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TRACE LEVEL ENRICHMENT AND CLEAN-UP OF β-LACTAM ANTIBIOTIC RESIDUES IN EDIBLE CHICKEN TISSUES AND FEATHERS BY SOLID PHASE EXTRACTION FOR QUANTITATIVE DETERMINATION UTILIZING LIQUID CHROMATOGRAPHY

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ABSTRACT. A simple analytical method for the extraction and determination of three β-lactam antibiotics in chicken tissues and feather samples was developed and validated using solid phase extraction (SPE) and liquid chromatography-ultraviolet, LC-UV, detection. The analytes were extracted with strong anion exchange (SAX) cartridges for the extract clean-up and enrichment before introduction into the LC system. The validation was carried out based on the recommendations from Commission Decision 2002/657/EC. Under optimised conditions, the proposed method offers correlation coefficients of $r^2 \geq 0.9991$ for all the studied analytes in both matrices. Percent recovery results were found to vary between 79.7–101.8% and 94.2–101.6% for chicken feathers and tissues, respectively, with the relative standard deviation, RSD < 8.4%. The limits of detection (LOD) were in the range of 0.1–0.13 μg kg^{-1} and 0.1–0.15 μg kg^{-1} while the limits of quantification (LOQ) were in the range of 0.3–0.38 μg kg^{-1} and 0.3–4.5 μg kg^{-1} in chicken feathers and tissues, respectively. The proposed method offers good sensitivity and selectivity and all the results demonstrating that the method would be widely used for determination of β-lactam antibiotics and other trace level antibiotics residues having similar physical and chemical properties in the matrices of varying origins.

KEY WORDS: Chicken tissues, Chicken feathers, β-Lactams, Solid phase extraction, LC-UV

INTRODUCTION

Raising healthy animals for food safety and affordability are important concerns and it requires using antimicrobials to treat their diseases [1]. Treating the animals not only controls pathogens affecting their own health, additionally it helps to control diseases transmitted to humans, and increases the effectiveness of animal production [2]. Excessive antimicrobial use in food animals, on the other hand, could have unexpected consequences, such as the development of resistant bacteria or a higher probability of detecting antibiotic residues in edible tissues or further in animal by-products [3, 4].

Chicken feathers are the by-products in poultry production and weigh up to 9% of mass of each live bird [5]. According to Nachman and coworkers [6] following poultry slaughter, feathers are converted by rendering into feather meal as a protein source ingredient in animal diets, processed and used as organic fertilizers in agriculture, as a raw material in biodiesel and as an ingredient in bio-plastics. The incidence of excessive antibiotics in chicken feathers can become the re-entry path for these drugs into the food chain and it has not been properly accounted for yet [5]. Therefore, feathers could be a potential reservoir of antibiotic residues and unintentionally expose to the environment.

In order to provide safe food and secure consumer health, antibiotic usage within the developed countries officially monitored through the materials collected on post mortem, such as muscles, liver, skin, fat in the food of animal origin using the established maximum residue limits

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(MRLs). While these limits have been set for most antibiotics for food producing animals, there are no regulatory limits specified MRLs for poultry feathers, as these structures are not used for direct consumption [7]. However, recent studies have detected different antimicrobial residues in animal by-products such as broiler chicken feathers. The researchers have found higher concentrations of antimicrobial residues in feathers than in edible tissue samples and they were persisted for longer periods [7–10].

In Ethiopia, β-lactam antibiotics, i.e., ampicillin, amoxicillin and penicillin are the most widely used class of medicines to treat respiratory tract infections, prostatitis, urinary tract infections, skin, and soft tissue infections of sensitive bacteria in poultry production [11–13]. High level of antibiotic residues were detected in meat and milk in studies conducted in Addis Ababa, Bishoftu, and Adama major cities in the country by microbiological or screening methods [14]. The report by Beyene and coworkers [15] indicated that the possible occurrence of residues of ampicillin, penicillin, streptomycin, and oxacillin are high and more common than other antibacterial drugs in Ethiopia due to irrational use of these drugs in food animals.

To date, several chromatographic separation techniques have been reported for the determination and quantification of antibiotics based on various detectors such as fluorescence (FLDs), diode array detectors (DADs), ultraviolet detectors (UVDs) and mass spectrometer (MS) [16–18]. The detectors have their own advantages and disadvantages, for example, FLDs have a good sensitivity and selectivity. However, the technique is applicable only for compounds that produce fluorescence and certainly requires derivatization to improve the fluorescence properties. DADs and UVDs are mainly used to detect antibiotics having ultraviolet absorbing groups, and have a high sensitivity, low noise level and wide linear range. Mass spectrometer is able to detect simultaneously more than 100 veterinary drugs in animal-derived foods; and give high recovery, high selectivity, good reproducibility, and low interference [19]. However, such highly specialized state-of-the-art instrumentations are not common in developing countries like Ethiopia because of high capital costs. Therefore, LC-UV was used as an alternative, cost-effective and simple instrumentation for the simultaneous detection of antibiotic residues from different matrices. Several antibiotics in diverse matrices were determined using LC-UV method and reported by several researchers [20–22]. However, the simultaneous analysis methods to multiple classes of antibiotics using LC-UV are still very limited.

The most time-consuming step in analytical method development is the optimization of the sample preparation that includes extraction and clean-up of antibiotics in complex matrices such as chicken tissues, animal manure, and others [23]. Some of the most common sample preparation techniques used in food analysis include solid phase extraction (SPE) [24], solid phase microextraction (SPME) [25], membrane extraction and others [26].

Antibiotic residues often occur in trace amounts in poultry foods, therefore, it is necessary to preconcentrate and purify analytes to bring the trace level residues to a detectable limit, close to or below the MRLs. SPE is very popular for the selective and quantitative sample preparation, clean-up and enrichment of organic compounds performed before chromatographic analysis to greatly reduce the occurrence of potential contaminants [27].

