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Method Validation for Detection of Sulphamethazine Residue in Chicken Meat Samples by Simple Rapid Liquid Chromatography Technique

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ABSTRACT

The occurrence of antibiotic residues in chicken meat constitutes a potential risk to the health of consumers. The present study describes the optimization and validation of a high-performance liquid chromatographic (HPLC) method for the determination of sulphamethazine (SMZ) in chicken meat using photo diode array detector (PDA) and C18 analytical column. The extraction method involving deproteinisation of the chicken sample followed by a solid phase extraction (SPE) clean-up of antibiotic residue has been optimized. The method was validated according to the European Commission Decision 2002/657/EC. The recoveries for the studied antibiotics ranged from 81.6–84% with relative standard deviations between 6.6 and 7.2%. The method was applied for the analysis of antibiotic residues in 16 raw chicken meat samples collected from Durg, Chhattisgarh, India. 3 samples found contaminated with antibiotic residues. The method has limit of quantification below the maximum residue limits (MRLs) and easy to perform, thus found suitable for performing routine analysis.

HIGHLIGHTS

- Study focused on the detection of sulphamethazine antibiotic residue in chicken meat samples.
- A rapid HPLC method with PDA detection was optimized for the detection of sulphamethazine residue.

Keywords: Antibiotic residue, HPLC-PDA, Maximum residue limits, Chicken meat

Indiscriminate use of antibiotics often leads to the evolution of antimicrobial resistance (AMR) in pathogens of both human and animal origin (Adebowale *et al.,* 2016; Manyi-Loh *et al.,* 2018). Prolonged exposure to antibiotic doses leads to the proliferation of resistant bacterial strains, which might transfer AMR genes to other species of bacteria, with difficulty in predicting consequences to human health (Dantas *et al.,* 2008). AMR is a global health threat because it renders many antibiotics ineffective and thus, simply treatable infections may become more virulent and even deadly to humans soon (WHO, 2015). Antibiotics are widely used in poultry sector for the treatment and prevention of diseases as well as for growth promotion and improving feed conversion efficiency. However, the injudicious use of antibiotics in poultry production system and non-compliance to withdrawal periods of antibiotic agents may persuade the presence of their residues in meat (Emami, *et al.,* 2012; Chattopadhyay, 2014). Globally, it

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is estimated that 50% of the antibiotics produced in the world are used in animals as growth promoters (De Briyne *et al.,* 2014). Poultry industry is blamed for the dramatic rise and spread of AMR in bacteria (Bhushan *et al.,* 2017; Patel *et al.,* 2018). In animal production system, AMR can lead to more severe outbreaks of diseases and mass deaths among animal and poultry populations with enormous economic losses (Manyi-Loh *et al.,* 2018). Besides the problematic situation of AMR, now a day's presence of various antibiotic residues in animal originated foods may represents great health risks to consumer health due to its several side effects including toxicity, carcinogenicity, and sensitivity (Wielinga and Schlundt, 2012).

Sulfonamides (SAs) are a group of synthetic antibiotics with a broad-spectrum activity against the majority of gram negative and gram positive bacteria. SAs have a bacteriostatic effect through binding ρ-aminobenzoic acid, which is essential for folic acid synthesis and hence, inhibit the bacterial DNA formation (Hela *et al.,* 2003). Among a number of derivatives, SMZ is one of the most commonly used SAs in animal medication and prevention as well as a growth-promoting agent in poultry (Awaisheh *et al.,* 2019). The low cost and high efficacy have resulted in the widespread use of SMZ in poultry sector, as an additive in water or feed (Barceló, 2007). Several reports indicated that SMZ comprised approximately 95% of total SAs residue detected in animal tissues (Van Boeckel *et al.,* 2015; Chen *et al.,* 2012).

