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# Validation of HPLC Multi-residue Method for Determination of Fluoroquinolones, Tetracycline, Sulphonamides and Chloramphenicol Residues in Bovine Milk

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## Abstract

The occurrence of antibiotic residues in milk constitutes a potential risk to the health of consumers. The present study describes the optimisation and validation of a high-performance liquid chromatographic (HPLC) method for the simultaneous determination of sulphadiazine (SDZ), sulphamethoxazole (SMX), oxytetracycline (OTC), doxycycline (DOX), tetracycline (TC), enrofloxacin (ENRO) and chloramphenicol (CLP) residues in bovine milk using colchicine (COL) as internal standard. The determination of these antimicrobials was carried out on $C_{18}$ analytical column using high-performance liquid chromatographic-diode array detection (HPLC-DAD). The extraction method involving deproteinisation of the milk sample followed by a solid-phase extraction (SPE) clean-up of antibiotic residues has been developed. The method was validated according to the European Commission Decision 2002/657/EC and applied for the analysis of antibiotic residues in 21 raw milk samples collected from Ludhiana, Punjab, India. The recoveries for the studied antibiotics ranged from 83.3–111.8% with relative standard deviations between 3.5 and 16.2%. The limits of quantification for these antibiotics, with the exception of chloramphenicol, were below the maximum residue limits (MRLs), making the method suitable for performing routine analysis.

**Keywords** Antibiotic residues · HPLC-DAD · Maximum residue limits · Milk · Solid-phase extraction · Validation

## Introduction

Antibiotics are extensively used in animal husbandry practices for treatment and prevention of diseases as well as for promotion of growth and improving production efficiency. However, the non-prudent use of antibiotics in dairy cattle management and non-compliance to withdrawal periods may persuade the presence of their residues in milk (Jahed-Khanik 2007; Andrew et al. 2009). The consumption of milk containing antibiotic residues may lead to allergic reactions, disorders of intestinal flora, immunopathological effects or emergence of resistant strains of bacteria (Moudgil et al. 2017; Nisha 2008). Antibiotic residues in milk are also of immense concern to manufacturers of milk products as these may interfere with starter culture for cheese, yoghurt and other dairy products resulting in significant monetary losses (Kebede et al. 2014).

Codex Alimentarius Commission (CAC), European Union (EU) and other regulatory agencies around the world have generated and enforced MRLs to ensure the limited presence of antibiotic residues in foods of animal origin and also restricting the usage of prohibited veterinary drugs (European Commission 2010; Codex Alimentarius Commission 2017). The presence of antibiotic residues in foods of animal origin is an issue of intense public health concern. Therefore, the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA), at its 40th Session of the CAC, updated the MRLs for veterinary drug residues in foods (Codex Alimentarius Commission 2017).

Sulphonamides, tetracyclines and fluoroquinolones are groups of synthetic antibacterial agents widely used in veterinary practice for the treatment and prevention of infections in food-producing animals (Jank et al. 2017; Martins et al. 2015). These three groups of antibiotics are most commonly used for therapeutic purpose in veterinary practice due to their low cost, easy over the counter availability and broad spectrum

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of activity (Martins et al. 2015). The chemical structures of the sulphonamides, tetracyclines, enrofloxacin and chloramphenicol are shown in Fig. 1. The EU/CAC has established a MRL of 100 μg/kg for sulphonamides, tetracyclines and fluoroquinolones in the foods and foodstuffs of animal origin. Chloramphenicol is an effective therapeutic agent for the treatment of mastitis in cattle because of its broad-spectrum activity, high efficiency and low cost (Bilandzic et al. 2011; Guidi et al. 2015). However, the potential risk of suspected carcinogenicity and aplastic anaemia in humans caused by its residues in foods, chloramphenicol is banned for use in food-producing animals in many countries. Therefore, it is very important to determine any non-prudent or extra label usage of this drug in food animals. For chloramphenicol residues in foods of animal origin, the EU has established a minimum required performance level (MRPL) of 0.3 ng/mL, the concentration that laboratories should be able to detect and confirm to ensure consumers safety (Guidi et al. 2015; European Commission 2002).

For fast screening of antibiotics in milk and other food commodities; microbiological tests, immunoassays and biosensors are widely used (Liu et al. 2009; Pikkemaat 2009; Raz et al. 2009). But these techniques are not able to distinguish between different types of antibiotics and may also lead to false-negative or false-positive results because of low sensitivity and specificity. While the fast screening methods are commonly used to detect the presence of antibiotics in milk, more accurate chromatographic methods such as high-performance liquid chromatography (HPLC) are required to identify and quantify antibiotics in foods.

