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Simple Multiresidue Method for Monitoring of Trimethoprim and Sulfonamide Residues in Buffalo Meat by High-Performance Liquid Chromatography

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A simple, specific, and rapid analytical method for the determination of trimethoprim (TMP) and three sulfonamide (SA) antimicrobial drug residues in buffalo meat is developed and validated. This method is based on a solid-phase extraction technique followed by high-performance liquid chromatography (HPLC)–photodiode array (PDA) detection. Target compounds were extracted from the meat by acetonitrile and water, cleaned up on a Bond Elute C_{18} cartridge column, and separated on a RP-C_{18} column during HPLC analysis. Acetonitrile along with water appears to be an excellent extractant as recovery of the analytes at maximum residues levels (MRLs) in spiked sample was in the range of 75–108%, with coefficient of variations (CVs) ranging between 1.34 and 22%. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.031 and 0.062 μg/g, respectively, for all of the compounds. Intra- and interday assay precisions of the method at 0.125 μg/g concentrations for any drug ranged between 3 and 4%. The linearities of the TMP, sulfadimidine (SDM), sulfadoxine (SDO), and sulfamethoxazole (SMX) were 0.9989, 0.9999, 0.9998, and 0.9997, respectively. For robustness, the analytical method was applied to 122 buffalo meat samples obtained from export meat processing plants.

KEYWORDS: Trimethoprim; sulfonamides; solid-phase extraction; buffalo meat; residues; liquid chromatography

INTRODUCTION

Sulfonamides (SAs) are chemotherapeutics most commonly used in veterinary practices, because of their inexpensiveness and wide-spectrum antimicrobial activity. SAs are used in combination with trimethoprim (TMP) in a concentration ratio of 5:1, and these combinations are commonly known as potentiated sulfonamides. SAs act by competing with bacterial *p*-aminobenzoic acid in the enzymatic synthesis of dehydrofolic acid, whereas TMP, which is a structural analogue of the pteridine dihydrofolic acid, is a competitive inhibitor of the enzyme dihydrofolate reductase. This combination of SAs and TMP exerts bactericidal effects by decreasing the availability of the reduced folates that are essential in the synthesis of nucleic acids in the bacteria (*1–3*). However, the presence of residues of TMP and SAs in meat is of toxicological and regulatory concern as some of them could be carcinogenic and cause allergic hypersensitivity reactions and therapeutic ineffectiveness in human beings (*4*). Therefore, in recent years, both legislators and consumers have shown increased interest in the safety of food products. Events such as the appearance of drug residues in food of animal origin have impelled governments in the United States, the European Union, Japan, India, and many other developed and developing countries in the world to set up monitoring programs (*5*). The Codex Alimentarius Commission (CAC) set maximum residue limits (MRLs) of 0.05 and 0.10 μg/g for TMP and SAs in cattle meat, respectively (*6*). Therefore, the analytical method for monitoring of SA residues in meat is required to be simple, rapid, precise, inexpensive, and capable of detecting residues below the MRL.

Many analytical methods have been developed for the determination of TMP and SAs in animal tissues (*5, 7–14, 16–26*). Earlier methods for measuring the total content of SAs in meat are colorimetric techniques using Bratton–Marshall reagent, which were found to be unsuitable due to lack of sensitivity and selectivity (*7*). Other analytical methods were mainly based on bioassay, TLC, GC, and HPLC (*8–10*). Although various GC and GC-MS methods have been developed, they required the polar SAs to be chemically derivatized due to their low volatility, and to overcome matrix interference, exhaustive cleanup was required (*11*). Indeed, high-performance liquid chromatography (HPLC), as an analytical technique, has been

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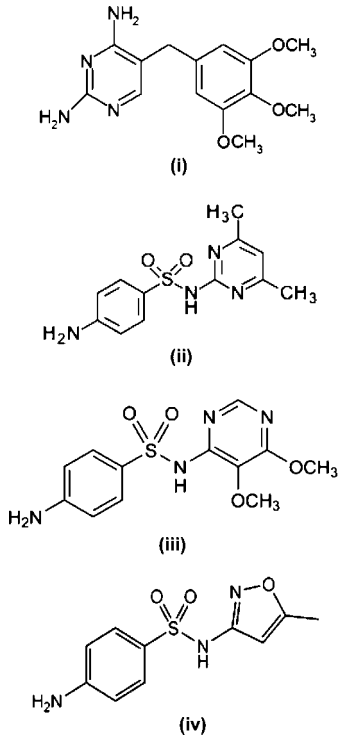

