

Analytical Methods

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3 **Validation of a new high throughput method for determination of chloramphenicol**
4 **in milk using liquid-liquid extraction with low temperature partitioning (LLE-**
5 **LTP) and isotope-dilution liquid chromatography tandem mass spectrometry (ID-**
6 **LC-MS/MS)**
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Abstract

Chloramphenicol (CAP) is an antibiotic banned for treatment of food-producing animals. The minimum required performance limit (MRPL) for analytical methods of 0.3 $\mu\text{g kg}^{-1}$ was set by the European Community for the detection of its residues in different matrices, including milk. A highly sensitive analytical method using isotope-dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) and liquid-liquid extraction with low temperature partitioning (LLE-LTP) was developed and validated for CAP residues in milk. Selected validation parameters such as selectivity, working range and linearity, trueness (recoveries), precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ) were evaluated. The validation procedures were based on the new Eurachem Guide and the European Union Commission Decision 2002/657/EC. The matrix effect was evaluated by ion suppression test and by comparison of matrix-matched analytical curve and the solvent standard analytical curve. A linear working range between 0.1 to 5.0 $\mu\text{g kg}^{-1}$ was observed. The homoscedasticity was demonstrated by the Cochran test, the coefficient of determination (R^2) was higher than 0.99 and the residual plot was free of trends. The limits of detection and quantification were 0.015 $\mu\text{g kg}^{-1}$ and 0.05 $\mu\text{g kg}^{-1}$, respectively. Mean recoveries evaluated at three levels (0.3, 1.0 and 3.0 $\mu\text{g kg}^{-1}$) were ranged from 94% to 114% with RSDs lower than 6.7% (repeatability). For intermediate precision, different analysts were compared and the RSDs were lower than 7.3%. The method was accurate and reproducible, and was successfully applied to the evaluation of CAP residues in milk samples.

Keywords

Chloramphenicol, milk, isotope-dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS), liquid-liquid extraction with low temperature partitioning (LLE-LTP)

1 Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic of amphenicols drugs family, isolated from *Streptomyces venezuelae*, which has historical use in veterinary medicine for treatments of several infections.^{1,2} CAP is also named d-(-)-threo-1-*p*-nitrophenyl-2-dichloroacetamido 1,3-propanediol and its chemical structure has both lipophilic and hydrophilic groups and substituents. Due to the presence of polar and nonpolar groups, CAP is slightly soluble in water and soluble in organic solvents as ethanol, methanol, acetonitrile and ethyl acetate. It is effective against a wide range of microorganisms and because of its low cost and ready availability, it has been extensively used worldwide since the 1950s in the treatment of animals, including food-producing animals.³⁻⁵ However, the ingestion of CAP by human beings can cause serious hemotoxic effects, such as the development of bone marrow suppression or aplastic anemia.^{1,6-8} Therefore, its use is reserved to the treatment of serious infections, when no other alternative is available⁷ and it was banned for the treatment of food-producing animals in the European Union, the United States and several other countries, including Brazil.⁹⁻¹³ Since the toxic effects from CAP are not dose dependent, an effect level could not be established and consequently a zero tolerance level was set for CAP residues in food.^{7,14} The minimum required performance limit (MRPL) for analytical methods of 0.3 $\mu\text{g kg}^{-1}$ was set by the European Community for the detection of CAP

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3 residues in different food matrices, including meat and milk.¹² Products intended for
4 internal consumption, importation or exportation must not exceed this mass fraction
5 level.
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10 However, CAP residues are still found in many foodstuffs despite its use
11 prohibition. According to the Rapid Alert System for Feed and Food (RASFF), a
12 database created by the European Commission, between 2002 and 2014, 449
13 notifications of CAP contamination in various matrices were reported in the RASFF
14 database, among which, 40 corresponded to dairy products. Data for the last three years
15 shows 51 notifications of CAP residues in crustaceans, meat, milk, feed additives and
16 others matrices. In 2014, CAP residues ($2.22 \mu\text{g kg}^{-1}$) were found in raw milk in
17 Poland.¹⁵ Therefore, the presence of prohibited substance CAP in foods is still a real
18 problem worldwide, and the development and validation of new methods for the
19 determination of CAP residues in foodstuffs is an important task to ensure reliable
20 analysis.
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36 Different analytical methods have been developed to determine CAP in milk and
37 food matrices using liquid chromatography (LC). Most of these employ a liquid-liquid
38 extraction (LLE) step with ethyl acetate^{1,4,7,16,17} and some of them include a clean-up
39 step using solid-phase extraction,^{3,14,18} solid-phase dispersion extraction^{2,4} and
40 molecularly imprinted polymers (MIPs),^{14,19} or hexane for fatty removal.^{14,17} A new
41 method of CAP extraction from milk using QuEChERS was also described.²⁰
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51 Recently, liquid-liquid extraction with low temperature partition (LLE-LTP) was
52 introduced for milk processing as an alternative extraction method and was shown to be
53 simple and cost-effective, since no clean-up step is necessary. The LLE-LTP was
54 described for the determination of pyrethroids in milk,^{21,22} water²³ and butter,²²
55 avermectins in milk^{24,25} and bovine muscle,²⁶ and aflatoxins and ocratoxin A in breast
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3 milk.²⁷ The LLE-LTP was used for qualitative screening of selected veterinary drugs in
4 porcine muscle such as tetracyclines, sulfonamides, penicillins, quinolones, macrolides
5 and benzimidazoles,²⁸ but to the best of our knowledge, it was not previously employed
6 for the determination of CAP in milk or dairy products. The LLE-LPT is based on the
7 miscibility of acetonitrile and water. To carry out this extraction, acetonitrile is added to
8 milk in a ratio of 2:1 (v/v). The resulting mixture is homogenized and only one liquid
9 phase containing water and solvent is formed. The liquid phase (in fact a suspension) is
10 cooled to -20 °C for some hours. Under these conditions, the aqueous phase is frozen,
11 whereas the acetonitrile rich phase that contains the analytes of interest, remains liquid,
12 and can be removed and concentrated.^{23,26,28,29} This simple and efficient method
13 employs reduced amounts of solvent, requires few manipulation steps and demands no
14 extra clean-up, allowing high recovery to be achieved.²⁸