HPLC is one of the most powerful tools in analytical chemistry with the ability to separate, identify and quantify the analytes present in food commodities. Its usage is increasing day by day in the field of residue analysis. 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

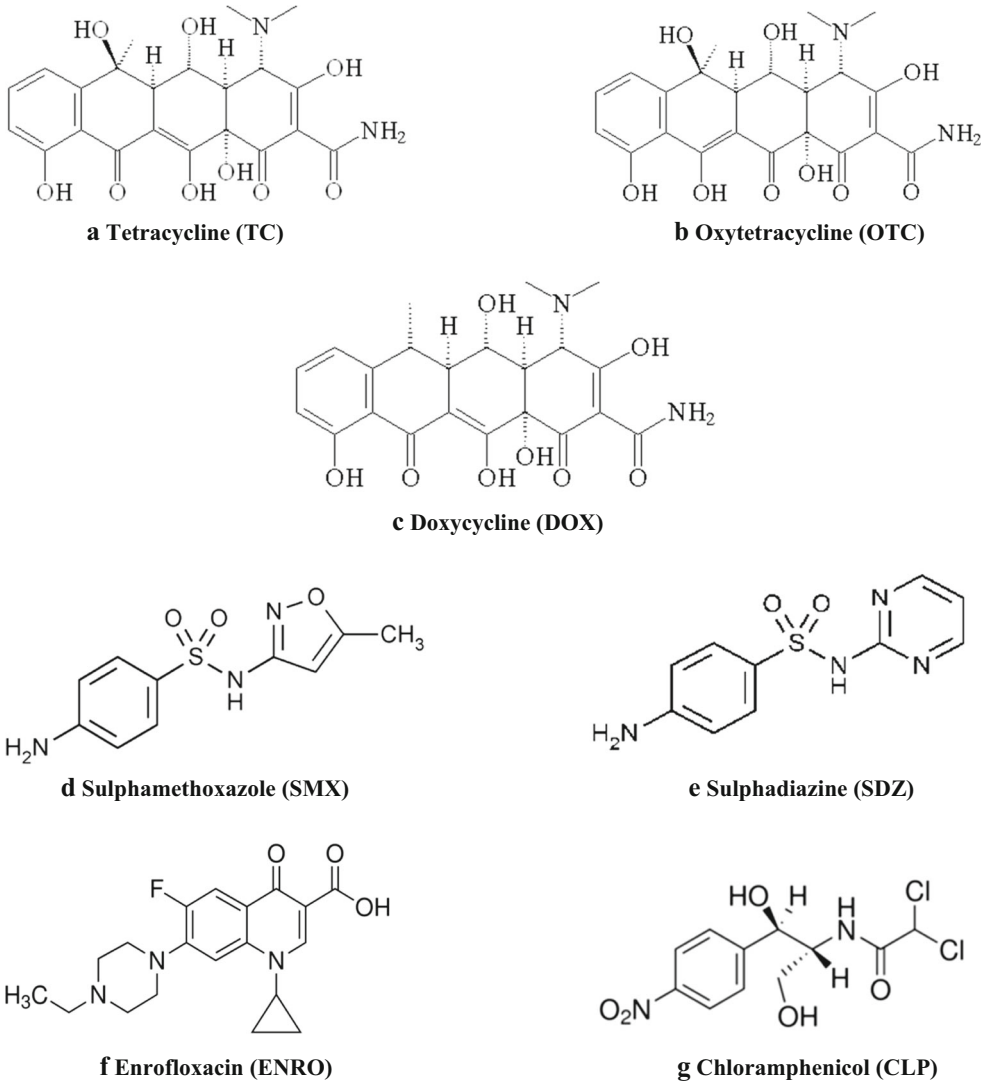



**Fig. 1** Chemical structures of antibiotics. **a** Tetracycline (TC), **b** oxytetracycline (OTC), **c** doxycycline (DOX), **d** sulphamethoxazole (SMX), **e** sulphadiazine (SDZ), **f** enrofloxacin (ENRO), **g**. chloramphenicol (CLP)

short time to produce results. Due to the escalating need to improve the speed as well as cost-effectiveness of the monitoring process for foods, there is demand for quantitative multi-residue and especially multiclass analytical methods (Kivrak et al. 2016). The use of HPLC multi-residue method is a way to improve the speed and cost-effectiveness by maximizing the number of analytes that can be determined by a single method.

Various HPLC-based methods targeting single antimicrobial group such as sulphonamides (Furusawa and Kishida 2001; Wu et al. 2007; Samanidou et al. 2008), tetracyclines (Andersen et al. 2005; Fritz and Zuo 2007; Samanidou et al. 2007), fluoroquinolones (Marazuela and Moreno-Bondi 2004; Hassouan et al. 2007) and chloramphenicol (Long et al. 1999) in milk have been developed and validated. Simultaneous determination of multi-residues of three antimicrobials groups (sulphonamides, tetracyclines and chloramphenicol) in milk using enrofloxacin as internal standard has been carried out by Mamani et al. (2009) in the past. However, the present study describes the simultaneous determination of multi-residues of four antimicrobial groups (sulphonamides, tetracyclines, enrofloxacin and chloramphenicol) using colchicine as internal standard. Also, the present study highlights simple sample preparation and extraction procedure as compared to method used by Mamani et al. (2009).