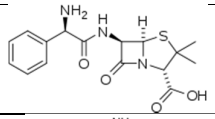
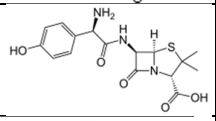
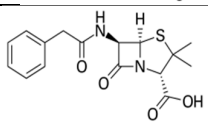
To the best of our knowledge, there are no works that have been carried out on the simultaneous extraction and analysis of β-lactam compounds in chicken tissues and feathers using SPE-LC-UV. The main goal of this study was to develop and validate SPE method for simultaneous extraction and determination of β-lactam antibiotic residues, i.e., amoxicillin (AMOX), ampicillin (AMPI) and penicillin G (PEN G) in chicken edible tissues and feathers using LC-UV. The study also aims to use chicken feathers as a suitable material for non-invasive alternative to tissue sampling and for surveillance of β-lactam antibiotic residue determination from chickens in locally produced poultry products. It has also been thought that determination of the antibiotic concentration in chicken feathers is useful to estimate environmental loads.

EXPERIMENTAL

Chemicals and reagents

The organic solvents used in this work, both acetonitrile and methanol (>99%) (HPLC grade) were purchased from Fisher Scientific (London, UK). Antibiotic standards with purity ≥99% of AMOX, AMPI and PEN G (Sigma–Aldrich, USA) were provided by the Ethiopian Food and Drug Administration (EFDA) and are listed in Table 1. Negative concentration chicken tissue control (Charm Scientific), and Isolute SPE cartridges (SAX, SCX, NH_2) with 50 mg/10 mL sorbent used for purification and enrichment were purchased from Sigma-Aldrich (USA) and were a kind donation from Ethiopian Public Health Institute (EPHI). Disodium hydrogen phosphates (Na_2HPO_4) (>99%), used for preparation of buffer solution, sodium hydroxide and orthophosporic acid (H_3PO_4) (>85%), used to adjust pH of the solutions, were purchased from Sigma-Aldrich (USA).

Table 1. The chemical structure, molecular formula, weight, Log K_{ow}, pK_a and solubility of the selected β-lactam antibiotics [28].

Compound	Chemical structure	Molecular formula, and weight ($g\ mol^{-1}$)	Log K_{ow}	pK_a	Solubility in water ($mg\ mL^{-1}$)
Ampicillin		$C_{16}H_{19}N_3O_4S$, 349.4	1.45	12.53, 2.7, 7.3	10.0
Amoxicillin		$C_{16}H_{19}N_3O_5S$, 365.4	0.87, 0.97	2.4, 2.8, 7.2	3.4
Benzylpenicillin (Penicillin G)		$C_{16}H_{18}N_2O_4S$, 334.4	1.85	2.7, 2.8	0.2

Log K_{ow} = octanol-water partition coefficient, pK_a = acidity constant.

Instruments and equipment

Shimadzu LC–20ad prominence with dual wavelength UV detector (Shimadzu Corporation, Kyoto, Japan) was used in chromatographic separation. Syringe membrane filters 0.2 µm Millex–HN (Millipore, Bed–ford, MA, USA) were utilized for filtration of the standards and samples. Other laboratory equipment used included: centrifuge, AX–320 (Tomy Seiko Co., Tokyo, Japan); vortex mixer, Vortex–Genie 2 (Scientific Industries Inc., Bohemia, New York, USA); ultrasonic machine, B5510J–DTH (Branson, Danbury, CT); Mill, M 20 Universal Mill, 120V, SKU: 1603603, IKA®–Werke GmbH & CO. KG Staufen/Germany, pH meter (Hanna Instruments Inc., Cluj-Napoca Jud, Cluj, Romania) used to measure and adjust the pH; Stuart SSL2/120V/60 laboratory scale linear reciprocating shaker, 120V, 50/60Hz, 50 Watts (Bibby scientific Ltd–Stone–Staffs–ST15OSA–UK). The solvents were purified using vacuum filtration assembly (Millipore filter cellulose nitrate gridded with 0.22 µ size and 47 mm diameter) (Sigma-Aldrich, USA). Water Still, 4 LPH, Double distilled, 240 VAC, 50/60 Hz from Stuart Aquatron (USA) was used to purify water.

Chromatographic operating conditions

Chromatographic separation was carried out using analytical column Hypersil BDS–C18 (3 µm, 100 mm × 4 mm) (Thermo Fisher Scientific, Phenomenex, USA) at 25 ºC. The separation was carried out in reversed phase and isocratic elution with 0.05 M Na_2HPO_4, acetonitrile and methanol (70:10:20) mobile phase composition, pH = 8, flow rate = 1.0 mL min^{-1}, wavelength = 230 nm. 20 µL was injected and the total analysis time was 14 min.

Preparation of standard solutions

Individual standard stock solutions of 1000 mg L^{-1} of each AMOX, AMPI and PEN G were prepared in 10 mL volumetric flask, by weighing 10 mg of the antibiotic standards and dissolving them in a 1:1 mL of MeOH:H_2O (v/v) and stored at −18 ºC. A series of mixed working standard solutions in concentration levels of 0.05, 0.5, 1, 1.5 and 2 µg L^{-1} were prepared freshly from 200 µg L^{-1} intermediate stock solutions, by transferring appropriate volume of the aliquot and diluting the a total volume to 10 mL with a mobile phase solution. 0.2 µm nylon syringe membrane filters were used to filter all solutions before injected into the LC system.