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32 An isotope-dilution liquid chromatography tandem mass spectrometry (ID-LC-
33 MS/MS) following the liquid-liquid extraction with low temperature partitioning (LLE-
34 LTP) of chloramphenicol (CAP) residues in milk was developed and validated. This
35 sample preparation method is quick and clean-up procedures (solid-phase extraction) or
36 defatting with solvent are not necessary. The goal of this work is to present the
37 development and validation of a reliable method to be used in routine analysis of CAP
38 in milk samples (fresh and powdered milk). This method is also being applied in the
39 certification study of a candidate certified reference material of chloramphenicol in milk
40 powder, currently under development in the Brazilian National Metrology Institute
41 (Inmetro). Isotope dilution mass spectrometry and gravimetry, which are both primary
42 methods, were used for sample and standard preparation. Together with the purity
43 assessment of CAP standard, these methods confer metrological traceability to the mol.
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2 Experimental

2.1 Reagents and solutions

Chloramphenicol (CAP) purity > 99% (Sigma-Aldrich, Saint Louis, Missouri, USA) and chloramphenicol-*d5* (CAP-*d5*) 100 $\mu\text{g mL}^{-1}$ in acetone (Dr Ehrenstorfer, Augsburg, Germany) were used as standards. The purity of CAP was determined in-house by quantitative proton nuclear magnetic resonance (^1H qNMR): 99.40 ± 0.65 % m/m ($k=2.77$; 95%). Acetonitrile HPLC grade (Tedia, Fairfield, Ohio, USA), methanol HPLC grade (J. T. Baker, Center Valley, Pennsylvania, USA) and ethanol Licrosolv (Merck, Darmstadt, Germany) were used. Ultra-purified water (Type I) was prepared through a water purification system (Millipore, Molsheim, France). All solutions and samples were gravimetrically prepared using an analytical balance with 220 g capacity and 0.01 mg resolution (Mettler Toledo, Greifensee, Switzerland).

2.2 Preparation of stock, calibration standards and QC solutions for CAP determination in milk samples

Sufficient amount of CAP was weighed and dissolved in ethanol to give a concentrated stock solution ($10.0\mu\text{g g}^{-1}$), which was stored at 4 °C until its use. A working standard solution of CAP in water (10.0ng g^{-1}) was prepared by gravimetric dilution of an appropriate aliquot of the stock solution.

All solution from an ampoule of chloramphenicol-*d5* (*d5*-CAP) 100 $\mu\text{g mL}^{-1}$ in acetone was gravimetrically diluted in sufficient ethanol to give a mass fraction of approximately $10.0 \mu\text{g g}^{-1}$ (internal standard stock solution). A working solution of *d5*-

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3 CAP in water, with a mass fraction of approximately $10.0 \mu\text{g kg}^{-1}$, was prepared by
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5 gravimetric dilution of an appropriate aliquot of the stock solution.
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8 Eight standard solutions of CAP in water (0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0 and
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10 $5.0 \mu\text{g kg}^{-1}$) containing $1.0 \mu\text{g kg}^{-1}$ of *d5*-CAP were gravimetrically prepared and used
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12 to obtain the analytical curve. Quality control (QC) samples at levels of 0.3, 1.0 and 3.0
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14 $\mu\text{g kg}^{-1}$ were also prepared using the blank matrix (milk).
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20 **2.3 Blank milk samples**

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24 For the initial method optimization, whole and skimmed milk samples purchased
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26 in local supermarkets were used. Afterwards, raw whole milk samples were employed
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28 to the method development and validation.
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32 A large amount of blank raw milk was obtained by hand-milking a dairy cow of
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34 the Sector of Bovine Milk Culture of the Rural Federal University of Rio de Janeiro.
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36 The animal had never been submitted to any treatment using CAP. After milking, the
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38 milk sample was stored at 4°C until pasteurization in the subsequent day using an ultra-
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40 high temperature (UHT) pasteurizer system (model FT25D Armfield, United Kingdom)
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42 operated at 75°C for 20 seconds. After pasteurization, the liquid milk was mixed in a
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44 blender with soy lecithin powder (emulsifier) to facilitate the posterior milk powder
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46 reconstitution. The milk powder was obtained using a spray dryer (Niro Atomizer,
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48 Denmark), operated with inlet and outlet temperatures of 190°C and 90°C , respectively.
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51 The whole milk powder material (blank) was vacuum-sealed, stored at -80°C and
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53 protected from the light. These samples were analyzed using the developed method and
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55 considered as blank samples for CAP. They were also used to prepare spiked quality
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57 control samples (QC) for validation purposes. Milk powder samples were reconstituted
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3 gravimetrically by adding 9 mL (~ 9 g) of ultrapure water to 1 g of milk powder. For the
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5 complete reconstitution, the resulting suspension was vortex-mixed for 1 min and kept
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7 in an ultrasonic bath for 20 min at 40°C. Each aliquot of 1 g of milk powder
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9 corresponded to approximately 10 g of fresh milk.
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20 Liquid-liquid extraction with low temperature partitioning (LLE-LTP) was
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22 employed for milk extraction. This method was applied in fresh milk (liquid) and in
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24 powdered milk (after reconstitution). A 2 mL milk aliquot was taken and weighed in a
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26 tube. An aliquot of 100 µL of internal standard working solution (*d5*-CAP) was
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28 transferred into the solution and it was weighed to determine the mass of internal
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30 standard added. An aliquot of 4 mL of acetonitrile was added to the tube and vortex
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32 mixed for 1 min. The milk protein was precipitated and centrifuged at 4000 rpm (1.600
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34 g) for 5 min at 20°C using a Z300 K, Hermle centrifuge. The supernatant single phase
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36 containing water and acetonitrile was transferred to another tube. The precipitated milk
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38 was submitted to another extraction step by the addition of 1 mL of water and 2 mL of
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40 acetonitrile. The tube was vortex mixed, centrifuged again and the liquid phase was
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42 combined with the first extract. The tube was cooled down to -20°C for at least 4 h for
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44 water freezing whereas the acetonitrile rich phase that contained the analyte remained
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46 liquid. To facilitate this phase removal from the tube, it was centrifuged at 4000 rpm
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48 (1.600 g) for 5 min at -20°C. The acetonitrile was removed from the tube using a
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50 Pasteur pipette before the ice melted. The sample was evaporated under N₂ flow until
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52 dryness at 60°C and reconstituted using 1 mL of the mobile phase. The resulting
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3 solution was filtered through a 0.22 μm disposable filter (13mm, Millipore) and
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5 analyzed by UPLC-MS/MS as described below.
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10 **2.5 Chromatographic and Mass Spectrometric conditions**

