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DETERMINATION OF CHLORAMPHENICOL IN MILK POWDER USING LIQUID-LIQUID CARTRIDGE EXTRACTION (CHEM ELUT) AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

Background. The European Union prohibits the use of chloramphenicol (CAP) as a veterinary drug in food-producing animals. Nevertheless, CAP have been detected in milk products (liquid milk and milk powder). Therefore, it is necessary to develop sensitive methods for determining CAP residues in milk powder.

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

Material and methods. Chloramphenicol was determined in milk powder using LC-ESI-MS/MS in negative mode. After fat removing milk powder sample was extracted/cleaned-up with a Chem Elut extraction cartridge. Separation was achieved on a Phenomenex Luna C-18 column with acetonitrile-water as a mobile phase. The mass spectrometer was operated in multiple reaction monitoring mode (MRM). Four transitions were monitored m/z 321→152, 321→194, 321→257 (CAP) and 326→157 (IS CAP-d5).

Results. Linearity, accuracy, precision, decision limit (CCα), detection capability (CCβ) and ruggedness were determined for m/z 321→152. The mean relative recoveries (inter standard-corrected) of CAP from whole milk powder spiked at levels 0.1, 0.2, 0.3 and 0.6 μg/kg were in the range 95 - 103%. Relative standard deviation (RSD%) of recoveries at all spiked levels were less than 14%. RSDs within-laboratory reproducibility calculated at fortification of 0.3 μg/kg was less than 16%. CCα and CCβ were below 0.1 μg/kg.

Conclusions. The developed LC-MS/MS method allows the determination of CAP in milk powder. The method was validated according to the Commission Decision No. 2002/657/EC requirements. This method can be applied to determination CAP in whole and skim milk powder.

Key words: *chloramphenicol, veterinary drug residues, milk powder, Chem Elut; LC-ESI-MS/MS*

STRESZCZENIE

Wprowadzenie. Unia Europejska zabroniła stosowania chloramfenikolu (CAP) jako leku weterynaryjnego u zwierząt, których produkty są przeznaczone do spożycia. Pomimo tego, CAP jest wykrywany w produktach mleczarskich (mleko i mleko w proszku).

Cel. Celem badań było opracowanie i zwalidowanie metody pozwalającej na oznaczanie CAP w mleku w proszku.

Materiał i metoda. CAP był oznaczany w mleku w proszku metodą LC-ESI-MS/MS w trybie jonizacji ujemnej. Po usunięciu tłuszczu próbka była ekstrahowana/oczyszczana za pomocą ekstrakcyjnych kolumienek Chem Elut. Do rozdziału CAP stosowano kolumnę chromatograficzną Phenomenex Luna C-18. Fazę ruchomą stanowił acetonitryl/woda. Spektrometr masowy pracował w trybie monitorowania wybranych reakcji (MRM). Cztery przejścia były monitorowane m/z 321→152, 321→194, 321→257 (CAP) i 326→157 (IS CAP-d5).

Wyniki. Liniowość, odzysk, precyzja, limit decyzyjny (CCα), zdolność wykrywania (CCβ) i odporność metody zostały wyznaczone dla 321→152. Średni względny odzysk CAP z mleka pełnego był wyznaczony dla próbek wzbogaconych na poziomach odpowiadających 0.1, 0.2, 0.3 i 0.6 μg/kg i mieściły on się w zakresie 95 - 103%. Względne odchylenie stadardowe (RSD%) dla wszystkich poziomów było mniejsze niż 14%. Powtarzalność wewnątrzlaboratoryjna (RSDs) obliczona dla poziomu wzbogacenia 0.3 μg/kg była mniejsza niż 16%. CCα and CCβ były poniżej 0.1 μg/kg.

Wnioski. Opracowana metoda LC-ESI-Ms/MS pozwala oznaczyć CAP w mleku w proszku. Metoda została zwalidowana zgodnie z wymaganiami decyzji Komisji nr 2002/657/WE. Metoda może być stosowana do mleka pełnego i odtłuszczonego.

