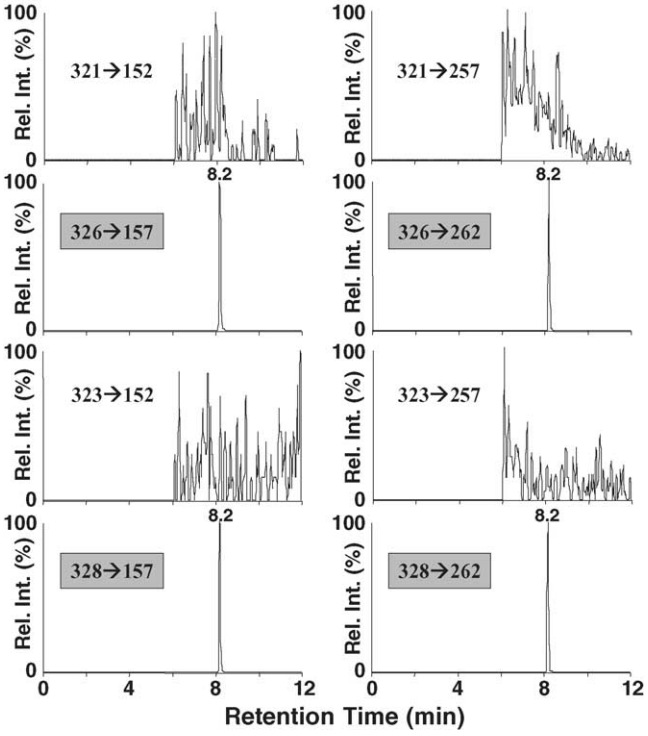

Fig. 2. LC–ESI-MS–MS chromatogram of a blank milk powder acquired in negative ionisation MRM mode. The different transition reactions of d_5-CAP spiked at 0.5 μg/kg are framed and shaded in grey.

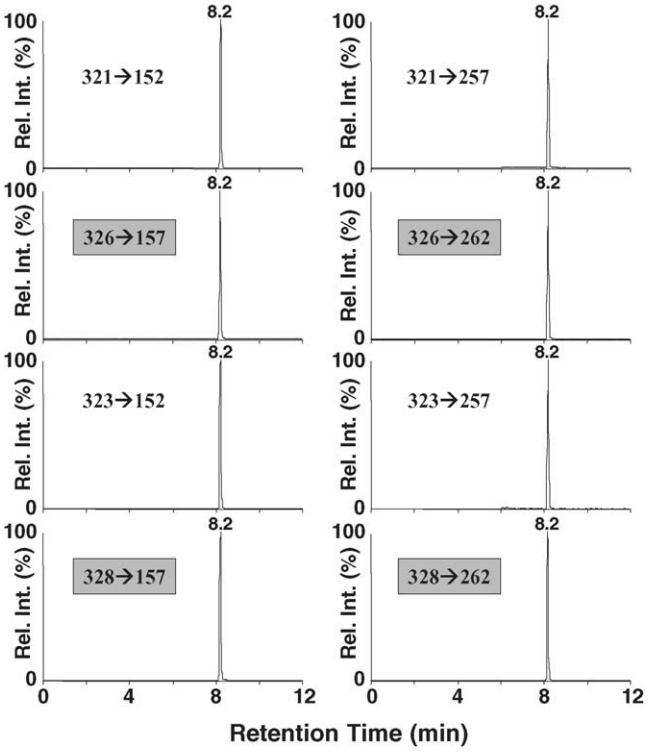

Fig. 3. LC–ESI-MS–MS chromatogram of a blank milk powder spiked at a concentration level of 0.1 μg/kg acquired in negative ionisation MRM mode. The different transition reactions of d_5-CAP spiked at 0.5 μg/kg are framed and shaded in grey.