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15 CAP was determined using a UPLC-MS/MS System (Xevo TQ, Waters, USA).
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17 Two different columns Acquity Uplc@ BEH C18 (50 mm x 2.1 mm, 1.7 μm , Waters,
18 USA) and Acquity Uplc@ HSS C18 SB (50 mm x 2.1 mm, 1.8 μm , Waters, USA) were
19 evaluated. The latter was selected for the method after a selectivity study. A mobile
20 phase composed of methanol and water (50:50, v/v), run in isocratic mode, at a flow
21 rate of 0.20 mL min^{-1} , allowed the best analytical conditions to be obtained. The
22 injection volume for standards and extracts were 10 μL . Mass spectrometric analysis
23 was performed by electrospray ionization operating in negative mode (ESI-). Multiple
24 Reaction Monitoring (MRM) was employed for quantitative analysis. The transitions
25 and collision energies were m/z **321.1>152.0** (18V) and m/z 321.1>257.0 (11V) for
26 CAP, and m/z **326.0>157.0** (18V) and m/z 326.0>262.0 (11V) for *d5*-CAP. The first
27 transitions (in bold) were used for the quantification and the others for the analyte
28 confirmation. The ESI parameters were: capillary voltage (1.0 kV), cone voltage (22
29 kV), desolvation temperature (600 $^{\circ}\text{C}$) and desolvation gas flow (900 L/Hr). All data
30 was acquired by MassLynx 4.1 software. The four identification points required by
31 2002/657/EC Decision¹¹ were obtained using LC-MS/MS with one precursor and two
32 products ions.
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58 **2.6 Validation method for CAP quantification by ID-LC-MS/MS**

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3 The analytical parameters of merit of the developed method were evaluated,
4 such as selectivity and matrix effect, working range and linearity, limit of detection
5 (LOD) and limit of quantification (LOQ), precision expressed as repeatability and
6 intermediate precision, and trueness evaluated through recovery studies. The validation
7 procedures followed the guidelines of the Eurachem Guide,³⁰ Commission Decision
8 2002/657/EC,¹¹ IUPAC Guideline,³¹ National Association of Testing Authorities of
9 Australia,³² US Food and Drug Administration,³³ and Brazilian National Institute of
10 Metrology, Quality and Technology Guide,³⁴ with is harmonized with international
11 regulations.
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27 **3 Results and discussion**

31 **3.1 Selectivity**

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36 Selectivity is the degree to which a method is able to quantify accurately the
37 analyte of interest being indifferent to the presence of the species capable of interfering
38 in its determination.^{30,31}
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44 First, the optimal signal for CAP was obtained by optimization of mass
45 spectrometry parameters as the capillary voltage, cone voltage, desolvation gas
46 temperature, desolvation gas and the collision energies with direct injection of CAP
47 solution at 1 mg kg⁻¹ on negative ionization mode (ESI-). One precursor and two
48 product ions were selected for CAP and *d5*-CAP to MRM experiments. The
49 chromatographic conditions were optimized and the retention time, separation
50 resolution of the chromatographic peaks and mass-spectrometric signals were observed.
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3 The signals of CAP and *d5*-CAP found under the chromatographic conditions employed
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5 are shown in Fig. 1.
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18 The first step of the selectivity study was the evaluation of interfering peaks in
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20 the matrix. Standard solutions, blank and spiked milk samples were analyzed in both
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22 columns by MRM. No peaks were observed for the blank milk samples in these
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24 experiments showing that they were free of CAP.
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28 The ion suppression of CAP resulting from the matrix effect was checked with
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30 the two UPLC columns and extracts of three types of milk: whole, skimmed and
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32 partially skimmed. With this purpose, a CAP solution was infused directly into the mass
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34 spectrometer generating a constant MRM signal, whereas the matrix extract (blank) was
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36 injected through the UPLC system. Under these conditions, a drop of the very selective
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38 MRM signal in the retention time of CAP would indicate an ion suppression effect.
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42 The ion suppression of CAP is shown in Fig. 2. The retention time of CAP was
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44 1.64 min (run time: 4 min) using the column Acquity UPLC@ BEH C18 (1.7 μ m X 2.1
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46 X 50 mm) (Fig. 2a). However, the injection of blank milk extract under TIC (Total Ion
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48 Chromatogram) conditions resulted in two peaks eluting with retention times similar to
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50 that of CAP (Fig. 2b), showing that some matrix compounds coeluted with CAP. This
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52 fact resulted in the suppression of CAP signal under MRM conditions (Fig. 2c). This
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54 fact was observed for all milk studied. These results indicated that despite the good
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56 chromatographic conditions obtained, a remarkable matrix effect was found using this
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3 column. The ion suppression was previously observed when using this column for
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5 analysis of CAP in chicken muscle.³⁵
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8 On the other hand, ion suppression was not observed using the Acquity UPLC@
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10 HSS C18 SB (1.8 μm x 2.1 x 50 mm) column evaluated in a similar set of experiments.
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12 The retention time (RT) of CAP was 1.04 min (run time: 3 min) (Fig. 2d). Whole,
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14 skimmed and partially skimmed milk were tested and no peaks were found in this part
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16 of the chromatogram (Fig. 2e) resulting in a constant signal of CAP (Fig. 2f) without
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18 any suppression of CAP ion signal. This fact was observed for all milk studied. These
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20 results showed that there were no matrix effects using this column, which was selected
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22 for the method development.
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36 **3.1.1 Matrix effect**