Słowa kluczowe: *chloramfenikol, pozostałości leków weterynaryjnych, mleko w proszku, Chem Elut, LC-ESI/MS/MS*

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INTRODUCTION

Milk is an important food in human nutrition because it contains essential constituents, such as proteins with high biological quality, carbohydrates useful for the nervous systems development, and essential fatty acids, vitamins and minerals. Milk powder can be contaminated with chloramphenicol (CAP). Human exposure to CAP could cause Grey syndrome, bone marrow depression and fatal aplastic anemia. Consequently, its use in food-producing animals and animal feed products is prohibited in European Union (EU). As a prohibited substance, zero tolerance applies. Currently, CAP has Annex IV classification under EU legislation [1]. No maximum residue limit (MRL) has been established for this antibiotic. Thus, the EU has assigned a minimum required performance limit (MRPL) for CAP in food of animal origin at a level of 0.3 μg/kg [2]. However, many companies introduced their own action limits forcing the development of sensitive assays able to detect 0.1 μg/kg.

A variety of method have been used to determine CAP in food of animal origin. Nowadays, as a screening test for animal-derived food, enzyme-linked immunosorbent assay (ELISA) method is used. ELISA method has a detection limit similar to that of liquid chromatography – tandem mass spectrometry (LC-MS/MS). However, commonly occurring problems with the ELISA method is one of false positive results [3, 17] Common confirmatory methods for the determination of CAP in animal food have been based on LC-MS/MS. Several LC-MS/MS methods have been developed for the determination of CAP residues in animal tissues [11], honey [12] liquid milk [8] and milk powder [9, 10, 13].

A detection of trace levels of CAP residues in rich matrix such as milk powder requires efficient sample preparation procedure (extraction and clean-up). Sample preparation procedures such as liquid-liquid extraction (LLE) [10] and solid-phase extraction (SPE) are common for extraction and clean-up. However, due to its ease of operation and environmental interest SPE has gained bigger popularity than LLE. Sorbent including C-18 [9, 16] Oasis HLB [3] and molecular imprinted polymer – MIP [7, 13] cartridges were successfully applied for the analysis of CAP in milk powder.

A potential drawback of SPE procedure is that it requires a large number of individual steps (column conditioning, sample application, washing and elution). In order to shorten the sample clean-up procedure Chem Elut extraction cartridge was applied. Chem Elut cartridge with diatomaceous earth instead of classical SPE methods was used to extract and purify the samples. The principle of SPE using diatomaceous earth is closely related to conventional liquid-liquid extraction. It involves the absorption of the aqueous phase on the diatomaceous earth, a porous material that acts as a support for the aqueous phase. This provides a large surface area for partition into an eluting solvent, which flows through the immobilized specimen under gravity, eluting the analytes of interest. However, large volumes of hazardous organic solvents are required. SPE with diatomaceous earth cartridges was previously applied for the determination of pesticide residues in fruit, vegetable samples and drugs in body fluids. Diatomaceous earth (Chem Elut, Extrelut) was also applied for the analyses of CAP by LC-MS-MS in urine [14, 15], plasma [15], shrimp tissue [4], liquid milk [8] and honey [6].

The aim of this work was to develop and validate LC-MS/MS method for determination and confirmation of the chloramphenicol residues in milk powder.

MATERIAL AND METHODS

Chemicals and reagents

Ultrapure water was obtained from Milli-Q system Millipore (Bedford, MA, USA). Acetonitrile LC-MS grade, ethyl acetate LC grade and hexane LC grade. Chem Elut extraction cartridges (5 ml, unbuffered) were provided from Varian (Part number 12198006, The Netherlands).

Chloramphenicol (CAP) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Internal standard deuterated chloramphenicol d5 (100 μg/mL in acetonitrile) was purchased from Cambridge Isotope Laboratories (FSD-117-100, 98%).

Standard solution

Individual stock solutions of CAP at 1 mg/ml was prepared in acetonitrile. This solution was diluted in acetonitrile to prepare an intermediate standard solution of 20 μg/ml. Working solution of 5.0 ng/ml was made by diluting intermediate standard with acetonitrile. Internal standard of CAP-d5 was prepared by dissolving the ampoule with 100 μg/ml in acetonitrile, which was adequately diluted to obtain a working solution of 3.0 μg/ml (IS). The stock standards solution (1.0 mg/ml) kept at –20°C were stable for 1 year. The intermediate standard solution (20 μg/ml) stored at –20°C was stable 3 month, while the working standard solution (5.0 ng/ml) stored at 4°C was stable for 3 months.

Samples preparation

The milk powder samples were collected by the Polish Veterinary Inspectorate at inspection points. Samples were stored at +4°C until analysis.