Thus, the current study describes the optimisation and validation of a simple HPLC-DAD method for the simultaneous multi-residue determination of SDZ, SMX, OTC, DOX, TC, ENRO and CLP in milk which could be applied to monitor these antimicrobials in routine analysis.

## Materials and Methods

### Equipment

HPLC measurements were carried out using a quaternary gradient chromatographic system from Varian, Inc. (USA), model Varian 920-LC, coupled to a photodiode array detector. Data acquisitions were performed by Varian Galaxie™ Chromatography Software.

Other equipment such as pH meter (Systronics® Digital pH Meter Type 355), electronic weighing balance (A&D Instruments, India, Pvt. Ltd.), refrigerated centrifuge (Eppendorf™, USA) and vacuum concentrator (Vacufuge® plus, Eppendorf™ AG, Germany) were also used in the present study.

### Chromatographic Column and Solid-Phase Extraction Cartridges

Chromatographic column and solid-phase extraction cartridges used by Mamani et al. (2009) were employed in the present study. The separation of the antibiotics by HPLC was performed on octadecyl X-Terra® C18 hybrid silica column (250 mm × 4.6 mm, 5 μm) from Waters Corporation (USA).

The solid-phase extraction procedures were carried out using polymeric cartridges (200 mg, 6 mL, OASIS HLB, Waters, USA). The cartridges were connected to 20 port solid-phase extractor (Waters Corporation, USA) for the clean-up of the sample which was further connected to the vacuum manifold for suction of samples by negative pressure.

### Standards and Reagents

Oxytetracycline hydrochloride (98–99%), tetracycline hydrochloride (98–99%), doxycycline hyclate (98–99%), enrofloxacin (98–99%), sulphdiazine (98–99%), sulphamethoxazole (98–99%), chloramphenicol (98–99%) and colchicine (≥ 95%) were purchased from Sigma-Aldrich (Fluka and Vetranal), Co, USA.

Analytical grade sodium acetate, calcium chloride, sodium ethylenediaminetetraacetate (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) 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 Solutions

Standard stock solutions of SDZ, SMX, OTC, DOX, TC, ENRO and CLP were prepared by dissolving 10 mg of each compound in 10 mL of methanol to give a final concentration of 1 mg/mL. The solutions were stored in dark glass bottles at 4 °C and were stable for a period of 1 month. Working solutions were prepared daily by appropriate dilution of aliquots of the standard stock solutions in HPLC grade water. The working solutions were used for sample spiking for preparation of calibration curves of concentration 50, 100, 200, 300 and 500 ng/mL.

The sodium EDTA-McIlvaine buffer (0.01 M) was prepared as described by Cinquina et al. (2003) by dissolving 11.8 g of citric acid monohydrate, 13.72 g of disodium hydrogen phosphate dehydrate and 33.62 g of ethylene diaminetetraacetic acid disodium salt in 1 L of water and pH adjusted to 4 with ammonium hydroxide. The solution was stored at 4 °C and was stable for a period of at least 90 days.

### Sample Preparation

An aliquot of 5 mL of blank or fortified milk 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-McIlvaine 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 milk flakes. The filtrate was then collected in a clean beaker and processed for further clean-up of sample.

The filter extracts were cleaned up over hydrophobic-lipophilic balanced SPE cartridges (Oasis HLB, Waters, USA) using a 12 port manifold at atmospheric pressure. 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 5 mL of methanol. Elute was collected in a clean beaker and concentrated to dryness at 35 °C in vacuum concentrator. The residues were dissolved in 1 mL of aqueous phase of the chromatographic mobile phase and filtered through a 0.22-μm syringe filter and stored in an auto sampler vials for further analysis. An injection volume of 50 μL was finally injected into the HPLC system.

### HPLC Analysis

The mobile phase composition described by Mamani et al. (2009) consisting of an aqueous phase of 0.075 mol/L sodium acetate, 0.035 mol/L calcium chloride and 0.025 mol/L sodium EDTA, pH 7.0 and an organic phase of methanol:acetonitrile (75:25, *v*/*v*) was used in the present study for optimisation of HPLC conditions to incorporate new compounds. Gradient elution of 90:10 aqueous phase:organic phase *v*/*v* to 50:50 aqueous phase:organic phase *v*/*v* (0–30 min) then to 90:10 aqueous phase:organic phase *v*/*v* (30–35 min) was used. The separation of antimicrobials was achieved on octadecyl X-Terra® C18 column. The flow rate was adjusted at 0.8 mL/min with temperature of HPLC column at 27 °C. Quantitative measurements were carried out by selecting the appropriate detection wavelength for each compound to attain maximum sensitivity. Therefore, the quantification was performed through internal standardisation at 254 nm (SMX, SDZ, COL), 311 nm (ENRO, CLP) and 355 nm (TC, OTC, DOX).