Various regulatory agencies like Codex Alimentarius Commission (CAC), European Commission (EC) and other agencies around the world have formulated and enforced MRLs to restrict the usage of prohibited veterinary drugs and ensure the limited presence of antibiotic residues in foods of animal origin (European Commission, 2010; Codex Alimentarius Commission, 2017).

The presence of antibiotic residues in foods of animal origin is an issue of immense public health concern. The EC report concerning the chemical residues in animal foods showed that the SAs, including SMZ, are one of the most occurring and contaminating drugs. SMZ is a suspected carcinogen (Baynes *et al.,* 2016) and has been detected and found in meat, fish, milk, and cheese (Mehtabuddin *et al.,* 2012; Mubito *et al.,* 2014). Furthermore, SMZ is more heat stable than other SAs, which indicates that it is less affected by different cooking conditions and more residues left in cooked food (Liman *et al.,* 2015). Therefore, the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) updated the MRLs for veterinary drug residues in foods of animal origin (Codex Alimentarius Commission, 2017). In animal tissues MRL of SMZ is set at 100 ppb (European Commission regulations, 1990).

HPLC is one of the most powerful tools in analytical chemistry with the ability to separately identify and quantify the different analytes present in food commodities. Therefore, in residue analysis, its usage is increasing day by day. The variety of mobile phases, the availability of wide range of column packings and the variation in modes of operations are the reasons for its high demand (Kebede *et al.,* 2014). It is an automated process with high specificity, sensitivity, accuracy and takes short time to produce results.

Thus, the current study describes the optimization and validation of a simple HPLC-PDA method for the determination of SMZ residue in chicken meat, which could be applied to monitor these residues in routine analysis.

MATERIALS AND METHODS

Equipment

HPLC measurements were carried out using a quaternary gradient chromatographic system from Waters, Inc. (USA) model Alliance® – e2695, coupled to a photodiode array detector, Waters® 2998. Data acquisitions were performed by Empower™ 3 Chromatography Software. Other equipment such as Sartorius electronic weighing balance, refrigerated centrifuge (Thermofisher™, USA), pH meter (pHTutor® Digital pH Meter), vacuum concentrator (Vacufuge® plus, Eppendorf™ AG, Germany) and Vortex Shaker (Spinix™ Tarson Instruments, India, Pvt. Ltd.) were also used in the present study.

Standards and reagents

SMZ (98–99%) analytical standard was purchased from Sigma-Aldrich Co. USA. Analytical grade sodium acetate, calcium chloride, sodium ethylenediamine tetraaceticacid (sodium EDTA), disodium hydrogen phosphate dihydrate,

citric acid monohydrate and sodium hydroxide were purchased from Sigma (USA). Hydrochloric acid, ammonium hydroxide, phosphoric acid, trichloroacetic acid (TCA), trifluroacetic acid (TFA) and HPLC grade methanol and acetonitrile were purchased from Merck (Germany). HPLC grade water was obtained from Milli-Q system from Millipore (USA).

Preparation of standard solution

Standard stock solution of SMZ was prepared at a concentration of 0.1 mg/ml, by dissolving an accurately weighed quantity of compound in 10 ml of methanol. The standard solutions were stored in dark glass bottles at -20°C and were stable for a period of 3 months. Working solutions were prepared daily by appropriate dilution of aliquots of the standard stock solutions in HPLC grade methanol. The working solutions were used for preparation of calibration curves of concentration 50, 100, 200, 300 and 500 µg/kg .

Sample extraction

An aliquot of 5g meat sample was transferred into 50 ml polypropylene centrifuge tube. Three milliliters of 20% TCA in acetonitrile was added to the sample to promote protein precipitation and vortexed for 5 min. To the vortexed sample, 15 ml of sodium EDTA-Mcllvaine buffer (pH 4) was added, sonicated for 10 min and centrifuged at 7500 rpm for 15 min at 4°C. The supernatant was filtered through a Whatman™ filter paper to remove any remaining flakes. The filtrate was then collected in a clean beaker and processed for further clean-up of sample.