A 5.0 ± 0.05 g of whole milk powder (26-28% fat) was weighed into a 100 ml plastic centrifuge tube and working solution (IS) was added (3 ng/ml CAP-d, 500 μl). Subsequently, 50 ml of water was added and

the tube was capped tightly. The mixture was placed in water bath at 40 °C and mixed about 10 min until a homogeneous ample was obtain. After cooling down to room temperature the mixture was placed in the freezer at temperature below –20°C for at least 10 min. Precipitated fat was removed by centrifuging at 4000 x *g* for 10 min at –4°C. After centrifugation supernatant was filtered through folded filters. The 5 ml sample was applied to 10 ml Chem Elut cartridge and left for 5 min. CAP was eluted from the cartridge in two stages with 10 ml and 8 ml of ethyl acetate. The ethyl acetate was evaporated to dryness under a stream nitrogen using a heating block at 45°C. The dry residue was dissolved in 1 ml of acetonitrile and 2x1 ml of hexane were added. The sample was mixed and hexane phase was discarded. Next, sample was evaporated once again to dryness under stream of nitrogen using a heating block at 45°C and residue was dissolved in acetonitrile/water (20:80, v/v, 250 µl) and filtered trough 0.45 µm PVDF filter into amber vial. An aliquot of 10 µl was injected into LC-ESI-MS-MS.

Matrix matched calibration curve

The milk powder matrix matched calibration curve was prepared and used for quantification. The calibration curve was built by spiking blank matrix samples of milk powder with CAP (levels from 0 to 0.6 µg/kg, five points). A fixed amount of internal standard was added to all samples (3 ng/ml CAP-d5, 500 µl). The equation was $y=a + bx$, where x was the injected amounts of CAP in µg/kg and y was the peak area ratio (CAP/IS). The calibration curve was obtained relating ratio CAP area m/z 321→152/326→157 (IS) with CAP concentration in µg/kg. Peak area (analyte to internal standard) ratios were calculated using Analyst 1.5.1 Software.

LC-ESI-MS/MS analysis

All analyses were performed on an Agilent 1200 series liquid chromatography interfaced to an Applied Biosystems/MDS SCIEX Q TRAP 5500 mass spectrometer (Concord, Ontario, Canada). The chromatographic separation was performed in a C18 column (150 mm x 2 mm id., 3 µm) (Phenomenex, Torrance, USA). The LC flow rate was set at 300 µl/min, the injection volume at 10 µl and the column temperature at 40°C. The mobile phase was acetonitrile (A) and water (B). The linear gradient program was: 0.0–0.1 min 0% A; 0.1–3.0 min 80% A; 3–12 min 80% A; 12–12.3 min 0% A; and 12.3–20 min 0% A. The LC flow was directed into the MS detector between 4 and 8 min using a VICI diverter (Valco Instrument Co. Inc., Houston, TX). The mass spectrometer was used in negative mode. The optimized source and gas parameters were as follows: curtain gas (CUR), 15 psi; collision gas (CAD), 7 psi; ion source temperature (TEM), 400°C; ion source gas 1 (GS1), 45 psi; ion source gas 2 (GS2), 60 psi; ion spray voltage, –3500 V and dwell time 150 ms. Nitrogen was used as collision gas. The instrument was operated in Multiple Reaction Monitoring (MRM) mode, using the following transitions: m/z 321→152, 321→194, 321→257 for CAP and m/z 326→157 for CAP-d5; with collision energies (CE) of 18, 14, 14 eV and 18 eV, respectively.

RESULTS AND DISCUSSION

Extraction and clean-up

Sample extraction/clean-up can be done either by applying a vacuum to pull the sample or solvent through the SPE cartridge, or by allowing gravity to pull the sample or solvent through. In our preliminary studies, extraction/clean-up procedure was investigated by using gravity cartridge Chem Elut (Varian) and Extrelut NT 3 (Part number 1.15095.0001, Merck). In this experiment, the whole milk powder samples were spiked CAP at 0.3 µg/kg. The sample was passed through the cartridge. CAP was eluted using of the ethyl acetate (section 'Sample preparation'). The extraction/clean-up was assessed through recoveries. The recoveries were calculated by comparing the area of analytes in speaking matrix to that of standard solution at the same contraction. Chem Elut cartridge and Extrelut cartridge allowed to achieving the recoveries of >90% and > 60%., respectively. The Chem Elut was selected in this work.