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41 Interferences may cause a bias by increasing or decreasing the analyte signal.
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43 The extent of the effect for a given matrix is usually proportional to the matrix signal
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45 and changes the slope of the calibration function.³⁰ To determine the extent of a matrix
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47 effect on an instrument response, the slopes of analytical lines were obtained by
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49 analyzing sets of standards in the same range of concentrations, prepared in matrix-free
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51 solvent and in the matrix by standard addition, and were compared for significant
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53 difference.³² Three sets of standards ranging from 0.1 to 5.0 $\mu\text{g kg}^{-1}$ were prepared
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55 gravimetrically and analyzed in triplicates at each level. The sets were prepared in water
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60 (solvent), raw whole milk and partially skimmed milk (a mixture of containing 50% v/v

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3 of raw whole milk and 50% v/v of commercial skimmed milk). The matrix standards
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5 were submitted to the preparation procedure described in Section 2.4.
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8 The parameters of the analytical curves and the coefficients of determination
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10 between the ratios of the areas of the peaks of CAP and *d5*-CAP and the ratios of their
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12 masses in the standard solutions were obtained by the least squares method. The slope
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14 of each matrix-matched analytical curve was compared with the solvent analytical curve
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16 (in water) by t-test for slope. The matrix-matched slopes were statistically equivalent to
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18 the aqueous standards slope ($t_{\text{calculated}} < t_{\text{critical}}$). The three analytical curves (Fig.
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20 3) were practically superposed. The curve parameters, their confidence intervals (at
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22 95%) and the t-test for slope results are shown in the Table 1. The first aspect to be
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24 highlighted is the excellent agreement among the curve parameters regardless of the
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26 standard compositions. The slopes of the lines were statistically equivalent as evidenced
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28 by the superposition of their confidence intervals obtained in water and milk extracts.
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30 The difference between slope values of milk extracts and aqueous standards were below
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32 2.2%, and satisfied the comparison criterion established by NATA³² that consider no
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34 significant difference when it is below 10%. In addition, the confidence intervals of the
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36 intercepts of the three curves included the origin (zero) demonstrating the absence of
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38 systematic errors. Thus, there is no need to compensate for matrix effects and the
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40 aqueous analytical curve was adopted for this method.
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60 3.2 Working range and linearity

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The working range is the interval over which a method provides results with an acceptable uncertainty, that can be demonstrated by suitable levels of precision, accuracy and linearity.^{30,32} The lower limit of the working range is the limit of quantification (LOQ) and the upper limit is defined by the concentration above which significant anomalies in the analytical sensitivity, expressed as angular coefficients, are observed.³⁰ On the other hand, the linearity of an analytical method is its ability to give test results directly proportional to the amount of analyte in the sample.³⁶

The linearity of the developed method was studied using aqueous standards gravimetrically prepared in the working range (0.1 to 5.0 $\mu\text{g kg}^{-1}$) leading to the parameters of the analytical curve shown in the Table 1. Aqueous standards were selected due to the results obtained in the selectivity studies. The determination coefficients (R^2) larger than 0.99, indicated a good adherence to a linear model. However, a Cochran test was applied considering a confidence of 95%, with 8 levels and 3 replicates per level. The calculated value ($C_{\text{calc}} = 0.4828$) was lower than the tabulated value ($C_{\text{tab}} = 0.5157$) demonstrating the homoscedasticity of the analytical curve. Moreover, the plot of residues was free of trends (Fig. 4). The intercept of the analytical curve close to zero (Table 1) also indicated an analytical system free of trends.

The method linearity was demonstrated in the working range evaluated. Therefore, analytical curves obtained using aqueous standards were adopted for the continuity of the method validation study.

Insert Fig. 4

3.3 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of quantification (LOQ) is the lowest level of analyte that can be determined with an acceptable performance, which includes good precision and trueness.³⁰ The LOQ was obtained experimentally by successive dilutions. It was estimated by a signal to noise ratio (S/N) of 10:1 and corresponded to the first calibration level of the analytical curve ($0.1 \mu\text{g kg}^{-1}$). The limit of detection (LOD) is estimated by a signal to noise ratio (S/N) of 3:1 and it is the lowest concentration of the analyte that can be detected by the method or differentiated from the background for a particular matrix (blank). In this study the LOD was estimated by dividing the LOQ by a factor of 10/3 or 3.33.