The filtered extracts were cleaned up over Oasis hydrophobic-lipophilic balanced SPE cartridges (Oasis HLB, Waters, USA) using a vacuum manifold. The SPE cartridges were preconditioned with 5 ml of methanol followed by 3 ml of HPLC grade water under gravity. The sample extracts were allowed to pass through and cartridges were washed with 3 ml of (3%) methanol in water. The retained analytes were then eluted with 3 ml of methanol and 2 ml (1%) TFA in acetonitrile. Elute was collected in a clean beaker and concentrated to dryness at 40°C in vacuum concentrator. The residues were dissolved in 1 ml of 20% methanol in water and filtered through a 0.22 µm syringe filter and stored in an HPLC auto sampler vials for further analysis. An injection volume of 50 µl was finally injected into the HPLC system.

HPLC analysis

HPLC-PDA analysis was performed on a Waters Alliance ® – e2695 HPLC system using the Agilent ZORBAX XDB-C18 (C18, 5 µm; 250 mm × 4.6 mm) reverse phase analytical column. SMZ elution was done under isocratic conditions with 0.01 M oxalic acid (pH 4.0), methanol and acetonitrile (70:10:20, v/v) as the mobile phase. All separation related parameter like injection volume, flow rate, column temperature and maximum absorption wavelength were optimized to obtain good separation of the SMZ compound. After injecting 50 µl of sample elute, SMZ was monitored by PDA at 270 and 280 nm. Empower 3® software was used for instrument control and data evaluation.

Method validation

The proposed method was validated for different performance criteria *viz*. linearity, intraday assay and interday assay, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ). The linearity response was examined by triplicate analysis of standard solution with SMZ at five levels (50, 100, 200, 300 and 500 µg/kg). The standard calibration curves were obtained by plotting concentrations (µg/kg) against peak area.

LOD and LOQ were calculated from the standard deviation (σ) of y-intercepts of regression analysis and the slope of calibration curve (m) using equations: 3 σ/m and 10 σ/m, respectively. The precision of the method consists of intraday assay precision and interday assay precision and expressed as % relative standard deviation (RSD) of peak area measurements. The intraday assay precision was determined by spiking three meat samples at a single concentration level of 200 µg/kg and evaluation was done through the results obtained with the method operating over 1 day under the same conditions. The inter-assay precision was determined at three fortification levels, 100, 200 and 300 µg/kg and the analyses were performed over the period of three consecutive days.

The accuracy of the method expressed as recovery %, was determined by triplicate analysis of spiked chicken meat samples at three fortification levels (100, 200 and 300

µg/kg). The recoveries were calculated by comparing the peak area of measured concentration to the peak area of the spiked concentration.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The chromatographic separation of SMZ compound was optimized using a 50 µL injection volume, adjusting the flow rate to 1 ml/min and column temperature to 30°C. The excellent separation for SMZ antibiotic standard with adequate resolution was achieved on ZORBAX XDB-column using PDA detector. The detection wavelength of 280 nm gave maximum absorption for this compound with adequate resolution.

Extraction of antibiotic residue from meat

The development of a good sample preparation method for extraction of SMZ antibiotic residue from the meat matrix is a difficult task due to unique physicochemical properties of SMZ compound. Also meat matrix co-extractants cause problem in detection of SMZ antibiotic residue. This requires pre-treatment, as well as clean-up of samples to eliminate specific interferences from meat matrix facilitating analytical determination of analyte with adequate resolution.

For the precipitation of proteins in milk and meat, use of TFA, TCA, oxalic acid, acetonitrile, acetone and hydrochloric acid have been described in the literature (Cinquina *et al.,* 2003; Zhao *et al.,* 2004; Camara *et al.,* 2013). In the present study, 20% and 30% TCA in acetonitrile were evaluated for precipitation of meat protein and best results obtained from 20% TCA in acetonitrile as it resulted in good protein precipitation. The sodium EDTA-Mcllvaine buffer has been used by many researchers for the extraction of SMZ residue from milk with good results (Camara *et al.,* 2003).