### Method Validation

The proposed method was validated according to the European Commission Decision 2002/657/EC using spiked milk samples (European Commission 2010). The performance criteria; linearity, sensitivity, specificity, intra-assay and inter-assay precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) were determined.

The linearity response was examined by triplicate analysis of milk samples fortified with SDZ, SMX, TC, OTC, DOX, ENRO and CLP at five fortification levels (50, 100, 200, 300 and 500 ng/ml) using 300 ng/mL of COL as internal standard. The standard calibration curves were obtained by plotting concentrations (ng/mL) against peak area ratio of analyte to internal standard.

LOD and LOQ were calculated from the standard deviation ($\sigma$) of *y*-intercepts of regression analysis and the slope of calibration curve ($m$) using equations: $3\sigma/m$ and $10\ \sigma/m$, respectively.

The sensitivity of the method, i.e. the change in analytical signal units per ng analyte, was represented by slope of the calibration curve. The specificity of the method was investigated by analysis of ten different blank milk samples to determine any interference from endogenous compounds.

The precision of the method consists of intra-assay precision and inter-assay precision and expressed as % RSD of peak area measurements. The intra-assay precision was determined by spiking five blank milk samples at a single concentration level of 200 ng/mL 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 ng/mL and the analyses were performed over the period of five consecutive days.

The accuracy of the method expressed as % recovery was determined by triplicate analysis of spiked milk samples at three fortification levels (100, 200 and 300 ng/mL) with 300 ng/mL of colchicine as internal standard. The recoveries were calculated by comparing the peak area of measured concentration to the peak area of the spiked concentration.

## Results and Discussion

### Optimisation of Chromatographic Separation

The chromatographic column and mobile phase composition described by Mamani et al. (2009) for simultaneous detection of sulphamethazine, sulphamethoxazole, sulphaquinoxaline, tetracycline, chlortetracycline, oxytetracycline and chloramphenicol in milk using enrofloxacin as internal standard was used for optimisation of HPLC conditions in the present study to incorporate new compounds. The mobile phase composed of aqueous phase of 0.075 mol/L sodium acetate, 0.035 mol/L calcium chloride and 0.025 mol/L sodium EDTA, pH 7.0 and an organic phase of methanol:acetonitrile (75:25, *v*/*v*) with gradient elution of 90:10 aqueous phase:organic phase *v*/*v* to 50:50 aqueous phase:organic phase *v*/*v* (0–30 min) then to 90:10 aqueous phase:organic phase *v*/*v* (30–35 min) was used.

In this study, the flow rate, column temperature and maximum absorption wavelength were optimized to incorporate sulphadiazine, doxycycline and colchicine. Thus, by adjusting flow rate to 0.8 mL/min and column temperature to 27 °C, excellent separation for targeted antibiotics; sulphadiazine, sulphamethoxazole, oxytetracycline, tetracycline, doxycycline, enrofloxacin and chloramphenicol in milk using colchicine as internal standard was achieved on X-Terra column using photodiode array detector (Fig. 2). Meclocycline sulphate and azithromycin were initially evaluated as internal standards but they eluted from the milk matrix at the same time as targeted antibiotics. However, COL was later employed for this purpose as it did not elute at the same time as targeted antibiotics from the milk matrix and also it is non-antimicrobial in nature.

Thus, the optimized method resulted in effective separation of all seven antibiotics belonging to four different groups in a single run with adequate resolution. The maximum absorption wavelength for each compound was optimized to attain maximum sensitivity. Thus, the detection wavelength of 254 nm for SMX, SDZ and COL, 355 nm for OTC, TC and DOX and 311 nm for ENRO and CLP gave maximum absorption for these compounds.

## Extraction of Antibiotics from Milk

The development of a unique sample preparation method for simultaneous extraction of antibiotics belonging to different groups from milk matrix is a difficult task due to their different physicochemical properties. This requires pre-treatment as well as clean-up of samples to eliminate specific interferences from milk matrix facilitating analytical determination of each analyte with adequate resolution.

The four main steps evaluated were precipitation of milk proteins, extraction of antimicrobials from milk matrix, clean-up using solid-phase cartridges and concentration of elute. For extraction studies, the known blank milk samples fortified with antibiotic standard solutions (1000 ng/mL) were employed.

For the precipitation of milk proteins, different solutions such as trifluoroacetic acid, trichloroacetic acid, oxalic acid, acetonitrile, acetone and hydrochloric acid have been described in the literature (Samanidou et al. 2008; Van Rhijn et al. 2002; Cinquina et al. 2003; Zhao et al. 2004; Camara et al. 2003). In the present study, 20% and 30% TCA in acetonitrile were evaluated for precipitation of milk protein with best results obtained from 20% TCA in acetonitrile as it resulted in complete protein precipitation. TCA was also used as milk protein precipitant in the studies conducted in the past, e.g. Toaldo et al. (2012) used 10% TCA in ultra-pure water, whereas Mamani et al. (2009) used 30% TCA in methanol for precipitation of milk proteins.