Sample collection

Most of the large scale commercial and private sector poultry farms are found around Debre Zeit; 60 km South-East of Addis Ababa which is the capital city of Ethiopia. The top three of the largest commercial poultry farms, located in this area include Elfora Agro-Industries, Alema and Genesis utilize modern production and processing facilities. In particular, Elfora delivers more than 420,000 chicken and over 34 million eggs annually to the market of Addis Ababa. Alema poultry farm is the second largest commercial poultry farms in the country, delivering nearly half a million broilers to the Addis Ababa market each year [29]. Large and small scale poultry meat suppliers are also distributing their products through retailers and supermarkets. The major consumers of the products are hotels, restaurants and various institutions with food catering services and households [30].

Two live chicken were bought for this study, in November 2020, from Shola Live Bird Retail Market, one of the largest markets found in Addis Ababa. The broiler chicken were kept in a cage under controlled conditions by feeding corn, wheat, and water for six months without giving any antibiotics. Then, muscle tissues and feather samples, which were free from antibiotic treatments, were collected from each slaughtered chicken and kept as a blank sample. Additionally, slaughtered and market ready nine chicken samples were purchased from randomly selected supermarkets in Addis Ababa, in April 2021, for the real antibiotic residue analysis. From the purchased chicken, four live chicken were immediately slaughtered in the market by the seller and the feathers were collected for real sample analysis. The samples were stored at −20 ºC until the analysis after arrival at the laboratory.

Sample preparation and extraction procedure

Chicken tissue samples were sliced with stainless steel knife, homogenized and ground in an industrial food processor before proceeding to the extraction process. As feathers are not homogeneous, exposed to micro- and macro-pollution, dust and other various environmental factors, they need to be prepared properly prior to analysis [31]. The contaminated individual samples that had traces of blood and feces on the feathers were excluded from the subsequent analyses. The quill or calamus part of the feather was removed using properly washed and alcohol wiped scissors. M 20 Universal Mill was used to ground the feather samples into tiny pieces (particle size of < 2 mm) for 5 min. Finally, all samples were individually packed and kept at −20 ºC.

In this study, extraction was based on liquid partitioning with mechanical shaking, ultrasonication, centrifugation of the matrix with organic extraction solvents and buffers, and purifying the extracts using SPE cartridges at ambient temperature. The techniques described previously by Maddaleno *et al.* [8] and Lakew *et al.* [32] were used to extract the selected β-lactams from the chicken tissue and feather samples.

Two grams chicken tissue, one gram for feather sample, was placed in a 50 mL polypropylene centrifuge tube and spiked with mixed standard solution. 10 mL ultrapure water was added and mixed manually and then shaken with a horizontal mechanical shaker for 15 min at 100 rpm. In order to equilibrate with the sample matrix, the mixtures were allowed to stand for 30 min at room temperature. 10 mL of extraction solvent (acetonitrile:methanol, 10:20 v/v) were added to the mixture and homogenized by vortex at 1000 rpm for 1 min and also sonicated for 15 min. After centrifuging for 5 min at 3500 rpm and ambient temperature, the resulting organic extract was purified using previously preconditioned SAX cartridges. The eluent was evaporated using a stream of nitrogen near to dryness and the resulting concentrated residues were redissolved in 2 mL MeOH and 20 µL aliquot was injected into the LC-UV system.

Solid phase extraction procedure

The extraction was optimised using chicken tissue and feather samples collected from slaughtered chicken that were kept under controlled conditions. The blank samples were spiked with a mixture of AMOX, AMPI and PEN G standards at the concentration of MRL, 50 µg L^{-1}. The SAX cartridge was preconditioned consecutively using methanol (5 mL), ultrapure water (5 mL) and finally 5 mL of pH 8 phosphate buffer (0.05 M). Then, the extracted sample was loaded onto the conditioned SAX cartridges at the flow rate of 2-3 mL min^{-1} in vacuum. The cartridges after loading were washed with ultrapure water (5 mL) and dried for 10 min. The extracted analytes were eluted with 5 mL methanol:acetonitrile (70:30 v/v).

Statistical analysis

Linear regression equation, correlation coefficients, graphical expression, recovery values, precision (as residual standard deviation) and β-lactam concentrations calculated as arithmetic mean of triplicates with standard deviation using Microsoft Excel 10. Fisher's statistical test for analysis of the variance (ANOVA) was applied to determine the significance of independent factors and the results are reported as mean ± SD.

RESULTS AND DISCUSSION

Optimisation of solid phase extraction conditions

The major aim of this study was to optimize and validate SPE method for the simultaneous extraction and determination of β-lactam antibiotics in chicken tissues and feathers. In order to get high extraction recoveries for the targeted compounds, various experimental parameters influencing the performances of SPE procedure were investigated and optimized; which include the effects of SPE sorbent type, the extraction time, sample pH, type and volume of elution solvents were optimized.

Effects of SPE sorbent type

The applicability of the SPE technique is mainly determined by the composition of the sorbent used in the extraction column. Specific selectivity for the compounds of interest can be achieved

by selection of the appropriate sorbents [33]. Basically, selection of the appropriate SPE sorbent requires understanding the nature of the sample matrix, analytes functional groups that influence its solubility, polarity, pH, and the analytes physicochemical properties and the sorbent responses to the changing extraction conditions [34, 35].

Preliminary clean-up experiments were carried out using extracted chicken tissue and feather samples in order to find the best sorbent for the solid phase extraction. In this study, performances of the available commercial SPE cartridges (strong anion exchange (SAX), strong cation exchange (SCX) and weak anion exchange (NH_2) were tested to extract and purify the selected antibiotic compounds from the sample matrices. The results indicated that extraction efficiencies varied considerably between the different sorbents and less interference observed when strong anion exchange (SAX) cartridge was used. This is because antibiotics exist in ionic and non-ionic forms in the solution and strong anion exchange bed sorbents such as SAX characterized by ionic interactions. Solid phase extraction using SAX sorbent has also been reported to be superior compared to other sorbents for the extraction and analysis of β-lactam antibiotics by other workers [34, 36]. Figure 1 show the mean recovery results obtained for each compound when applying the extraction methods described in sections 2.6 and 2.7 to the chicken muscle and feather samples. Better extraction recovery was obtained in the order of SAX > NH_2 > SCX. Therefore, SAX was selected as a good clean-up sorbent cartridge.

