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Analytical Method Validation for Quantification of Chloramphenicol Residues in Poultry Meal Using a Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: Quantification of Chloramphenicol (CAP) residues in complex matrices such as poultry meal is a tedious analytical procedure. In this study, a rapid and precise liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous detection and quantification of CAP residues in poultry meals. The chromatographic separation of the CAP was performed at 40°C column temperature on a reverse-phase C18 column using a binary gradient pump mode and quantification of CAP was performed by LC-MS/MS in electrospray mode. Mobile phase constituents were solvent (a) deionized water, and (b) acetonitrile. The flow rate was 0.35 mL/min and the entire run time was 5 min. The method was validated according to 2021/808/EC guidelines, and acceptance criteria for specificity, linearity, recovery, and precision were met in all the cases. The relative standard deviation (RSD) for precision was < 11% for all the cases. The linearity of the calibration curves was excellent ($R^2 > 0.999$) at concentrations of 0.25, 0.50, 0.75, 1.0, 2.0, and 5.0 µg/kg for matrix-matched CAP standard, and the range of linearity of this method was 0.0-5.0 µg/kg with R^2 value greater than 0.99. The decision limit (CCα) and detection capability (CCβ) were 0.29 µg/kg and 32 µg/kg respectively, and the recovery percentages ranged between 94% and 100 %. The obtained results of the proposed method met the validation criteria and this method could be a precise and highly desirable analytical procedure for rapid and accurate quantification of chloramphenicol residues in poultry meal.

Keywords: Chloramphenicol Residues, Poultry Meal, LC-MS/MS, Method Development & Validation

1. Introduction

The use of antibiotics in veterinary medicine is huge especially to promote growth and increase feed efficiencies in food-producing animals [1]. The indiscriminate use of antibiotics may lead to the development of resistance, allergic reactions, and carcinogenic or teratogenic effects [2] through pathways that include the accumulation of antibiotic residues in the human food chain. Among the various antimicrobials, chloramphenicol (CAP) is a broad-spectrum antibiotic frequently used in livestock feed and poultry meals due to its excellent antibacterial and pharmacokinetic properties and low price [3]. However, Chloramphenicol is implicated in the generation of aplastic anemia in humans and causes reproductive and hepatotoxic effects in animals [4]. So, the use of the CAP is illegal for the administration of food-producing animals in many countries worldwide including Bangladesh [5]. In order to ensure consumer health, some countries completely banned the use of chloramphenicol for the treatment of animals used for food production with a zero tolerance for chloramphenicol residues.

Although it is prohibited, CAP is still used in livestock production through feed, food enzyme products, and poultry meal. The poultry meal is an important source of animal protein used to feed domestic animals, and birds, prepared from the ground, rendered, clean parts of the carcass of slaughtered poultry such as heads, necks, undeveloped eggs, gizzards, intestines, and feet. In addition to its unlawful use,

products of animal origin can contain CAP residues due to their prevalence in the environment. According to some studies, CAP can still be found in several food matrices, indicating its continued use [6-9]. Due to the risk of drug residue occurrence in foods and foods of animal origin, many sensitive and specific methods including immunoassay, biosensor, and chromatographic techniques were optimized and validated for the qualitative and quantitative determination of different antibiotics and their residues in food products [10-14] with the different analytical conditions. Confirmatory methods, typically based on gas chromatography-mass spectrometry (GC–MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques, have been developed for the determination of chloramphenicol residues in a wide range of sample types.

Previous researches have set forth diverse forms of pretreatment techniques for CAP residues in foods before chromatographic determination, which include liquid-liquid extraction, solid-phase extraction, or the QuEChERS technique [14, 15]. Traditional methods of extracting organic analytes from food samples usually consist of a long liquid-liquid extraction procedure with a homogenization step followed by one or more purification steps and purification of the extract to remove co-extractants, before the sample is subjected to chromatographic separation [16].

However, there are numerous techniques for analyzing CAP residues in foods of animal origin, however method for analyzing CAP residues in poultry meal are nonetheless rare. Therefore, the present study aimed to develop and validate a rapid and precise analytical method for the simultaneous identification and quantification of chloramphenicol and its residues in poultry meal using a Liquid Chromatography-tandem Mass Spectrometry.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile (MS grade), and ethyl acetate employed in this study were obtained from Honeywell, Germany, and authorized reference standards of Chloramphenicol and Chloramphenicol D5 (Internal standard) were purchased from LGC Labor GmbH (Augsburg, Germany). Double deionized (DI) water used in this study was acquired from a water deionization plant (ePure-D4642-33, ThermoFisher Scientific, USA). All solutions were sonicated and filtered through a 0.22 µm filter using a vacuum filtration unit (Welch, Pall Scientific, USA) prior to use.