Matrix effects

Matrix effects of ion suppressing was checked, which is a common problem of ESI technique. The matrix effects may result as positive or negative responses depending on the level of ion suppression and can greatly affect the method accuracy and reproducibility. The ion suppression effects were evaluated by typical experiment system. The blank whole milk powder sample was prepared as described in section Sample preparation. The dry extract was dissolved in mobile phase. The mobile phase was mixed with CAP solution in mobile phase to the final concentration of 0.1, 0.3 and 0.6 µg/kg. Then, spiked samples were compared to standard in mobile phase (0.1, 0.3 and 0.6 µg/kg). The signals intensities (peak areas) of m/z 321→152 and 326→157 CAP-d5 (IS) transitions were observed. The signals intensities for the CAP were lower than the standard solution. The matrix effects were in the range –29% to –37% for 321→152 and –22% to –28% for 326→157 ("–" represents a loss of analyte signal - ion suppression). These results revealed that determination of CAP was affected by the interferences from real samples to some extent. Therefore, to provide reliable

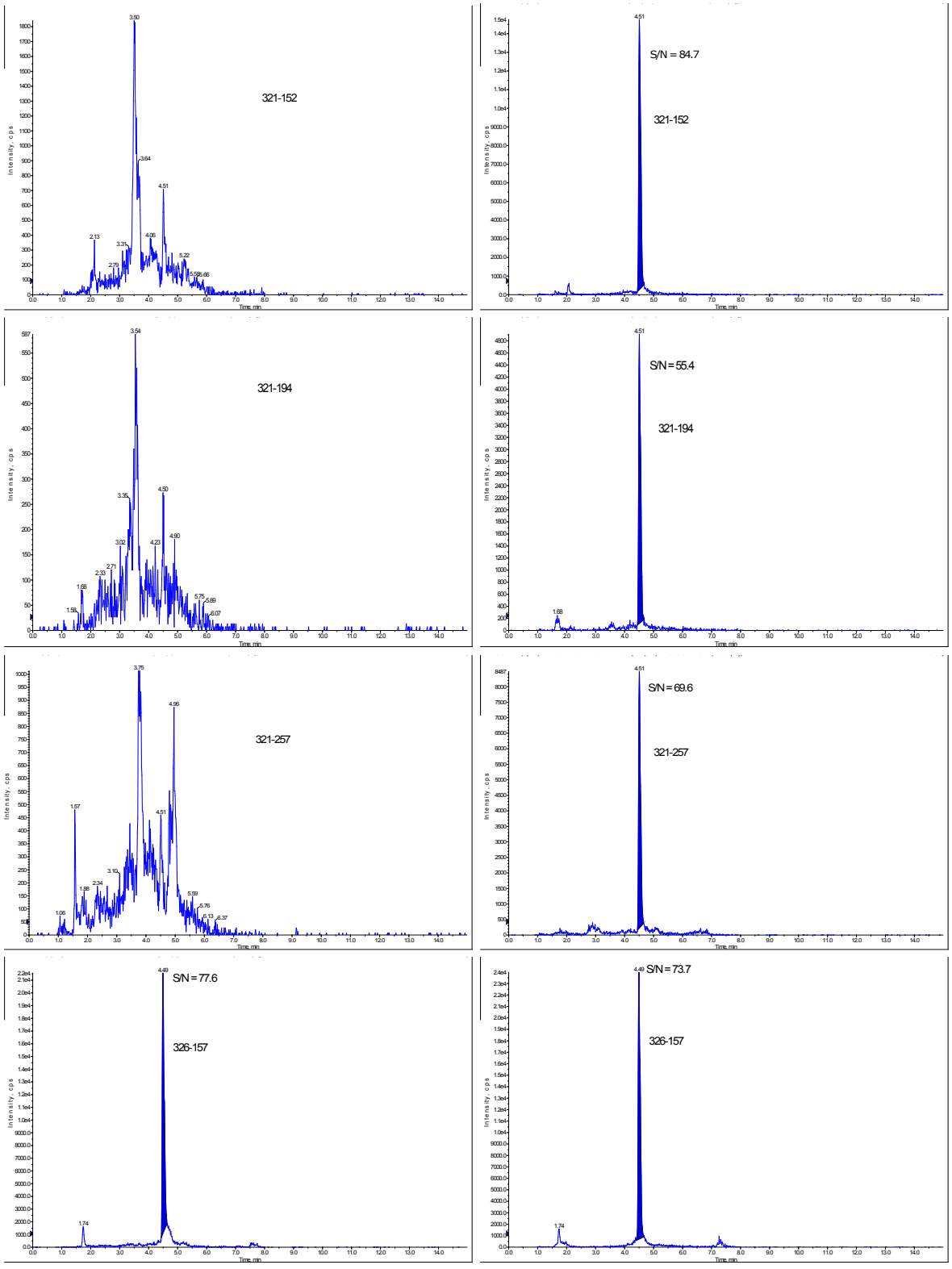

Figure 1 LC-ESI MS/MS chromatograms of blank and spiked whole milk samples at 0.1 µg/kg.

results, matrix-matched calibration curves were chosen the roughout this study.

Identification

The performance of analytical method is evaluated by checking the identification criteria: the signal-to-noise ratio (S/N) of diagnostic ions have to be grater then three, the relative ratio of retention time of the analyte to that of the IS corresponded to that of the calibration solution within a ±2.5% tolerance for liquid chromatography and the relative peak area ratios of the various transition reactions within the tolerances set by the EU criteria [1]. All these were fulfilled with the samples

spiked at 0.1, 0.2, 0.3 and 0.6 μg/kg. At the lowest spiked level (i.e. 0.1 μg/kg), the S/N was greater than 3.

All the chromatograms obtained throughout the validation study showed very good stability of the relative retention time for the spiked samples and for the positive samples with relative deviations always better than ±2.5%. The peak area ratios of the various transition reactions were calculated from the spiked samples. The ratios vary from 30 to 35% for 194/152 and 55 to 69% for 257/152. The mean ratios for 194/152 and 257/152 was 32 (6.1) ±25% (peak are ratio (RSD) ± tolerance given in Decision 2002/657/EC) and 56 (7.9) ±20%, respectively. A similar results were observed for pure standard solution prepared in water. The mean ratios for 194/152 was 31% and for 257/152 was 59%.