For preliminary extraction of antimicrobials from milk matrix, various extraction solvents and buffers described in the literatures such as phosphate buffer at pH 7, phosphate citrate EDTA buffer at pH 4 and 7 and sodium EDTA-Mcllvaine buffer at pH 4 and 7 were evaluated with best results obtained from sodium EDTA-Mcllvaine buffer at pH 4 that resulted in simultaneous extraction of all targeted antibiotics from milk with good recoveries. The sodium EDTA-Mcllvaine buffer has been used by many researchers for the extraction of antibiotic residues from milk with good results (Camara et al. 2003; Brtio and Junqueira 2006; Ahlberg et al. 2006).

The extracts obtained after protein precipitation and extraction were cleaned up using polymeric Oasis HLB cartridges.

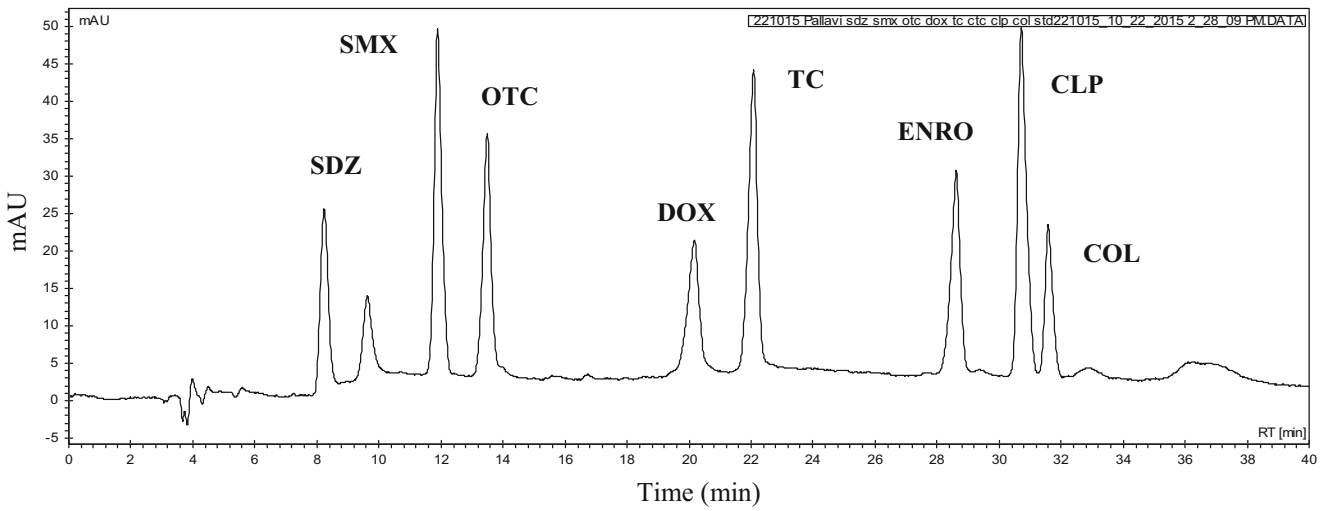



**Fig. 2** Chromatogram of SDZ, sulphadiazine; SMX, sulphamethoxazole; OTC, oxytetracycline; DOX, doxycycline; TC, tetracycline; ENRO, enrofloxacin; CLP chloramphenicol; and COL colchicine (1000 ng/mL). Flow rate: 0.7 mL/min. 254 nm (SDZ, SMX, COL), 311 nm (ENRO, CLP) and 355 nm (OTC, TC, DOX)

Various researchers have reported higher recovery values using polymeric Oasis HLB cartridges for clean-up of extracts (Andersen et al. 2005; Huelamo et al. 2009; Wang et al. 2015). Also, the higher recovery values for antibiotics belonging to three different groups (sulphonamides, tetracyclines and chloramphenicol) using Oasis HLB cartridges and lower interferences near the retention time of each analyte agree with the previous study of Mamani et al. (2009).

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 enhanced interferences and lower recovery values for antimicrobial residues. 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 each analyte and improved the recovery values for antimicrobials under study.

The final elution step was carried out using 5 mL of methanol as it has high elution strength because of high polarity. The residues were concentrated to dryness in vacuum concentrator and reconstituted in 1 mL of aqueous part of mobile phase before injecting into the HPLC system.

The representative chromatograms of blank milk samples and samples spiked with 100 ng/mL of antibiotic standards and 300 ng/mL of internal standard are shown in Fig. 3a–f.