2.2. Instrumentation and Chromatographic & MS/MS Conditions

The liquid chromatographic-mass spectrometry system is equipped LC (UPLC- I Class) pump with binary gradient mode, and an MS detector (Xevo TQS-Micro, and Nitrogen NM32LA, Waters Corporation USA; Peak Scientific) with MassLynx software. The separation of CAP was accomplished in chromatographic system using a C18 reversed-phase LC column (Acquity UPLC BEH C18 1.7 µm, 2.1x 100 mm) operating at column oven temperature of 40°C. Deionized water and acetonitrile were used as a mobile phase conducted in an isocratic elution condition. The mobile phase flow rate was 0.35 ml/min and the injection volume was 10 µl for standard and samples. The mass spectrometry analysis mode was a negative scan mode for determination of CAP with the following conditions: temperature of the source and desolvation was 150°C, and 600°C respectively, and gas flow of Cone and desolvation was 50L/hr, and 1000 L/hr respectively. ES Negative multiple reaction monitoring (MRM) of 321.2 >152.2 for quantification of CAP, and MRM of 326.2>157 for quantification of Internal Standard (CAP-D5) were used at retention time 5.0 min.

2.3. Preparation of Standard Solution

Stock standard solution of 1000 µg/mL was prepared by weighing 10 mg of the Chloramphenicol and Internal standard (CAP-D5) in a 10 mL amber color volumetric flask separately and diluted to volume with MS grade acetonitrile. These prepared solutions were used as reference stock standard solutions and kept in a refrigerator at -20°C for further use. Intermediate standard solutions of 100 µg/mL of CAP and CAP-D5 were prepared from stock standard solution in acetonitrile. Working standard solutions were prepared daily from intermediate standard solutions. The standard solutions were filtered through a 0.20 µm polyvinylidene fluoride (PVDF) syringe filter prior to injection into the liquid chromatography system.

2.4. Preparation of Sample Solution

Weighed portions (poultry meal: 2 ± 0.01 g) of blended sample in 50 ml screw-capped plastic falcon tube. Spiked standard and working internal standard solution to all tubes. Vortex for 5 min and wait for 15 minutes. Added 10 ml ethyl acetate and vortex for 10 minutes. The solution was centrifuged at 6500 rpm for 10 minutes at 10°C temperature. Collected the upper layer (ethyl acetate-5 ml) and transferred it to the 15 ml screw-capped tube, and repeated the same procedure a second time. Then, evaporated the solvent (ethyl acetate) under N_2 gas at 40°C temperature. Reconstituted the remaining portion attached to the bottom with 2 ml of 50% ACN. The solution was vortex for 3 minutes and centrifuged for 5 minutes at 10°C temperature. Finally, collected the supernatant and filtered with a 0.22 µm PVDF filter and transferred to the sample vial for analysis with LC-MS/MS.

2.5. Method Validation Parameters

Method validation of the current study was performed by assessing the essential parameters of the validation process like specificity, linearity and calibration curve, recovery, precision, and decision limit. The validation parameters were evaluated in accordance with 2021/808/EC guidelines [17].

2.5.1. Linearity and Calibration Curve

To determine the linear range and calibration curve six

spiked poultry meal samples (starting from 0.25 to 5.0 µg/kg) have been prepared. Then run the spiked matrix-matched standard solution. The matrix-matched standard calibration curve was prepared with all data and was linear in the concentration range of 0.0-5.0 µg/kg.

2.5.2. Selectivity

Demonstration of the absence of interference from the ingredients in the Poultry meal sample by LC-MS/MS. The selectivity of the test method in this study was evaluated by measuring the peak area of reagent blank solution, matrix blank solution, standard solution, and spiked sample solution.

2.5.3. Recovery

Three sets of spike samples at 1.0, 2.0, and 3.0 times the LCL (lowest calibrated level) level have been prepared and analyzed, each level of each set contained six replicate samples. The LCL of the linearity curve was 0.25 µg/kg. Therefore, three sets of spike samples were 0.25 µg/kg, 0.50 µg/kg, and 0.75 µg/kg. Raw data were calculated using the following equation-

Recovery (%) = (measured content / fortification level) X100.