Validation

For validation specificity, linearity, accuracy, precision (repeatability, and reproducibility), decision limit (CCα), detection capability (CCβ) and ruggedness were determined according to Commission Decision 2002/657/EC [1].

The specificity of the developed method was evaluated by analyzing 20 different blank and spiked whole milk powder samples at 0.1 μg/kg in order to investigate possible interference retention time. Figure 1 shows a blank sample and a spiked milk powder at 0.1 μg/kg. All blank milk powder extracts exhibited no significant interferences at retention times of target and internal standard. Good chromatographic signals were obtained for CAP and CAP-d5 (IS) in powder milk treated with described method. The typical LC retention time of CAP and CAP-d5 (IS) was around 4.8 min.

The linearity was evaluated by analyzing the calibration curves of spiked whole milk powder in the 0.0–0.4 μg/kg (five calibration points, six curves). These samples were randomly chosen from previously analyzed CAP free samples. The matrix calibration curves were linear in the range 0.0 – 0.6 μg/kg. The correlation coefficient and slope were within the range 0.994 – 0.999 and 3.51 – 3.65 respectively.

The accuracy and precision-repeatability of CAP were measured in blank spiked samples of whole milk powder that were spiked at 0.1, 0.2, 0.3 and 0.6 μg/kg. The samples were analysed on different days close to each other, with the same instrument and same operators. Six replications were obtained for each concentration. Accuracy was assessed through relative recovery. The relative recovery (%) were calculated against matrix matched standard curves using internal standard added before sample preparation. The relative standard deviation (RSD, %) was calculated for each level. The relative recovery and precision (repeatability) date obtained from the analyses of blank spiked samples at four contraction levels are reported in Table 1. The results show good relative recoveries ranged between 95 and 103% with a good RSD, less than 14%. The high relative recoveries and the use of internal standard ensure that the method is suitable for determination of CAP in milk powder at the level required.

Absolute recovery (%) of analytes was determined by comparison of peak areas from blank whole milk powder samples spiked with known amounts of CAP (0.1, 0.2, 0.3 and 0.6 μg/kg) before the preparation procedure to peak area from matrix extract spiked after it The results were summarized in Table 1. The absolute recoveries were in the range of 69 to 77%. The mean absolute recover was 72%.