## Validation of Method

The HPLC-DAD method was validated for the determination of tetracyclines, sulphonamides, enrofloxacin and chloramphenicol by assessment of following parameters: linearity, sensitivity, specificity, intra-assay and inter-assay precision, accuracy, LOD and LOQ. The results are presented in Table 1.

The linearity and sensitivity of the proposed method were determined for all the test antibiotics from calibrations curves by plotting the peak area ratio of analyte to internal standard against increasing concentrations of each analyte under study (50,100, 200, 300 and 500 ng/mL). The analysis was performed in triplicates using 300 ng/mL of colchicine as internal standard. Linear regression data showed good linearity for all the analytes with correlation coefficient ($r$) in the range of 0.998–0.999.

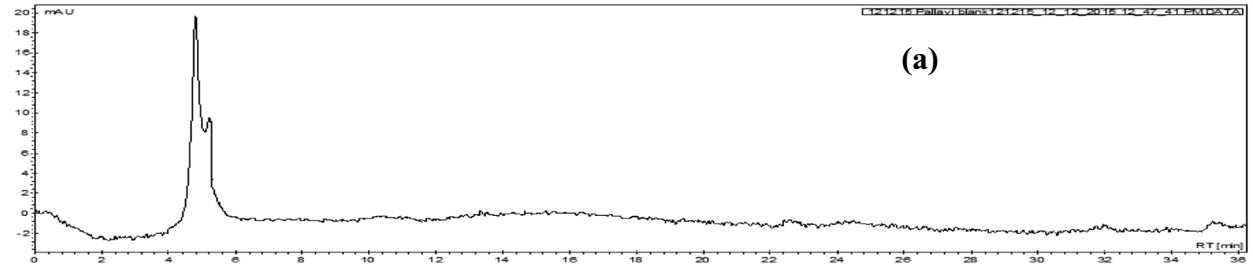
The application of the method to ten different blank milk samples in order to verify the method specificity demonstrated that no potential interferences from endogenous compounds were detected at 311 nm near to the retention time of ENRO and CLP and at 355 nm near to the retention time of OTC, DOX and TC. Also, at 254 nm, no interferences were observed near to the retention time of SMX and COL; however, some interferences were observed near to the retention time of SDZ but none of them have any effect on its detection.

Since no characteristic interferences were detected at retention time of each analyte, the optimized method presents adequate selectivity for the determination of targeted antibiotics. The characteristic chromatograms from blank and fortified milk samples at three different wavelengths (254 nm, 311 nm and 355 nm) are presented in Fig. 3a–f.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the standard deviation ($\sigma$) of $y$-intercepts of regression analysis and the slope of calibration curve ($m$) using equations 3 $\sigma/m$ and 10 $\sigma/m$, respectively. As presented in Table 1, the LOD for the studied antimicrobials ranged from 17.2 to 24.9 ng/mL. The detection limit (LOD) of method is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. The quantification limit (LOQ) of method is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy under the stated operational condition of the method. The LOQ for the studied antimicrobials ranged from 51.5 to 74.6 ng/mL and the values are below MRL established by the European Commission (EC), with the exception of CLP, for which LOQ was recorded as 64.9 ng/mL. For chloramphenicol residues in foods of animal origin such as milk, meat and eggs, EC has established a mimimum required performance level (MRPL) of 0.3 ng/mL to ensure consumers safety (European Commission 2002). The optimized method is not suitable for detection of CLP residues in milk below MRPL.

The precision of the method was evaluated as intra-assay and inter-assay and expressed as % RSD of peak area measurements. The intra-assay precision was determined by analysis of five blank milk samples in one single day spiked at a single concentration of 200 ng/mL. The RSD for studied antimicrobials was in the range of 3.5–6.2%. The inter-assay precision was determined by analysis over five consecutive days of blank milk samples at three fortification levels; 100, 200 and 300 ng/mL. The RSD for targeted antimicrobials was in the range of 4.1–16.2%. According to the European Commission Decision 2002/657/EC (European Commission 2002), the intra-assay precision and inter-assay precision should be lower than 15% and 23%, respectively, and the observed values are in agreement with the EU guidelines.