Therefore, the instrument LOD and LOQ were estimated at 0.03 and $0.1 \mu\text{g kg}^{-1}$ respectively. The trueness and precision of the LOQ were checked with replicates of spiked milk samples at this concentration. The mean recovery was 101% and the relative standard deviation of 3.1%. The LOD was checked with spiked milk samples at its level and the difference of CAP signal from the background of blank samples was proved, showing LOD was determined appropriately. However, considering that the sample treatment leads to an extract with a concentration factor of 2 (Section 2.4) it is possible to estimate the LOD and LOQ of milk samples using the developed method is the half of instrumental limits, ie 0.015 and $0.05 \mu\text{g kg}^{-1}$, respectively. This method achieves the MRPL of $0.3 \mu\text{g kg}^{-1}$ established to CAP measurements in food.¹²

3.4 Accuracy: Trueness and Precision

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3 The accuracy of a measurement result describes how close it is to its true value
4 and includes the effects of trueness (systematic error) and precision (random error).³²
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6 Trueness is the closeness of agreement between the average of an infinite number of
7 replicate measurements and a reference value.³⁷ However, due to the impossibility of
8 taking an infinite number of measurements, the trueness cannot be estimated in this
9 way. A practical trueness determination is expressed quantitatively in terms of bias.³⁰
10 Precision is the closeness of agreement between measured values obtained by replicate
11 measurements of the same or similar objects under specified conditions. It can be
12 estimated under different conditions (repeatability, intermediate precision or
13 reproducibility) and it is usually expressed numerically by the standard deviation,
14 variance, or coefficient of variation.³⁷ Precision is generally dependent on analyte
15 concentration, and it should be determined at a number of concentrations across the
16 range of interest.³⁰
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33 Recovery experiments were used for bias evaluation in this study, since
34 Reference Materials for CAP in milk were not available in the market. The experiments
35 were carried out using replicates of spiked raw whole and partially skimmed milk
36 samples taken as blank matrices, at three different levels (0.3, 1.0 and 3.0 $\mu\text{g kg}^{-1}$), one
37 of which corresponded to the MRPL of CAP. The precision was evaluated by
38 repeatability studies across the range of interest during the recovery experiments.
39 Intermediate precision was evaluated comparing the results of samples spiked at the
40 same levels using different analysts at different times.
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53 Independent triplicates for each level were prepared using raw whole and
54 partially skimmed milk samples. Each sample was treated as described above (Section
55 2.4) and the resulting extracts were analyzed in triplicates in the UPLC-MS/MS system
56 leading to the recoveries and relative standard deviations presented in Tables 2 and 3.
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3 The method showed good recoveries (>90%) and repeatability (RSD <10%) for the
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5 three levels tested for both matrices.
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20 Acceptable recovery is related to analyte concentration and to the purpose of the
21 analysis.³³ The recoveries found with matrices in this study (Tables 2 and 3) met the
22 performance criteria of acceptable limits of 60 to 120% for concentrations ranging from
23 1 to 10 $\mu\text{g kg}^{-1}$ and 50 to 120% for concentrations < 1 $\mu\text{g kg}^{-1}$ established by the Codex
24 Alimentarius,³⁸ and 70 to 110% for concentrations ranging from 1 to 10 $\mu\text{g kg}^{-1}$ and 50
25 to 120% for concentrations < 1 $\mu\text{g kg}^{-1}$ as recommended by 2002/657/EC Decision.¹¹
26 The criteria established by FDA³³ and AOAC Guidelines³⁹ is also achieved, which is 70
27 to 125% at 10 $\mu\text{g kg}^{-1}$. The use of gravimetric preparations and few preparation steps of
28 the developed method certainly contributed to the excellent recoveries. The method
29 repeatability and intermediate precision, expressed as relative standard deviation
30 (RSD(%)),¹¹ was performed simultaneously with the recovery evaluation (Tables 2 and
31 3). The RSD(%) were $\leq 6.7\%$ for the three levels assayed and both matrices.
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48 According to 2002/657/EC Decision,¹¹ the coefficient of variation (CV or RSD)
49 must be as small as possible in these concentrations, and no reference value is
50 established for concentrations below 100 $\mu\text{g kg}^{-1}$. However, the AOAC Guideline⁴⁰
51 established criteria for the repeatability expressed as RSD(%) of 30% and 21% at 1 and
52 10 ppb, respectively. This performance criteria is also achieved.
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3 The values shown in Tables 2 and 3 also met the criteria previously established by
4 this manuscript's authors: the recovery ranging from 85 to 115%, and repeatability
5 with the maximum relative standard deviation of 10%. These limits values were
6 established considering its intended application in the certification process of a
7 reference material of CAP in milk.
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15 The intermediate precision of the method was evaluated comparing the data of the
16 repeatability study and the results of spiked milk samples obtained by a different analyst
17 in a different day, at the same levels (0.3, 1.0 and 3.0 $\mu\text{g kg}^{-1}$). The mean recovery was
18 90% (RSD 7.3%) for 0.3 $\mu\text{g kg}^{-1}$, 96% (RSD 6.9%) for 1.0 $\mu\text{g kg}^{-1}$ and 97% (RSD
19 4.6%) for 3.0 $\mu\text{g kg}^{-1}$. All RSD(%) values found were below 10%, showing good
20 stability of the method. In methods used to support the Codex Maximum Residue Limit
21 for Veterinary Drugs, RSD(%) up to 30% are accepted, at working concentration range
22 (1 to 10 $\mu\text{g L}^{-1}$).³⁸
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34 The analytical figures of merit presented above demonstrated the analytical
35 performance of the proposed method for the determination of analyte of interest and
36 satisfied the criteria established by international agencies for the evaluation of trace
37 levels of food contaminants, specifically, of CAP in milk, meeting also its MRPL.
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45 46 **3.5 Comparison of methods** 47