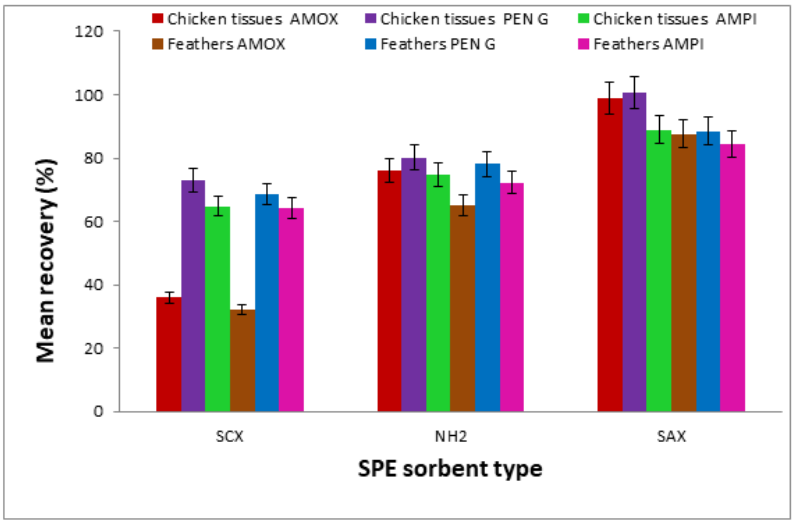


Figure 1. The effect of sorbent type on the recovery of β-lactam compounds in chicken tissue and feather samples.

The effect of sample pH on the analytes extraction

Antibiotics have some polar functional groups and the extraction solution pH has great importance as it affects the extent of ionization, extraction efficiency as well as solubility of the analytes [33]. The sample solution or extraction solvent pH influences the degree of ionization and extraction efficiency differently for each compound. For example, β-lactams are easily decomposed in acidic and basic media, but they are stable at neutral or slightly basic conditions. Benito Pena and his coworkers [34] reported that the best results were obtained in pH ranges of 7.5–8.0 for Oasis MAX cartridges with recoveries ranging between 99 and 107%. Under these conditions, when pH

> pK_a, the basic penicillin can be retained in the sorbent phase by anionic exchange and hydrophobic interactions. The recoveries slightly lowered at higher pH = 9.0 probably due to hydrolysis of the β-lactams in basic media. The retention behaviour of AMOX and AMPI can be explained on the basis of their acid-base or zwitterion properties (Table 1). At low pH the β-lactams in their acidic form show low retention, however due to the presence of the positive charge, they can be quantitatively recovered at pH 7.5 (zwitterion forms).

The influence of sample pH on extraction efficiency was evaluated by adjusting the sample pH in the range of 3 to 9, using sodium hydroxide or orthophosphoric acid. The acidic solution medium was not favourable for all β-lactam compounds to retain in the sorbent as shown in Figure 2. The recoveries were increased by raising the pH of the sample solution to about 5.0 and continued up to 8.0 and then started to decrease on further increase to 9. The highest analytical signals were obtained at the sample pH of 8 with good reproducibility and these results fell within the manufacturer pH specified range. Therefore, the sample pH of 8 was selected as the optimum value for the extraction and used in the subsequent experiments.

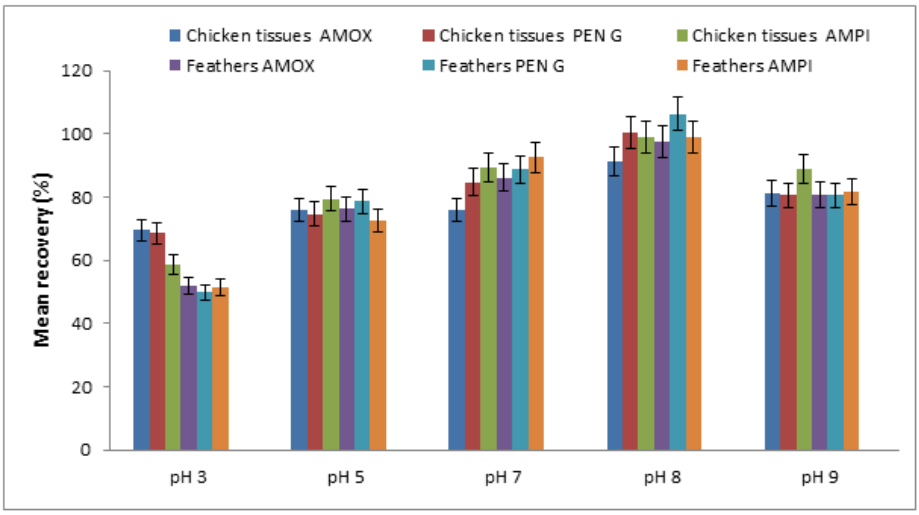


Figure 2. The effect of sample pH on the recovery of β-lactam compounds in chicken tissue and feather samples.

Effect of elution solvent type

Elution solvent type, volume and composition were selected according to the physicochemical properties of the antibiotic drugs. The choice of elution solvent is so critical and should be carefully considered because too powerful solvents will be eluted out with more interference and if the elution solvent strength is not enough, a larger elution volume will be needed that will dilute the sample and giving lower detection sensitivity [37].

To select the best eluent for eluting the analytes from the sorbent, water-miscible organic solvents including ACN, MeOH, and mixtures of their different proportions (80:20, 70:30, 50:50 v/v) were tested. These solvents were selected based on the fact that they could be suitable to desorb the targeted antibiotic analytes from the sorbent using minimum volume [38]. Figure 3 shows the results of the effect of the elution solvents on the recovery of the targeted compounds. The optimum elution solvent ratio with maximum mean recoveries was observed at the ratio MeOH:ACN (70:30 v/v) and chosen for further experiments.