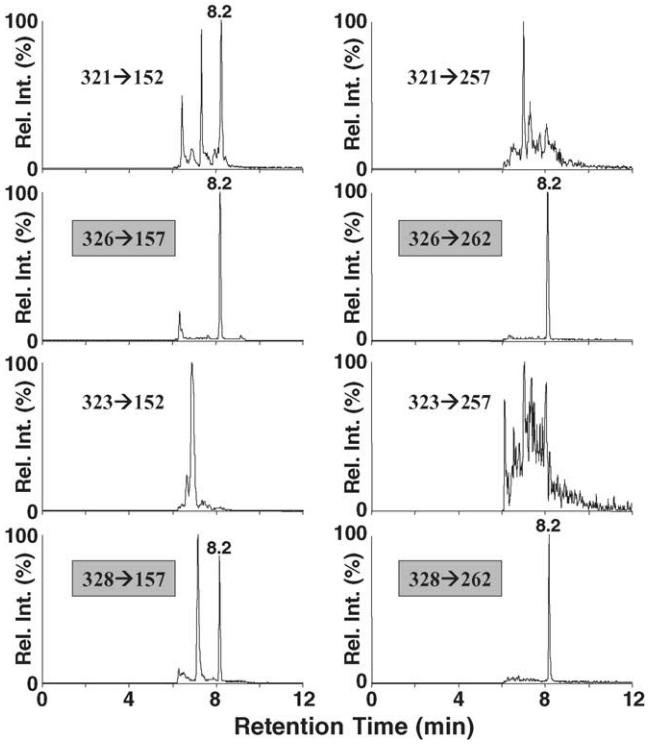

Fig. 4. LC–ESI-MS–MS chromatogram of a blank milk powder acquired in negative ionisation MRM mode. The different transition reactions of d_5-CAP spiked at 0.5 μg/kg are framed in grey.

and accepted in milk extract samples at 0.33 ± 20% and 0.65 ± 15%, respectively. The same comparison of ion ratios (*m/z* 328 → 262 versus 326 → 262 and *m/z* 328 → 157 versus 326 → 157) was performed for the d_5-CAP to confirm that interfering chemicals did not pollute the response of the internal standard. Fig. 4 depicts LC–ESI-MS–MS chromatograms of a blank milk extract. The importance of monitoring several MS–MS transition reactions is evident. Indeed, the peak eluting at a retention time of 8.2 min observed for *m/z* 321 → 152 may be erroneously attributed to CAP in the matrix, but its presence is obviated by the absence of the characteristic signals at *m/z* 323 → 152, 321 → 257 and 323 → 257.

Repeatability was calculated from the analysis of six blank milk powders spiked with CAP at each of three fortification levels (0.1, 0.2 and 0.5 μg/kg) and performed by one operator on three separate occasions. According to 2002/657/EC, the repeatability needs to be calculated on fortified matrix at concentration equivalent to 1, 1.5 and 2 times the MRPL (i.e. 0.3 μg/kg corresponding to 0.3, 0.45 and 0.6 μg/kg). However, no MRPL was defined at the time when our method was validated, so the fortification levels used in this work do not exactly agreed with the ones expected but still give some indication on this parameter. The repeatability at the 95% confidence level was then deduced from the within-day precision using an expansion factor of 2.77. Accuracy and within-laboratory reproducibility were obtained following the same protocol, but three operators carried out analyses on one occasion. The within-laboratory reproducibility was

Table 1
Performance data of the LC–ESI-MS–MS method for the analysis of CAP in milk powder

	Fortification levels (μg/kg)					
	0.10		0.20		0.50	
Transition reaction (*m*/*z*)	321 → 257	321 → 152	321 → 257	321 → 152	321 → 257	321 → 152
Under repeatability conditions[a]:						
Overall mean ± S.D. (*n* = 18)	0.11 ± 0.01	0.11 ± 0.01	0.21 ± 0.03	0.21 ± 0.02	0.50 ± 0.02	0.55 ± 0.03
r (μg/kg)[b]	0.02	0.03	0.07	0.04	0.04	0.10
Under within-laboratory reproducibility conditions[c]:						
Overall mean ± S.D. (*n* = 18)	0.10 ± 0.01	0.11 ± 0.02	0.20 ± 0.02	0.21 ± 0.03	0.49 ± 0.05	0.53 ± 0.08
Overall accuracy (%)	104	112	101	106	98	106
iR (μg/kg)[d]	0.02	0.05	0.06	0.08	0.15	0.21