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50 In addition to validation procedures, this method was compared with the sample
51 preparation procedure more frequently described in literature, which employs liquid-
52 liquid extraction of milks using ethyl acetate for CAP extraction.^{4,16} With this purpose, a
53 2 mL milk aliquot was taken and accurately weighed. The internal standard solution
54 (d5-CAP) was added to the sample and vortex-mixed. Subsequently, formic acid 10
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3 (mmol L⁻¹) and sodium sulfate were added to the sample and vortex-mixed. Two
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6 extractions steps using ethyl acetate (4 mL) were carried out and the mixture was
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8 centrifuged at 4000 rpm (1.600 g) for 5 min at 20°C. The supernatant was transferred to
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10 another tube and evaporated under N₂ flow to dryness. Ultrapure water (1 mL) was
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12 gravimetrically added to dissolve the solid. The solution was filtered through a 0.22 µm
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14 filter and analyzed under the same UPLC-MS/MS conditions.
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18 Reasonable recoveries of spiked samples were found analyzing commercial milk
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20 samples (101 to 126% with RSD% < 8.7%), which are similar to those obtained by
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22 others authors: 80.6 to 107.7% with RSD% < 16.8%,¹⁶ and 89.3 to 98.6% with RSD% <
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24 12.4%.⁴ However, compared to the commercial milk samples, worst recovery values
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26 (85 to 148% with RSD% < 16%) were obtained when analyzing raw milk, possibly
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28 because of the high fat content. Milk composition changes regarding cow's lactation
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30 period and milking time. Fat content higher than 3%, which is the standard value for
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32 commercial whole milk in Brazil, is often found. The preparation of raw milk with this
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34 method leads to a visible fat into the tube after evaporation of extract. The proposed
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36 liquid-liquid extraction with low temperature partitioning (LLE-LTP) procedure showed
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38 no fat interference with clear and transparent extracts and excellent recoveries (Tables 2
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40 and 3). Furthermore, the addition of acetonitrile leads to milk protein denaturation and
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42 the liquid phase can be easily removed because the fat and water are separated in the
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44 freezing process. Moreover, this method of sample preparation is quick and clean-up
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46 procedures are not necessary. These results also show the feasibility of the extraction
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48 method (LLE-LTP) for the determination of CAP in milk.
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3.6 Analysis of real samples

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The proposed method was applied to CAP evaluation in commercial milk samples acquired in the local market, but CAP contamination was not found in any of them. This method was also employed to evaluate an incurred milk material obtained after treatment of a dairy cow with chloramphenicol succinate (20 mg/kg) intramuscularly. Milk samples were collected before and after the drug application by the hand-milking technique. CAP residues were found in milk at the first hours after treatment. Forty-four samples of raw milk obtained from the treated cow were analyzed daily (morning and afternoon milkings) during 22 days, until complete clearance and total excretion of CAP (residues $< 0.015 \mu\text{g kg}^{-1}$). These results also demonstrated the applicability of the developed method to determine CAP in cow milk below its MRPL.

4 Conclusions

A high-throughput and sensitive analytical method using liquid-liquid extraction with low temperature partitioning (LLE-LTP) and isotope dilution mass spectrometry for determining chloramphenicol (CAP) residues in milk was developed and validated. The LLE-LTP was applied with successful results shown by the excellent recovery at very low levels, including the MRPL ($0.3 \mu\text{g kg}^{-1}$). The developed LLE-LTP method for the extraction of chloramphenicol from milk allowed the extraction and the clean-up to be carried out in the same step, avoiding additional purification of the extracts. This method also showed good results for raw milk, because fat interference was avoided. The gravimetric preparation of samples and standards solutions certainly contributed to the good analytical figures of merit (selectivity, linearity, recovery, precision, limit of detection and limit of quantification) obtained in this study. Therefore, the presented method can be applied for the routine analysis of CAP in liquid milk and milk powder

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3 using volumetric preparations. The use of fast and reliable methods for CAP
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5 determination is very important to ensure CAP-free dairy products for domestic
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7 consumption and exportation, since CAP is banned for treatment of food-producing
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9 animals in the EU and several other countries, including Brazil.
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12 The Brazilian National Metrology Institute (Inmetro) is using this method in a
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14 certification study of a candidate certified reference material of CAP in milk powder
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16 and for routine analysis of fresh and powdered milk samples.
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19 As far as we are aware, this is the first study showing the application of LLE-
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21 PBT for CAP determination in whole and partially skimmed bovine milks.
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29 **Acknowledgements**

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50 support on pasteurization and spray dryer of raw milk.
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Figure Captions

Fig. 1 Multiple reaction monitoring chromatograms of chloramphenicol standard at 1.0 $\mu\text{g kg}^{-1}$: (a) transition for quantification of CAP (321.1 > 152); (b) transition for confirmation of CAP (321.1 > 257); (c) transition for quantification of *d5*-CAP (326 > 157); (d) transition for confirmation of *d5*-CAP (326 > 262).

Fig. 2 Ion suppression test results using Acquity Uplc@ BEH C18 (column 1) and Acquity Uplc@ HSS C18 SB (column 2): a) CAP standard chromatogram on column 1 (retention time of 1.64 min); b) TIC of blank milk extract on the ion suppression test, showing two interfering peaks (column 1); c) MRM (321.1>152) showing the signal suppression of CAP using a blank milk extract (column 1); d) CAP standard chromatogram on column 2 (retention time of 1.04 min); e) TIC of a blank milk extract on the ion suppression test, showing no interfering peaks (column 2); (f) MRM (321.1>152) signal after injection of a blank whole milk extract, with no suppression effect (column 2).

Fig. 3 Analytical curves of CAP in water, raw whole milk and partially skimmed milk.

Fig. 4 Residual Plot for the analytical curve of CAP obtained using aqueous standards.