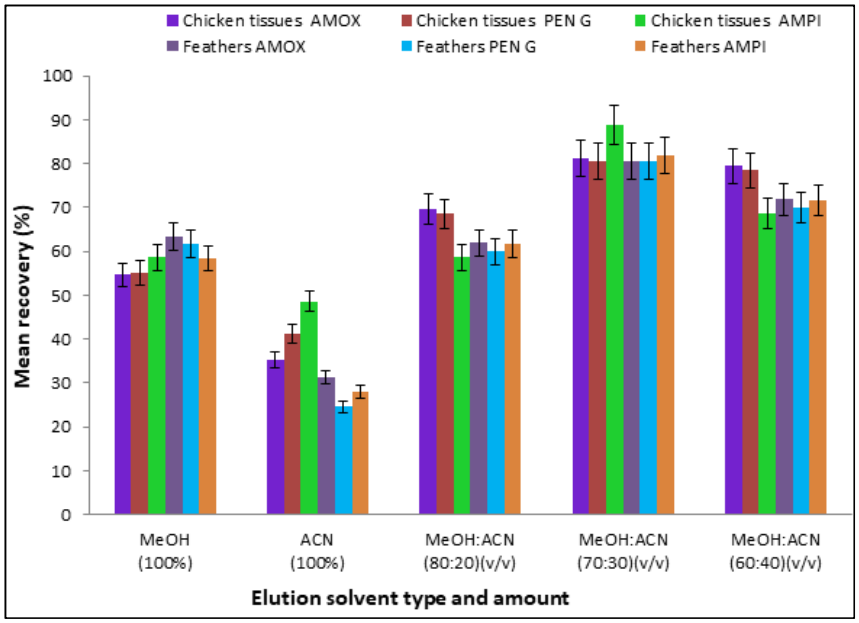


Figure 3. Effect of elution solvent type and composition on the recoveries of the tested β-lactam antibiotics.

Effect of elution solvent volume

Attempts were made to minimize the elution solvent consumption and sample preparation time by optimizing the elution solvent volume. The effects of different elution solvent volumes (3, 5, 10 and 15 mL) of methanol:acetonitrile (70:30 v/v) were tested. As shown in Figure 3, higher extraction recoveries were obtained at a volume of 5 mL. Therefore, 5 mL was selected as the optimum elution solvent volume for further experiments. Beyond this volume, the recovery decreases, and elution solvent beyond 15 mL was not tested due to the dilution effect, cost and environmental issues.

Effect of extraction time

Mass transfer is a time dependent process and one of the most important factors in most of the extraction procedures [39]. The exposure time between aqueous phase and sorbent facilitates the equilibrium and increase the precision and sensitivity of the extraction process. The effect of extraction time was evaluated by varying the time in the range of 3–15 min. The results indicated that the extraction recovery was enhanced by increasing the sorption time from 3 to 10 min and then remained nearly constant with further increase to 12 min. As can be seen from Figure 4, longer extraction time (≥ 12 min) resulted in the gradual decrease in peak area of the analyte, owing to the fact that adsorption of the analyte into the sample matrix [39]. Therefore, 10 min was selected as an optimum extraction time for the subsequent experiments.

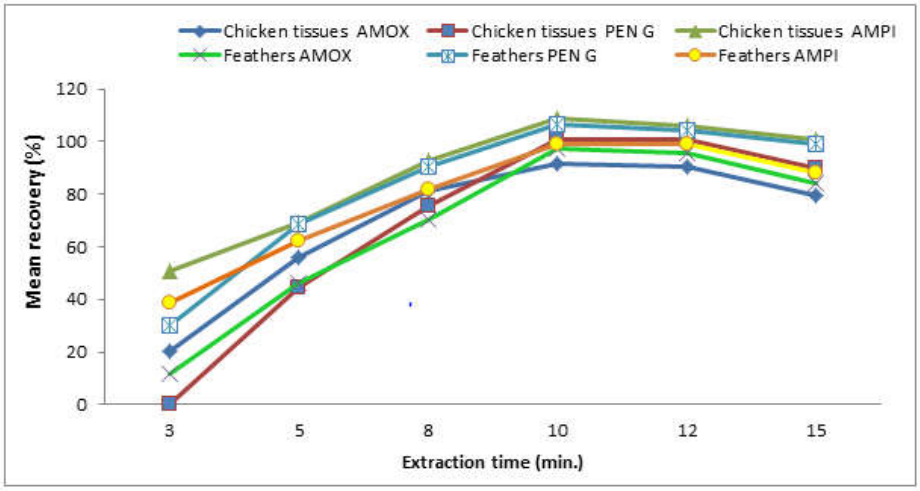


Figure 4. Effect of extraction time on the recoveries of β-lactam antibiotics.

Method validation

The validation of the developed method was performed according to the guidelines of the European Commission Decision 2002/657/EC [40] and ICH Guidance [41]. Validation parameters for the determination of β-lactams in chicken tissue and feathers were evaluated for linearity, specificity, precision (repeatability and reproducibility), recovery, limit of detection (LOD), limit of quantification (LOQ**),** decision limit (CCα), and detection capability (CCβ).

Linearity of the analytical method

A calibration plot is a regression mode used to predict the unknown concentrations of an analyte based on instrument response to the known concentrations of an analyte (standard) [42]. Because sample matrices tend to affect (either reduce or enhance) the ion intensities of the target analytes, matrix-matched calibration plots were used to determine the antibiotic concentrations. The method linearity was evaluated by assessing the signal responses of the targeted analytes from matrix-matched chicken tissues and feather samples by spiking at five concentration levels, i.e., 0.1, 0.5, 1, 1.5 and 2 µg kg^{-1} and proceeded for the entire extraction procedure. As shown in Table 2, the calibration plots exhibited good linearity with regression coefficients (R^2) 0.9991–0.9998 for all the studied compounds.