2.5.4. Repeatability Precision

For repeatability, precision checks three sets of samples have been prepared to spike at 1.0, 2.0, and 3.0 times the LCL level, and analyzed as before. The mean concentration, standard deviation, and the coefficient of variation (%) of each level of fortified samples have been calculated. Finally, the overall mean concentrations and CVs for the fortified samples have been calculated.

2.5.5. Within-Laboratory Reproducibility

For within-laboratory reproducibility precision check, three sets of samples have been prepared to spike at 1.0, 2.0, and 3.0 times the LCL level, and analyzed by the second analyst as before. The mean concentration, standard deviation, and the coefficient of variation (%) of each level of fortified samples have been calculated.

2.5.6. Decision Limit (CCα)

To determine the decision limit (CCα), 20 blank poultry meal samples have been fortified with chloramphenicol at the LCL level (0.25 µg/kg) and analyzed. The decision limit (CCα) was calculated using the following equation

CCα = CLCL + 2.33 x SD20 representative samples spiked at LCL level

2.5.7. Detection Capability (CCβ)

To determine the detection capability (CCβ), 20 blank poultry meal samples have been fortified with the chloramphenicol at the LCL level (0.25 µg/kg) and analyzed. The detection capability (CCβ), was calculated using the following equation

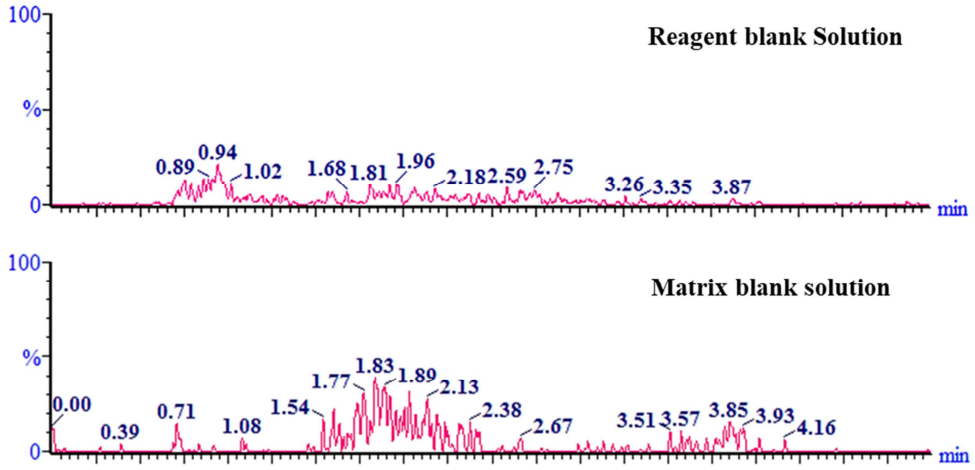
CCβ = CCα + 1.64 x SD20 representative samples spiked at LCL level