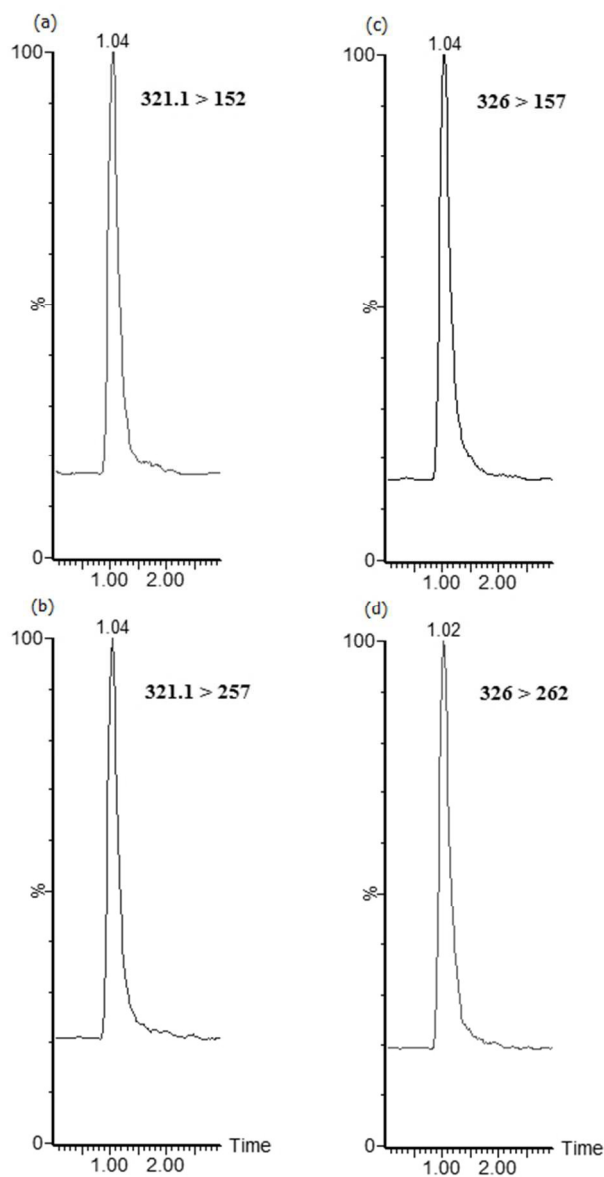


Fig. 1 Multiple reaction monitoring chromatograms of chloramphenicol standard at 1.0 µg kg⁻¹: (a) transition for quantification of CAP (321.1 > 152); (b) transition for confirmation of CAP (321.1 > 257); (c) transition for quantification of d5-CAP (326 > 157); (d) transition for confirmation of d5-CAP (326 > 262). 136x231mm (96 x 96 DPI)

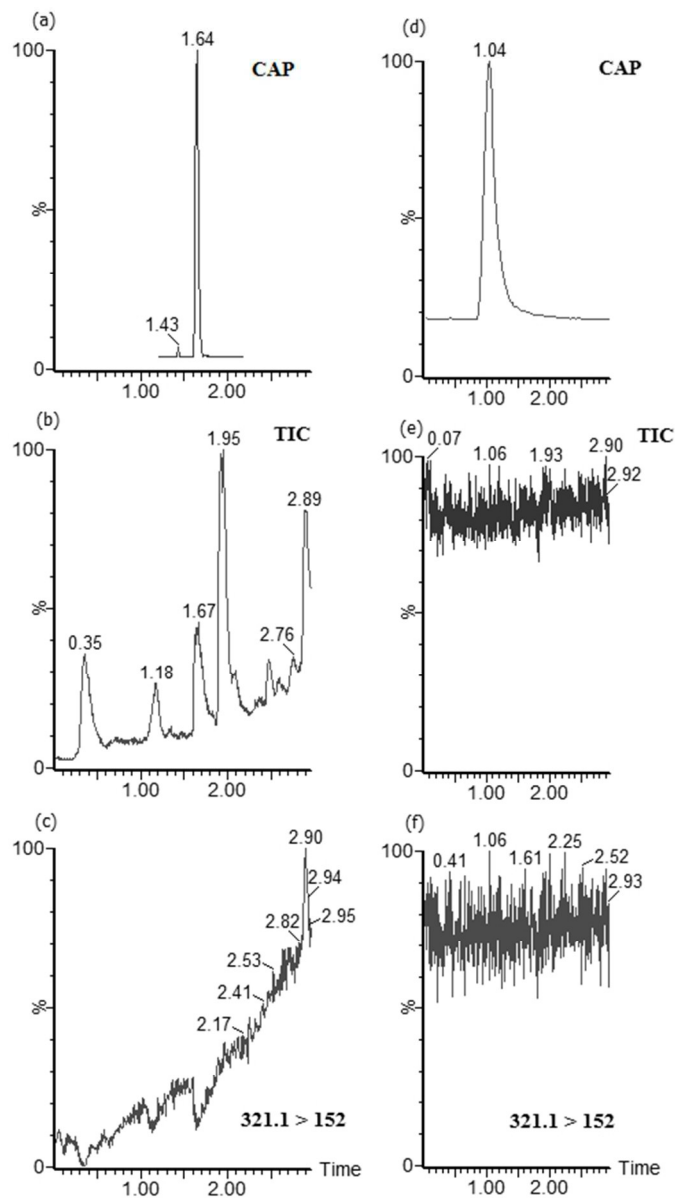


Fig. 2 Ion suppression test results using Acquity Uplc@ BEH C18 (column 1) and Acquity Uplc@ HSS C18 SB (column 2): a) CAP standard chromatogram on column 1 (retention time of 1.64 min); b) TIC of blank milk extract on the ion suppression test, showing two interfering peaks (column 1); c) MRM (321.1>152) showing the signal suppression of CAP using a blank milk extract (column 1); d) CAP standard chromatogram on column 2 (retention time of 1.04 min); e) TIC of a blank milk extract on the ion suppression test, showing no interfering peaks (column 2); f) MRM (321.1>152) signal after injection of a blank whole milk extract, with no suppression effect (column 2).
138x231mm (96 x 96 DPI)