Limit of detection and limit of quantitation

The LOD is defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence [42]. The criteria for selecting the LOD of this method was evaluated from a response of a signal-to-noise ratio greater than 3:1, whereas the LOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy [42]. Under the stated experimental conditions, the LOQ was determined by the signal-to-noise ratio greater than 10:1 on the basis of 10 spiked blank samples of each matrix. As shown in Table 2, the LOD of the method ranged from 0.10 to 0.15 µg kg^{-1} for chicken tissues and 0.10- 0.13 for feathers. The LOQ were 0.3 to 0.45 µg kg^{-1} for chicken tissues and 0.30-0.38

μg kg^{-1} for feathers which were low enough to determine the maximum residue levels of the selected β-lactams in the chicken tissue and feather samples specified by ICH Guidance [41]. It can be concluded that the proposed method allows good detectability, enabling the application of the proposed method to real samples.

Table 2. Linear equations, correlation coefficients and quantification limits of the targeted β-lactam antibiotics results of the proposed method.

Chicken tissue	Antibiotics		
	AMOX	AMPI	PENG
Linear equation	y = 93726x + 11648	y = 33158x + 3613.5	y = 32393x + 3139.9
Correlation coefficient (R^2)	0.9995	0.9995	0.9991
LOD (μg kg^{-1})	0.13	0.10	0.15
LOQ (μg kg^{-1})	0.40	0.30	0.45
%RSD	1.84	2.39	4.40
CCα (μg kg^{-1})	50.82	53.44	51.97
CCβ (μg kg^{-1})	52.30	58.04	55.41
Feathers			
Linear equation	y = 97275x + 4050.4	y = 33728x + 3175.7	y = 48473x + 2466.3
Correlation coefficient (R^2)	0.9994	0.9998	0.9992
LOD (μg kg^{-1})	0.13	0.10	0.10
LOQ (μg kg^{-1})	0.38	0.29	0.30
%RSD	4.46	2.02	7.15
CCα (μg kg^{-1})	53.77	50.98	53.15
CCβ (μg kg^{-1})	57.22	62.63	56.56

Precision and accuracy

The precision of the method were evaluated using reproducibility and repeatability studies. The repeatability of the method (intra-day precision) was conducted by analysing six replicates of β-lactams spiked chicken tissue and feather samples at three concentrations levels, that is 0.5, 1 and 1.5 μg kg^{-1} times the MRL of chicken tissues, on the same day. The reproducibility (inter-day precision) was measured by using three sample sets, spiked at the same concentration levels and analysed on three different days. The reproducibility of the method, expressed in terms of the relative standard deviations was below 8.4% which is less than 20% and within the recommended limits provided by UNODC 2009 [42].

The accuracy was evaluated by carrying out recovery assays following the recommendations of the International Conference on Harmonisation (ICH) [41]. Nine determinations over three concentration levels were determined and the results were reported as percent recovery. The relative recoveries of the analytes were between 94.2–101.6% for chicken tissues and 79.7–101.8% for feathers (Table 3) which were within the recommended limits of the ICH, 70–120% at all concentrations with RSD <20%. Therefore, the obtained results confirmed that the extraction is efficient, reproducible and accurate.

Decision limit and detection capability

The decision limit (CCα) and detection capability (CCβ) values were calculated in accordance with the guidelines of the European Commission Decision 2002/657/EC [40]. The decision limit is defined as the limit above which it can be stated with an error probability of 5% that a sample is not compliant with a probability of error equal to α. While the detection capability is the lowest

content of the target species that can be detected, identified and quantitatively determined in a sample with an error probability equal to β.

For substances that have no permitted limit, CCα can be established by the calibration curve procedure according to the ISO 11843 (SANCO/2004/2726 revision 4 document) [43]. In order to establish the CCα parameter, 20 representative blank samples of each matrix (chicken and feathers) were spiked at the corresponding MRL; 50 µg kg^{-1} for AMOX, AMPI and PENG, and analysed following the developed method according to the described procedure. Even though β-lactam MRL is not set for feather samples, these were spiked following that of the chicken tissues MRLs (50 µg kg^{-1}) [40] for AMOX, AMPI and PENG and proceeded the extraction procedures. The observed values of error α on the limit of decision (CCα) vary from 0.82-3.77 µg kg^{-1} while the corresponding error β on the detection capability (CCβ) values ranged from 1.48 to 9.51 µg kg^{-1} as shown in Table 2.

Table 3. Repeatability (intra-day precision), reproducibility (inter-day precision) and relative standard deviation (RSD %) of the method.