Figure 1. Chemical structures of trimethoprim (i), sulfadimidine (ii), sulfadoxine (iii). and sulfamethoxazole (iv).

given much attention in this field. HPLC with photodiode array (PDA) detection has been successfully applied to determine several SA residues in animal tissues (*16*, *17*). Several pretreatment methods, such as solid-phase extraction (SPE) (*12*), liquid–liquid extraction (LLE) (*9*), and matrix solid-phase dispersion (MSPD) (*20*), have been applied for the extraction of SA antimicrobials from meat and milk. However, all of the methods could resolve the problem in the same way as GC and GC-MS methods, through a complex extraction and cleanup procedures, or required large volumes of sample and solvent or the use of toxic solvents such as chloroform, dichloromethane, and *n*-hexane. Although at present many confirmatory LC-MS-MS methods are quoted in the literature (*5*, *12*, *13*), the cost involved in the required instrumentation is high. As in animal tissues, various interfering substances exist and residues of SAs are very low in quantity, which increases the need for the development of low-cost but precise analytical methods that are capable of rapidly assaying the presence of residual drugs in foods.

The present study describes the simultaneous determination of residues of TMP and three SA (sulfadimidine, sulfadoxine, and sulfamethoxazole) antimicrobial (**Figure 1**) residues in buffalo meat; these drugs are commonly used in veterinary practice throughout the world. A Bond-Elute C_{18} cartridge column was used for cleanup of sample extract as this has been successfully used in the analysis of drug residues in biological samples (*14*). This method required only small amounts of toxic solvents.

MATERIALS AND METHODS

Chemicals and Reagents. Pure standards of trimethoprim (TMP, 99.5%), sulfadimidine (SDM, 99.7%), sulfadoxine (SDO, 99.8%), and sulfamethoxazole (SMX, 99.8%) were obtained from Sigma-Aldrich. A Bond-Elute C_{18} cartridge column was procured from Supelco Co. All of the solvents and chemicals used in this study were of HPLC grade and were procured from E. Merck and Rankem, India. For HPLC grade water, water was obtained by using a Milli-Q water purification system (Milli-Q gradient, Millipore).

Table 1. Recoveries ($n = 5$) of Trimethoprim and Sulfonamides Spiked into Buffalo Meat Samples

	recovery (CV %)		
compound	low (0.03 μg/g)	medium (0.12 μg/g)	high (1.0 μg/g)
TMP[a]	77.47 (22.33)	79.67 (11.35)	88.46 (6.11)
SDM	81.93 (12.32)	94.08 (3.52)	103.08 (1.34)
SDO	75.0 (11.95)	93.76 (11.64)	107.72 (4.22)
SMX	91.61 (12.62)	96.64 (4.96)	102.40 (2.74)

[a] The low, medium, and high concentrations for TMP were 0.03, 0.25, and 2.0 μg/g, respectively.

Standard Preparation. The standard stock solutions at 1 mg/mL free base concentration of each standard were prepared by dissolving pure standards in HPLC grade acetonitrile, and solutions were maintained at 4 °C. Composite working standard solutions of 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.62, and 0.3 μg/mL of each drug were prepared daily in Milli-Q water and were used to spike the blank meat sample.

Sample Collection. A total of 122 buffalo meat samples composed of 92 Longissimus dorsi (LD) and 30 silver sides (SS) were collected from four different export meat processing plants located across the country. Samples were collected over a 12 month period. The samples were collected from the deboning table where the chilled carcasses were cut, deboned, trimmed, and packed. About 200 g of the sample was cut aseptically from LD or SS randomly at different periods of deboning operations and transferred to self-sealing colorless polyethylene bags. The bags were labeled and deep-frozen and brought to the laboratory under frozen conditions in a foam box containing chiller packs. Both types of samples were stored at −20 °C before analysis, separately.