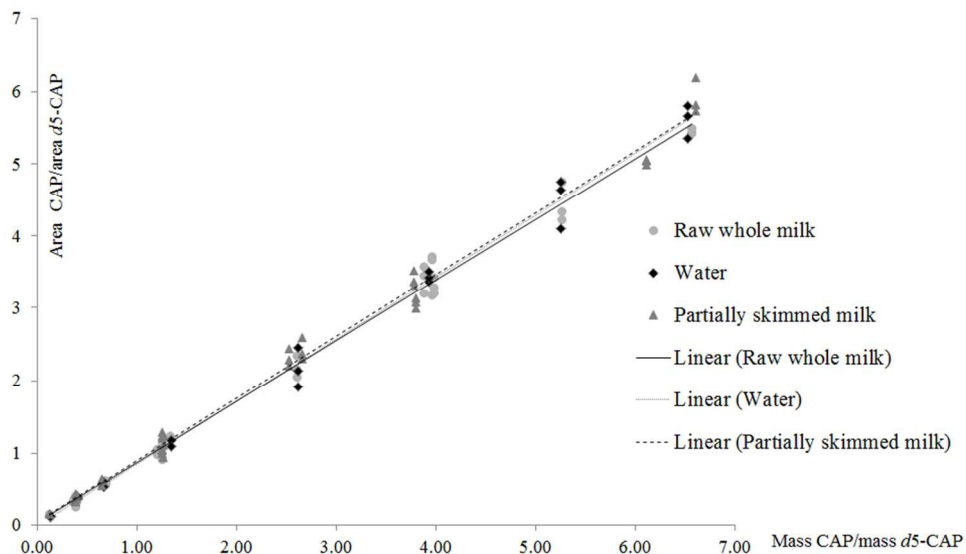


Fig. 3 Analytical curves of CAP in water, raw whole milk and partially shimmed milk.
263x151mm (96 x 96 DPI)

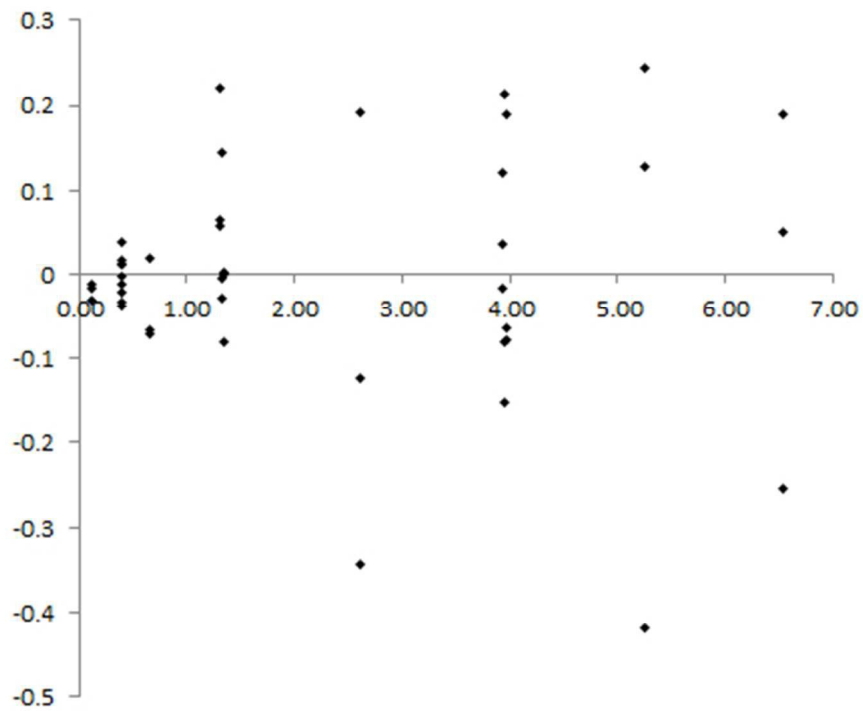


Fig. 4 Residual Plot for the analytical curve of CAP obtained using aqueous standards.
117x97mm (96 x 96 DPI)

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Table 1 Evaluation of matrix effects by comparison of analytical curves

Parameter	Water	Raw whole milk	Partially skimmed milk
Slope (Confidence Interval)	0.8582 (0.8285 a 0.8879)	0.8400 (0.8193 a 0.8607)	0.8529 (0.8289 a 0.8770)
Intercept (Confidence Interval)	-0.0027 (-0.1049 a 0.0995)	0.0273 (-0.0358 a 0.0904)	0.0545 (-0.0193 a 0.1283)
R ²	0.9939	0.9941	0.9925
t-test for slope with curve in water	-	t _{calc} (1.073) < t _{crit} (1.999) Without matrix effect	t _{calc} (0.2846) < t _{crit} (1.9996) Without matrix effect

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Table 2 Evaluation of recoveries and repeatability of spiked samples of spiked raw whole milk

Level ($\mu\text{g kg}^{-1}$)	Recoveries (Mean)	Overall Mean	RSD(%)
0.3	104%	101%	3.6%
	97%		
	101%		
1.0	106%	101%	4.5%
	99%		
	98%		
3.0	102%	101%	3.8%
	104%		
	97%		

Table 3 Recoveries and repeatability of spiked samples of partially skimmed milk

Level ($\mu\text{g kg}^{-1}$)	Recoveries (Mean)	Overall Mean	RSD(%)
0.3	111%	113%	1.5%
	112%		
	114%		
1.0	105%	104%	2.5%
	101%		
	106%		
3.0	107%	102%	6.7%
	105%		
	94%		

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