2.6. Statistical Analysis

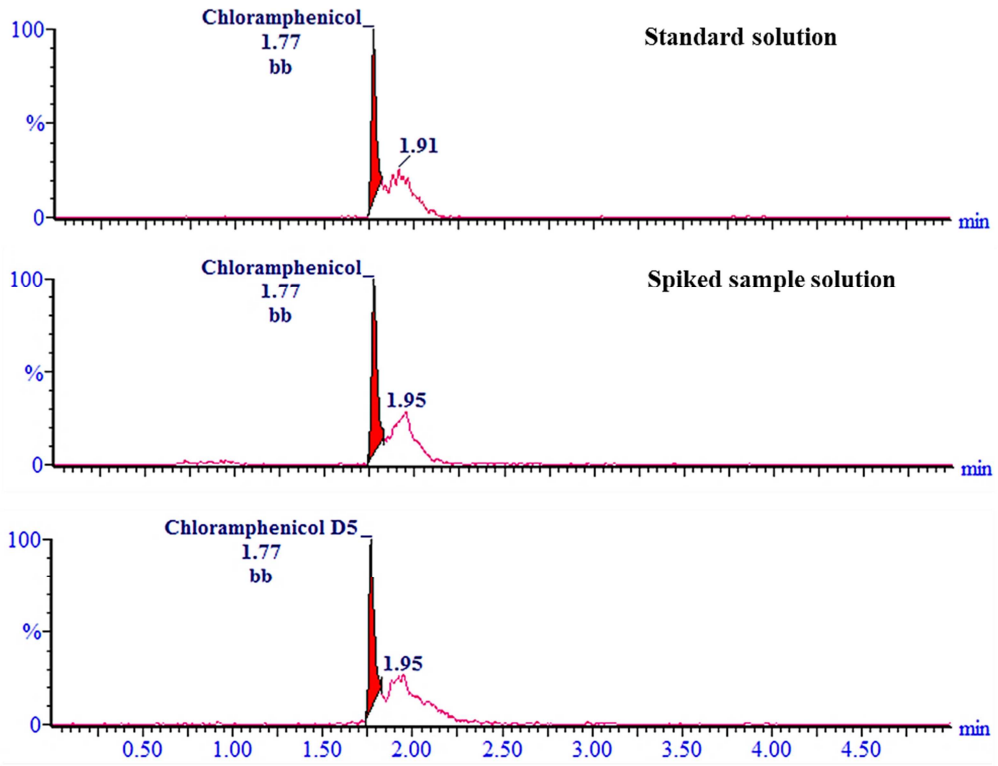
The data analysis of this study was performed with the Masslynx software and Statistical Package for the Social Sciences version 16 (SPSS-16) statistical package by one-way analysis of variance, and we used least-squares method for regression analysis.

3. Results and Discussion

This study demonstrates the development and validation of a particular analytical method in which validation criteria [17] are met in all cases. Typical chromatograms of the standard solution and sample solution spiked with standard and internal standards are shown in Figure 1.



a.



b.

Figure 1. *Typical chromatograms of the matrix-matched solution spiked with standard.*

The retention time of both CAP and internal standard (CAP-D5) was 1.77±0.01 min. The selectivity test results (Figure 1 and Table 1) of the assay method demonstrate the absence of interference with the elution of CAP, and CAP-D5 in the matrix blank sample.

Table 1. *Selectivity test results.*

Sample Name	Retention Time (min)	Response/Peak area
Reagent blank Solution	Nil	Nil
Matrix blank solution	Nil	Nil
Standard solution (0.25 µg/kg)	1.77	445
Spiked sample solution (0.25 µg/kg)	1.77	315

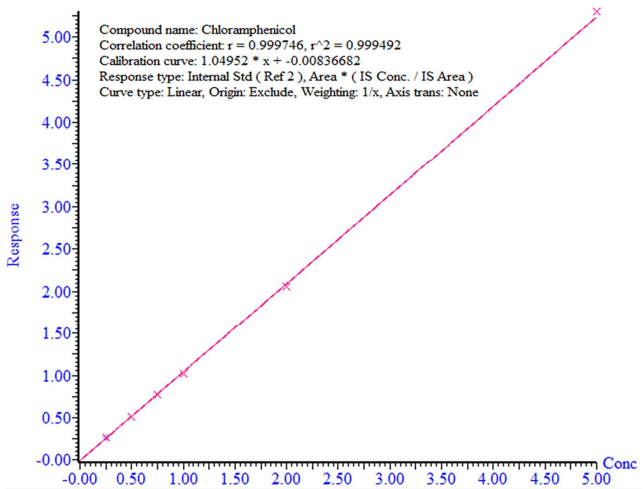


Figure 2. *Matrix- matched calibration curve of chloramphenicol.*

From Figure 2, demonstrates the excellent linearity (R^2>0.999) within the concentration range of 0.25-5.0 µg/L. The range of linearity of this method was 0.0-5.0 µg/L with an R^2 value greater than 0.999.

The trueness of the method was determined by recovery percentage and the values are between 94 % and 100% (Table 2), which imparts that this method is accurate and also indicates that the commonly used excipients present in the poultry meal formulations do not interfere with the proposed method.

Table 2. *Recovery of the method for the chloramphenicol in poultry meal.*

Replicate	Recovery (%)		
	0.25 µg/kg	0.50 µg/kg	0.75 µg/kg
1	91.9	91.2	109.5
2	101.5	96.7	86.1
3	93.2	97.9	93.9
4	96	92	108.9
5	91.8	99.2	106.7
6	113.6	92.6	95
Average	98	94.93	100.02

The precision for the method and analyst was evaluated which is shown in Table 3 and Table 4. The results demonstrate that the RSD value for both cases is <12%, which suggests that the proposed method has an excellent reproducibility. The decision limit (CCα), and detection capability (CCβ) for CAP are 0.29 µg/kg, and 0.32 µg/Kg respectively.