The extracts obtained after protein precipitation were cleaned up using polymeric Oasis HLB cartridges. After solid-phase clean-up of extracts, washing step of cartridges was evaluated. Initially, washing was carried out using 5 ml of ultra-pure water but it resulted in interferences and lower recovery values for antibiotic residue. Addition of 3% methanol to ultra-pure water and reduction of washing volume from 5 ml to 3 ml lowered the interferences near the retention time of the analyte and improved the recovery values for antibiotic residue under study.

Method evaluation

The linear range, LOD and LOQ were obtained from five point solvent matched calibrations curve by using SMZ standard at five dilution levels 50, 100, 200, 300 and 500 µg/kg with triplicate analysis. The calibration curve for SMZ was found linear in the range of 50-500 µg/kg with correlation coefficient (r^2) of 0.999. The LOD and LOQ for SMZ were found to be 12.8 µg/kg and 38.8 µg/kg, respectively. The LOD and LOQ values were below the MRL (100 µg/kg) established by EU for SMZ in meat.

Table 1: Method validation parameters for sulphamethazine

Method Validation Parameters	Values
Linear range (µg/kg)	50–500
Linearity (R^2)	0.99
Linear regression (Equation)	y = 167.75x – 809
LOD (µg/kg)	12.80
LOQ (µg/kg)	38.80
Intraday assay precision, n = 5 (%RSD) at 200 µg/kg	6.68 %
Inter day assay precision, n = 5 (%RSD)	
100 µg/kg	7.20%
200 µg/kg	6.63%
300 µg/kg	6.74%
Recovery %	
100 µg/kg	81.8%
200 µg/kg	81.6%
300 µg/kg	84.0%

LOD - Limit of detection, LOQ - Limit of quantitation, RSD - Relative standard deviation.

The application of the method to ten different blank meat samples in order to verify the method specificity demonstrated that no interferences from endogenous compounds were detected at 280 nm at the retention time of SMZ. The intraday-assay precision was found to be 6.6% at spiking concentration of 200 µg/kg whereas interday-assay precision was found to be 7.2, 6.6 and 6.7 % at spiking concentration of 100, 200 and 300 µg/

kg, respectively. The values observed were in agreement with EU guidelines. The average recoveries of SMZ at the concentration of 100, 200 and 300 µg/kg were found to be 81.8, 81.6 and 84% respectively and were in accordance with the EU guidelines. The assay validation parameters for SMZ are presented in Table 1.

In a study carried out by Mehtabuddin *et al.* (2012) the average recoveries for SMZ from spiked meat samples were found to be 60-90%. These recovery values were less as compared to recoveries detected in the present study. Contrary to this, the studies conducted by Cheong *et al.* (2010) the mean recovery for SMZ was 82% which was comparable to the recoveries found in the present study.

Analysis of marketed raw chicken meat samples

The procedure was applied to the analysis of SMZ residues in 16 marketed raw chicken meat samples, collected nearby chicken meat shops of Durg city, Chhattisgarh, India. Analysis showed that the 3 samples were positive for SMZ residues with levels below the LOQ of the method. The purpose of conducting analysis of the raw chicken meat samples is to corroborate the performance of the method and not to perform quality control testing.

CONCLUSION

The extraction and cleanup method coupled with HPLC–PDA was presented for the extraction, identification and quantification of SMZ in chicken meat samples. The above method offered a number of features including good linearity, high recovery, and short analysis time, simple operation process, cost effective and environmentally friendly. The method has advantages of simplicity, easy operation and consumption of low volume of the less hazardous organic solvents. Therefore, the developed method can be utilized as an attractive method for the determination of SMZ residue in chicken meat samples.

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