**Fig. 3** Chromatograms: **a** Blank milk sample, detection λ: 254 nm; **b** Blank milk sample fortified with SDZ, SMX and COL, detection λ: 254 nm; **c** Blank milk sample, detection λ: 355 nm; **d** Blank milk sample fortified with OTC, TC, DOX and COL detection λ: 355 nm and 254 nm; **e** Blank milk sample, detection λ: 311 nm; **f** Blank milk sample fortified with ENRO, CLP and COL, detection λ: 311 and 254 nm. Fortification levels: 100 ng/mL of SDZ, SMX, OTC, DOX, TC, ENRO and CLP and 300 ng/mL of COL (internal standard)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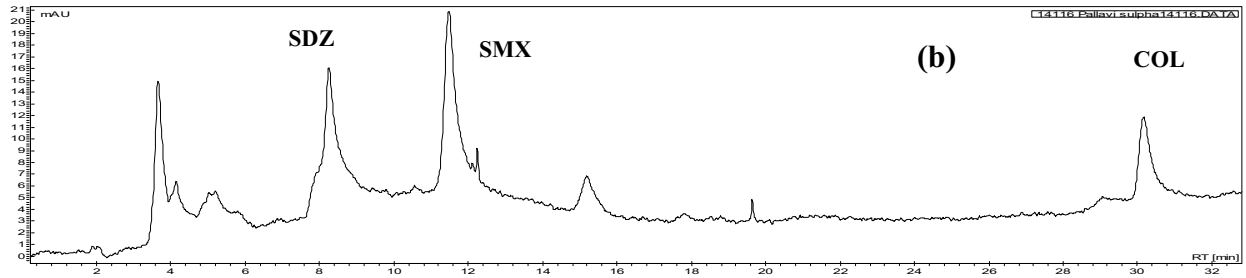
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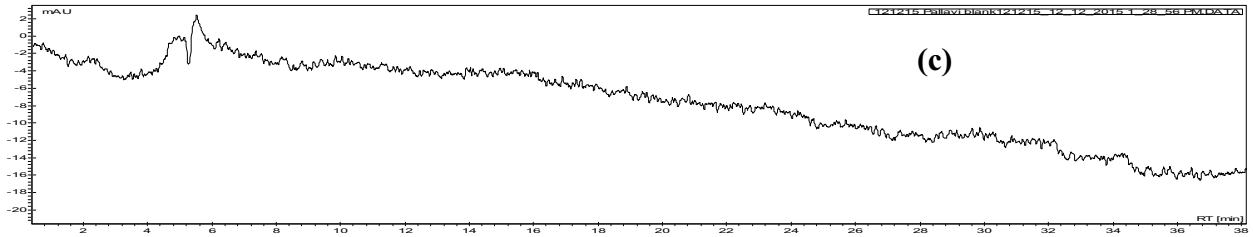
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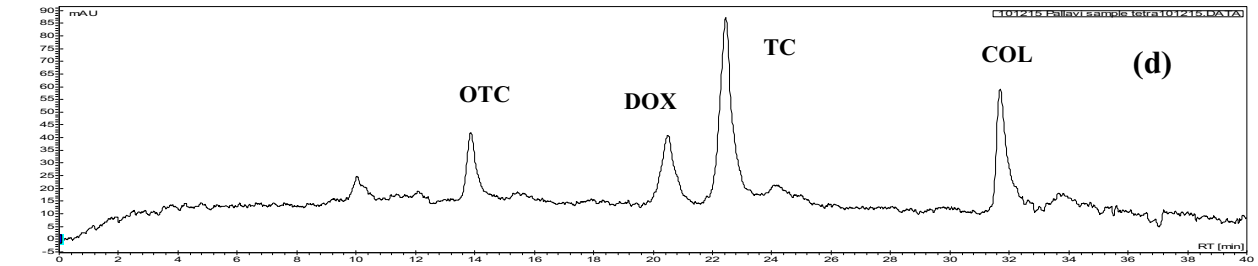
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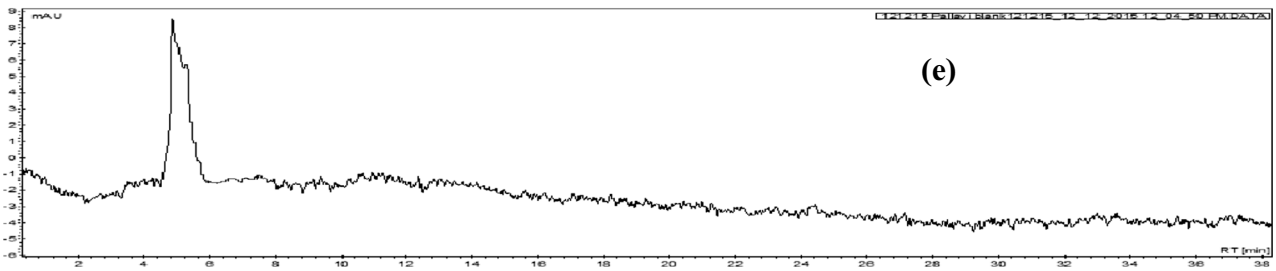
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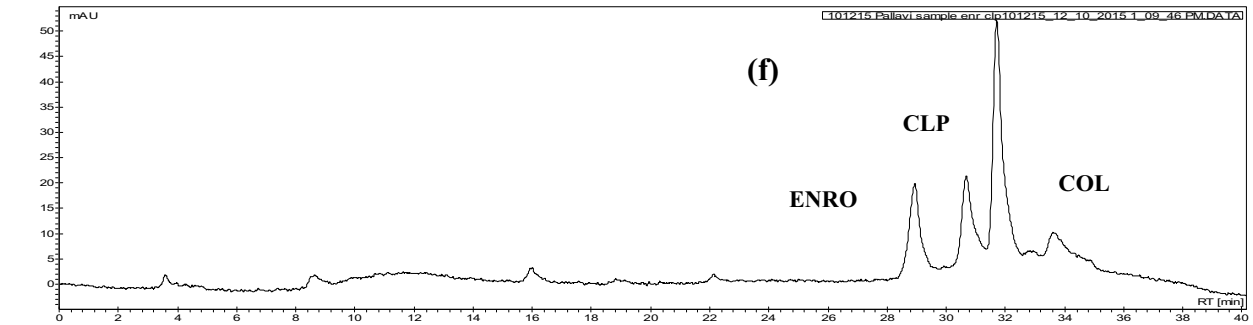
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CLP
ENRO
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**Table 1** Validation parameters for HPLC-DAD method optimized for the determination of SDZ, SMX, OTC, DOX, TC, ENRO, and CLP in milk