Sample	Analyte	Added (µg L^{-1})	Within-day (n = 6 determinations)			Between-day (n = 6, and 3 days determinations)		
Tissue			Measured ± SD* (µg L^{-1})	RSD (%)	Recovery (%)	Measured ± SD* (µg L^{-1})	RSD (%)	Recovery (%)
	AMOX	25	24.8 ± 1.4	6.7	98.7	25.5 ± 2.9	7.3	101.6
		50	49.2 ± 0.5	1.6	97.9	48.2 ± 0.9	1.3	97.3
		75	72.1 ± 3.0	2.7	97.5	72.4 ± 2.7	4.9	94.2
	AMPI	25	23.3 ± 0.8	7.5	95.5	24.7 ± 1.3	5.2	99.1
		50	48.5 ± 1.2	2.9	98.1	49.3 ± 2.1	4.1	98.9
		75	70.8 ± 1.1	1.4	96.3	75.7 ± 1.9	2.2	101
	PEN G	25	24.8 ± 1.6	6.4	99.4	23.7 ± 1.8	7.7	95.4
		50	49.6 ± 2.1	4.3	99.1	50.5 ± 2.8	7.8	100.6
		75	75.8 ± 2.1	3.0	100.2	76.7 ± 3.2	4.2	101.5
Feathers	AMOX	25	23.7 ± 2.2	3.2	96.2	23.7 ± 0.3	3.8	95.5
		50	59.9 ± 2.3	3.7	99.8	49.1 ± 2.1	4.5	98.2
		75	74.5 ± 2.7	1.1	99.7	74.3 ± 1.1	2.8	97.5
	AMPI	25	24.9 ± 1.1	5.1	98.9	24.2 ± 2.1	8.4	97.9
		50	49.5 ± 0.6	3.3	95.8	50.1 ± 3.4	2.3	100.8
		75	74.6 ± 2.0	2.2	99.8	73.1 ± 1.9	2.4	95.8
	PEN G	25	25.1 ± 1.3	6.1	100.1	24.1 ± 1.9	7.5	96.3
		50	45.9 ± 1.9	1.7	91.8	50.5 ± 5.8	6.5	100.9
		75	75.5 ± 2.4	2.3	100.6	76.3 ± 2.1	3.8	101.8

SD* = Standard deviation.

Selectivity of the analytical method

The selectivity/specificity is a measure of the ability of the method to identify or quantify the particular analytes in the presence of other substances, without interferences from the other components [42]. The selectivity of the method can be evaluated from the final chromatograms obtained by implementing the optimum sample extraction and clean-up, and then applying all the optimized separation and detection conditions. The selectivity/specificity of the method was assessed by analysing blank samples of chicken tissue and feather samples spiked with 100 µg kg^{-1} β-lactam standards. No interfering peaks were observed around the retention times of all the tested antibiotics which can be seen from the resulted chromatograms before and after the spiked sample extract, as shown in Figure 5 (A–E).

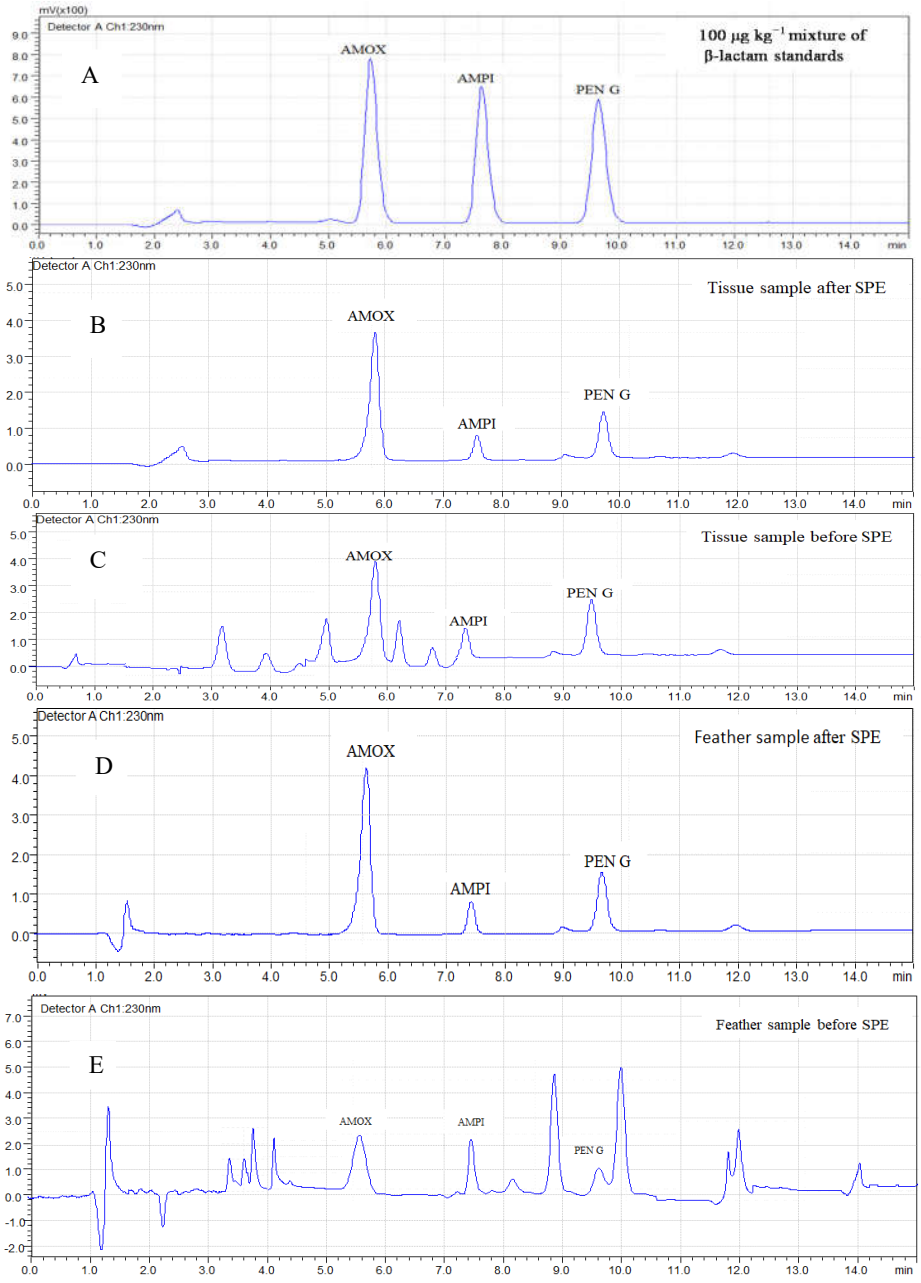


Figure 5. Chromatograms of AMOX, AMPI and PENG standards 100 µg kg^{-1} (A), spiked chicken tissue before clean-up (B), after clean-up (C); spiked feather sample before clean-up (D), after clean-up (E) with SAX cartridge.