[a] Six negative milk powders were spiked at each of the three fortification levels and analysed on three separate occasions by the same operator using the same equipment over a 2 week period.
[b] Repeatability at the 95% confidence level.
[c] Six negative milk powders were spiked at each of the three fortification levels and analysed by three operators using the same equipment over a 1 month period.
[d] Within-laboratory reproducibility at the 95% confidence level.

obtained by multiplying the within-laboratory precision by an expansion factor of 2.77 (95% confidence level). The performance data of the described procedure are summarised in Table 1 and show that both transition reactions sets give comparable results.

The new analytical limits, namely the decision limit (CCα) and the detection capability (CCβ) were calculated from the within-laboratory experiments as previously described [26]. Both transition reactions gave similar results for the decision limit (CCα) calculated at 0.02 μg/kg for both *m*/*z* 321 → 152 and 321 → 257, and the detection capability (CCβ) calculated at 0.03 μg/kg and 0.04 μg/kg for *m*/*z* 321 → 152 and *m*/*z* 321 → 257, respectively. Taking into account the amount of starting material (5 g) and the recovery mean value (30%, *n* = 4), the CCα and CCβ values based on the MRM response *m*/*z* 321 → 257 are estimated on-column at 30 pg (93 fmol) and 60 pg (186 fmol), respectively.

3.4. Measurement of uncertainty

The estimation of measurement uncertainty is based on the results of in-house testing of spiked and QC samples. Its significant relevance corresponds to the range over which our analytical result will fall provided that the analytical system is "under control". The analytical parameters taken into account are precision (repeatability, within-laboratory reproducibility), trueness and calibration data (standard preparation, linear regression). Each step involved in the sample preparation (i.e. weight of test portion, preparation and dilution of internal standard, volumes, injection, etc.) was assigned to a defined uncertainty and summed as a final value. This uncertainty was calculated using an expansion coefficient of 2, which represents a confidence interval of 95%. The measurement uncertainty (U±) of CAP for *m*/*z* 321 → 152 and *m*/*z* 321 → 257 at each fortification level (i.e. 0.1, 0.2 and 0.5 μg/kg) is shown in Table 2. The uncertainty values and precision data obtained from the within-laboratory reproducibility (Table 1) compare well, except at the higher fortification level of 0.5 μg/kg (*U* = 10 and 15% against 9 and 11% for *m*/*z* 321 → 152 and 321 → 257, respectively).

Table 2
Measurement of uncertainty

Fortification levels (μg/kg)	Uncertainty on CAP at 95% confidence level	
	m/*z* 321 → 152	*m*/*z* 321 → 257
0.1	0.10 ± 0.02	0.10 ± 0.04
0.2	0.20 ± 0.05	0.20 ± 0.07
0.5	0.50 ± 0.1	0.50 ± 0.15

Any loss of the analyte during the analytical procedure (sample pre-treatment) will be compensated by the same behaviour of its deuterated surrogate standard, consequently minimising the final uncertainty value. Therefore, the precision values obtained from the within-laboratory reproducibility data encompass by large those of the uncertainty measurements.

4. Conclusion

A quantitative single residue method using isotope dilution LC–ESI-MS–MS for determining trace levels of chloramphenicol in milk powders has been developed and validated according to the new EU criteria for the analysis of veterinary drug residues. The method clearly demonstrates good accuracy, sensitivity, selectivity, and the ability to quantify with adequate certainty the presence of residues of chloramphenicol in the sub part-per-billion range (CCβ calculated at 0.03 μg/kg and 0.04 μg/kg for *m*/*z* 321 → 152 and *m*/*z* 321 → 257, respectively). This method is suitable for a wide range of different milk powders, and can be employed as a quality monitoring tool especially in those countries/regions where the use of illegal drugs is not under adequate control.

Further work will be conducted in our laboratory aimed at incorporating CAP within a multiresidue method for the simultaneous detection of several veterinary drug residues (e.g. tetracyclines, aminoglycosides, macrolides) in a common extraction and analytical run.

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