| Parameters | SDZ | SMX | OTC | DOX | TC | ENRO | CLP |
|---|---|---|---|---|---|---|---|
| Linear range (ng/mL) | 50–500 | 50–500 | 50–500 | 50–500 | 50–500 | 50–500 | 50–500 |
| Linearity ($r$) | 0.998 | 0.998 | 0.999 | 0.998 | 0.998 | 0.998 | 0.998 |
| Sensitivity (ng/mL) | 0.0088 | 0.0098 | 0.0078 | 0.0064 | 0.0084 | 0.0125 | 0.0069 |
| LOD (ng/mL) | 22.4 | 20.2 | 17.2 | 24.9 | 21.0 | 18.9 | 21.4 |
| LOQ (ng/mL) | 68.1 | 61.2 | 51.5 | 74.6 | 63.7 | 57.3 | 64.9 |
| Intra-assay precision, $n = 5$ (%RSD) 200 ng/mL | 4.0 | 3.5 | 6.2 | 6.2 | 4.7 | 4.1 | 6.1 |
| Inter-assay precision, $n = 5$ (%RSD) | | | | | | | |
| 100 ng/mL | 12.0 | 11.7 | 16.2 | 14.6 | 6.7 | 8.3 | 14.6 |
| 200 ng/mL | 6.8 | 9.2 | 13.2 | 10.8 | 9.2 | 5.7 | 11.1 |
| 300 ng/mL | 7.0 | 7.9 | 9.6 | 8.6 | 7.4 | 4.1 | 8.8 |
| Accuracy, $n = 5$ (%recovery) | | | | | | | |
| 100 ng/mL | 87.0 | 89.1 | 102.4 | 83.3 | 90.2 | 86.0 | 88.8 |
| 200 ng/mL | 107.2 | 106.7 | 105.6 | 93.5 | 101.3 | 110.5 | 95.8 |
| 300 ng/mL | 104.8 | 111.8 | 99.2 | 87.2 | 108.1 | 105.6 | 105.8 |

The accuracy of the method was determined by triplicate analysis of spiked milk samples at three fortification levels (100, 200 and 300 ng/mL). The recovery for studied antimicrobials ranged from 83 to 112%. The results are presented in Table 1. The recovery values are in accordance with the EU guidelines, which established a range of 80–120% for these concentration levels (European Commission 2002).

### Analysis of Milk Samples

In order to verify the performance of proposed method, 21 samples of pooled raw milk procured from randomly selected dairy farms located in adjoining villages of Ludhiana city, Punjab, India, were analysed for the presence of targeted antibiotics. Analyses showed that the milk samples were positive for oxytetracycline and enrofloxacin residues with levels below the LOQ of the method for these antibiotics and thus below the established MRL values (European Commission 2002). The purpose of conducting analysis of the milk samples is to corroborate the performance of the method and not to perform quality control testing.

## Conclusions

A high-performance liquid chromatographic method with photo diode array detection was presented for the simultaneous extraction, identification and quantification of SDZ, SMX, TC, OTC, DOX, ENRO and CLP in bovine milk. The method satisfies all the performance criteria included in the European Commission Decision 2002/657/EC for monitoring of residues of these antimicrobials in milk. For CLP residues, for which MRPL is 0.3 ng/mL, the proposed method is not adequate for monitoring this antimicrobial in milk for quality control programs.

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### Compliance with Ethical Standards

**Conflict of Interest** Pallavi Moudgil declares that she has no conflict of interest. Jasbir Singh Bedi declares that he has no conflict of interest. Rabinder Singh Aulakh declares that he has no conflict of interest. Jatinder Paul Singh Gill declares that he has no conflict of interest. Amit Kumar declares that he has no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals.

**Informed Consent** Not applicable.

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