Comparison of the method with previously reported results

The analytical performance of the developed extraction method has been compared with the previously reported methods for multiresidue analysis of chicken tissue and feather samples using GC-MS, LC-MS and LC-UV detection systems. As shown in Table 4, the comparison of methods focused on parameters such as type of SPE sorbent used, the simplicity of extraction and detection methods and elution solvents used, recovery and limit of detection. The results show that the method developed in this work is comparable or in some cases better than the reported methods in terms of using less extraction solvents with simple LC-UV detector. In the other developed solid phase extraction methods, complex extraction procedures and elution solvent combinations have been used to extract the antibiotics.

Table 4. Comparisons of the performance of the developed extraction method with some other previously reported methods.

Antibiotics	Sample	Type of SPE sorbent and extraction solvents	Method	Recovery (%) and RSD (%)	LOD, LOQ (μg kg^{-1})	Ref
TCs, (4)	Feathers, muscle and liver	Sep-Pak® C18, hexane , acetonitrile, oxalic acid, methanol	HPLC–MS/ MS	Feathers: 92–107 muscle: 94–108 liver: 93–108 and RSD<0.011	LOD = 20 for all Feathers: 21.5– 24.2, muscle: 21.2–21.6, liver: 25.0–27.7	[7]
LYc, (4)	Feathers, muscle, and liver	Chromabond® Florisil® cartridge, methanol, hexane, ethyl acetate	LC–MS/MS	98–101 and RSD<23	Feathers: 19, 62 muscle: 22, 73 liver:10, 34	[8]
CAP, TAP, FF and FFA	Poultry and porcine tissues	HLB, ethyl acetate ammonium hydroxide, hexane, methanol, sodium chloride. Derivatized with N,O-bis-(trimethylsilyl) trifluoroacetamide and 1% trimethylchlorosilane	GC–MS	78.5–105.5 and RSD<17	Poultry: 0.1–0.5, porcine tissues: 0.25–2.0	[44]
β-Lactams (3)	Feathers and chicken tissues muscle	SAX, acetonitrile and methanol	HPLC–UV	Feathers: 80–102 muscle: 94–102 and RSD<8.4	Feathers: 0.1–0.13, 0.3–0.38 muscle: 0.1–0.15, 0.3–4.5	This method

CAP–chloramphenicol, TAP–thiamphenicol, FF–florfenicol, FFA–florfenicol amine, DMSO–dimethylsulfoxide, TCs–Tetracyclines, LYc–Lincomycin.

Application to real samples

The optimized and validated SPE extraction method was applied to the extraction of AMOX AMPI and PEN G in chicken tissues and feather samples collected from some supermarkets and retail markets in Addis Ababa, Ethiopia. Identification of the target antibiotic compounds in real samples was performed based on matching the retention times obtained in the sample extract with the β-lactam standards.

The experimental results showed that all the analytes in chicken tissue samples were detected at the concentration level below the MRL established values set by the European Union legislation [40]. A number of the targeted compounds were not detected (nd) for AMPI and PEN G in chicken tissue samples, this might be either the chicken tissues are free from antibiotic residues or have concentrations below the detection limits. The concentrations of β-lactam antibiotics in all the feather samples were detectable and they were much higher in the range 218.2–2,375 μg kg^{-1} as listed in Table 5. The obtained results were about 10 times less than the values reported by Pokrant and his co-workers [5], i.e. 2,859 μg kg^{-1} for sulfachloropyridazine in feather samples and 20.54 μg kg^{-1} in edible tissues.

Table 5. β-Lactam antibiotic residues found in real chicken tissues and feather samples.

	β-Lactam residues (μg kg^{-1})						
	Chicken tissues			Feather samples			
ID	AMOX	PEN G	AMPI	ID	AMOX	PEN G	AMPI
C1	0.03	nd	nd	F1	2371.3	1843.3	411.9
C2	0.05	nd	nd	F2	2292.1	2296.5	900.4
C3	0.06	nd	nd	F3	1658.3	308.4	2352.4
C4	0.08	0.03	nd	F4	1791.9	218.2	2374.8
C5	0.02	0.03	0.08				
C6	0.06	0.03	0.04				
C7	0.02	0.02	0.18				
C8	0.05	nd	0.08				
C9	0.08	0.09	0.09				

*n.d. = not detected; the MRL for each AMOX, AMPI and PEN G = 50 μg kg^{-1}; the retention time: AMOX = 5.79, AMPI = 7.58 and PEN G = 9.65.

The applicability of the developed method was further evaluated by extracting real chicken tissues and feather samples spiked at 100 μg kg^{-1} concentrations. The mean recovery was ranged from 84-98% and RSD% ranged from 0.11 to 3.4% (n = 3). The samples peak areas were compared with standard peak areas at the same concentration levels to assess the influence of matrix effects and the results confirmed that the matrices of the real chicken tissues and feather samples do not have obvious effects on the proposed extraction method.

CONCLUSION

The present study describes the optimisation and application of solid phase extraction with a simple analytical method followed by the LC-UV detection for the determination of β-lactam antibiotic residues. The optimized experimental method was successfully applied for the determination of amoxicillin, ampicillin, and penicillin G in chicken tissue and feather samples. Parameters affecting the performances of SPE procedure were optimized and the obtained results indicated that strong anion exchange (SAX) cartridge exhibited good selectivity for the selected antibiotic compounds from chicken tissue and feather samples. The validation was carried out based on the recommendations from Commission Decision 2002/657/EC and the proposed method offers good sensitivity and selectivity. The developed method can therefore be used as an alternative for the simultaneous extraction and quantitative determination of trace level β-lactam antibiotics and other antibiotic residues having similar physical and chemical properties in the matrices of varying origins.

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