Table 1. Relative recovery, precision and absolute recovery for whole milk powder (n=6)

Spike levels (μg/kg)	0.1	0.2	0.3	0.6
Relative recovery (%)	103	97	102	95
RSD (%)	12.8	9.0	8.7	13.9
Absolute recovery (%)	71	72	77	69

For an evaluation of reproducibility only within-laboratory reproducibility was considered. The precision within-laboratory reproducibility was calculated in spiked samples at concentration of MRPL (0.3 μg/kg). They were analysed on three different days (3x6), with the same instrument but different operators. The overall RSDs was calculated as within-laboratory reproducibility. The average recovery was 67% with RSDs 15.8%.

To comply with Commission Decision 2002/657/EC [1] the CCα and CCβ were determined. Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α=0.1% that the sample is non-compliant in case there is a non-zero test limit. Detection capability (CCβ) means the smallest content of the substance that may be detected, identified or quantified in a sample with the error probability of β=95% .

Decision [1] defines two methodologies for determination of CCα and CCβ during method validation. The first method is based on determination of signal to noise (S/N) ratios in blank samples and matrix material spiked at the CCα. The second method refers to the international standard ISO 11843-2 [5]. In this work analytical limits CCα and CCβ were calculated by applying the matrix calibration curve procedure according to case 1 of ISO 11843-2 - constant standard deviation. These method parameters are to be used instead of the familiar limit detection (LOD) and limit of quantification (LOQ). The CCα and CCβ were calculated using two calibration curves (at five levels 0.0, 0.1, 0.2, 0.3 and 0.6 μg/kg) from six different experiments on different whole milk powder matrix and different days. Curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte. CCα and

CCβ were calculated for m/z 321→152/ 326→157 (IS) ion transitions. The mean values CCα and CCβ were 0.026 μg/kg and 0.033 μg/kg. These values were below MRPL of 0.3 μg/kg [2].

The ruggedness was tested by introduction of seven small but deliberate changes in operation parameters (variables) and by the consequent assessment of their influence of the method results. We developed 8 testes in accordance with *Youden* approach [1], using a matrix whole milk powder spiked at 0.3 μg/kg. Seven independent sample preparation parameters examined are reported in Table 2. *Student t*-test at 95% confidence limit was used to compare the high level with varied low level. No significant differences on the performance were observed, indicating good ruggedness of the method.

Table 2. Sample preparation parameters setting applied in the robustness experimental design

Sample preparation parameters	High level	Low level
Water bath temperature (°C)	45	35
Mixing time (min)	15	5
Freezing out time (min)	15	5
Elution mode	after 10 min	immediate
Defating mode	1 x 2 ml	2 x 1 ml
Extract evaporation temperaturę (°C)	50	40
% ACN in final mixture	22	18

Application to skim milk powder

The present procedure was also applied to analyse CAP in skim milk powder, containing about 1-2 % fat. LC-MS/MS was therefore applied, without any modification to whole milk powder treated milk spiked with the CAP at the same contraction levels as the whole milk samples. In all instances, basically the same results were obtained as for whole milk powder for parameters such as linearity, repeatability and recovery. No interfering peaks showed up in any of the LC-MS/MS trace. CCα and CCβ were similar to those obtained for whole milk powder. CCα and CCβ were 0.024 and 0.031 μg/kg, respectively. Table 3 shows the relative recovery, repeatability and absolute recovery obtained from skim milk samples.

Table 3. Relative recovery, precision and absolute recovery for skim milk powder (n=6)

Spike level (μg/kg)	0.1	0.2	0.3	0.6
Relative recovery (%)	94	105	107	99
RSD (%)	10.7	7.4	6.8	11.2
Absolute recovery (%)	80	78	76	74

CONCLUSIONS

The developed LC-ESI-MS/MS method using Chem Elut extraction cartridge, validated according to European Commission criteria, could be applied to control chloramphenicol in whole milk powder. RSD was lower than 14% and 16% for repeatability and reproducibility. The *Youden* ruggedness test applied to the milk powder preparation procedure showed that the selected potential critical parameters do not significantly affect the assay results. This method can be also applied to skim milk powder. The developed method may be used for the detection of CAP at 0.1 μg/kg.

Conflict of interest

The authors declare no conflict of interest.

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