Table 3. *Precision under repeatability conditions (n=6).*

Replicate	Conc (µg/Kg)	Conc (µg/Kg)	Conc (µg/Kg)
Inj-01	0.23	0.45	0.67
Inj-02	0.25	0.46	0.82
Inj-03	0.23	0.48	0.65
Inj-04	0.24	0.49	0.70
Inj-05	0.23	0.46	0.82
Inj-06	0.28	0.50	0.80
Average	0.25	0.47	0.74
SD	0.02	0.02	0.08
% RSD	8.66	4.20	10.58

Table 4. *Within-laboratory reproducibility (n=6).*

Replicate	First analyst			Second analyst		
	% RSD (0.25 µg/kg)	% RSD (0.50 µg/kg)	% RSD (0.75 µg/kg)	% RSD (0.25 µg/kg)	% RSD (0.50 µg/kg)	% RSD (0.75 µg/kg)
Inj-01	0.23	0.45	0.67	0.26	0.52	0.74
Inj-02	0.25	0.46	0.82	0.35	0.55	0.73
Inj-03	0.23	0.48	0.65	0.30	0.55	0.69
Inj-04	0.24	0.49	0.70	0.25	0.55	0.72
Inj-05	0.23	0.46	0.82	0.27	0.59	0.74
Inj-06	0.28	0.50	0.80	0.30	0.51	0.80
Average	0.25	0.47	0.74	0.29	0.54	0.73
SD	0.02	0.02	0.08	0.03	0.03	0.04
% RSD	8.66	4.20	10.58	11.57	5.21	4.89

Previously some LC-MS/MS methods have been published concerning the simultaneous determination of CAP in various food and feed samples [18-22] with the different chromatographic conditions, longer run time, and poor recoveries [23-26]. Although, these methods were reported for quantification of CAP in different sample matrices they are incompetent to analyze poultry meal. The method we developed and validated is a more precise quantification of CAP residues in poultry meal with good selectivity, linearity, precision, and high recovery that met all the criteria of the validation parameters (Table 5). Moreover, the application of the method to test samples showed no false negative or false-positive results, which was further confirmed in proficiency tests (fapas proficiency testing, Fera Science Ltd., UK) with z-scores between -2.0 and +2.0.

Table 5. *Summary of acceptance criteria and obtained results.*

Parameters	Acceptance criteria	Obtained results
Selectivity	The excipient compounds must not interfere with the analysis of the targeted analyte.	Chromatography shows- (i) the existence of peak area in standard solutions and real sample; (ii) absence of peak area in blank and matrix solutions.
Linearity	$R^2 > 0.99$	$R^2 > 0.999$
Trueness by Recovery	Recovery should be between 50 to120 %	94 to 100 %.
Precision (Repeatability/ Within-laboratory reproducibility precision)	Repeatability: RSD ≤ 20 % Within-laboratory reproducibility: RSD ≤ 30 %	Repeatability: 4.20 to 10.58 % Reproducibility precision: 4.89 to 11.57 %
Decision Limit (CCα)	CCα = CLCL +2.33xSD20	0.29 µg/Kg
Detection capability (CCβ)	CCβ = CCα + 1.64 x SD20 representative samples spiked at LCL level	0.32 µg/Kg

A simple sample extraction procedure and a short run time of less than 5 minutes make the procedure more convenient. Therefore, the proposed method could be a simple, accurate, and rapid analytical technique for simultaneous detection and quantification of CAP residues in routine and quality analysis of poultry meals with wide application in modern poultry production.

4. Conclusion

This study developed and validated a simple and fast confirmatory method based on HPLC–MS/MS for the simultaneous identification and quantification of the residue of chloramphenicol in poultry meal with excellent linearity, accuracy, and precision. It meets the criteria set out in 2021/808 / EC guidelines. The sample preparation procedure in our proposed method is simple and quantification of chloramphenicol in the real samples is also comparable with excellent recovery. The proposed method could be used for the effective routine analysis of chloramphenicol residues in poultry meal samples.

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