Sample Preparation, Extraction, and Cleanup. Frozen meat samples were thawed overnight in a refrigerator. The muscle samples (100–150 g) were diced into small pieces after being trimmed of external fat and fascia and then blended in a high-speed (15000 rpm) tissue blender (York Scientific Industries Pvt. Ltd., New Delhi, India; S. no. 293) for 2 min. Ten grams of blended tissue sample was taken into a 100 mL polypropylene centrifuge tube, and 10 mL of Milli-Q water was added; the mixture was homogenized for 1.5 min using an Ultra-Turrex T_{25} tissue homogenizer (Janke and Kenkel, IKA, Labor Technik).

For extraction, 0.5 g of meat homogenate was accurately weighed in a glass test tube by dispensing homogenate with the help of a micropipet of 500−5000 μL capacity. Then 1 mL of acetonitrile was added to it, and the tube was held for 10 min at room temperature (27 ± 1 °C), vortexed at high speed for 10 min, and finally centrifuged (Biofuge, Heraeus) at 3500 rpm for 10 min. Supernatant was collected into a separate test tube, and the residues were re-extracted with 1 mL of an acetonitrile/water mixture (1:1) followed by centrifugation once again as above. Both of the supernatants were pooled together and then passed through a Bond-Elute C_{18} cartridge column preconditioned with 5 mL of HPLC water followed by vacuum drying. After completion of extract loading, SA compounds were eluted with 3 mL of acetonitrile in a graduated tube at the flow rate of 1 mL/min and then evaporated under a gentle stream of nitrogen at 40 °C. The residue was dissolved with 500 μL of an acetonitrile/water mixture (1:1) and filtered through a 0.22 μm nylon membrane filter; 20 μL of the aliquot was injected into the HPLC system.

HPLC-PDA Conditions. For the speciation analysis of SA compounds, a high-performance liquid chromatograph (Shimadzu Corp., Kyoto, Japan) composed of an LC-10 AT quaternary gradient pump, a Rheodyne manual loop injector with a 20 μL loop, a column oven CTO-10AS vp, and a PDA detector was employed. Separation of TMP, SDM, SDO, and SMX was achieved using a reverse phase octyldecylsilane C_{18} (RP-C_{18}) stainless steel column; 250 × 4.6 mm i.d., 5 μm particle size, 100 Å pore size (Phenomenex, Torrence, CA) with matching guard column as stationary phase and a mixture of 50 mM ammonium acetate buffer (pH 4.65)/acetonitrile (73:27 v/v) as mobile phase. The eluent was monitored at a wavelength of 265 nm with a flow rate of 0.5 mL/min at a column oven temperature of 40 °C. The data collected were analyzed with class-vp 6.12 version software, taking into account the peak heights of analytes.

Table 2. Precision Data on Analysis of Trimethoprim and Sulfonamide Drugs in Buffalo Meat Samples at Different Concentrations

	intraday precision (CV%, $n = 5$)			interday precision (CV%, $n = 5$)		
compound	low (0.03 μg/g)	medium (0.12 μg/g)	high (1.0μg/g)	low (0.03 μg/g)	medium (0.12 μg/g)	high (1.0 μg/g)
TMP[a]	10.94	7.39	6.11	16.48	13.83	8.09
SDM	8.54	3.42	2.62	12.33	3.59	2.89
SDO	9.98	5.29	3.93	11.79	10.20	4.24
SMX	10.26	6.59	2.03	11.29	6.80	2.84

[a] The low, medium, and high concentration for TMP were 0.031, 0.25, and 2.0 μg/g, respectively.

Table 3. Linear Regression Data for Trimethoprim and Sulfonamide Drugs in Buffalo Meat Samples[a]

		regression line				
compound	linear dynamic range (μg/g)	slope	intercept	r^2 value	LOD (μg/g)	LOQ (μg/g)
TMP[a]	0.031–2.0	−0.0094	1.5366	0.9989	0.031	0.062
SDM	0.031–2.0	−0.0035	0.3429	0.9999	0.031	0.062
SDO	0.031–2.0	0.0023	0.5855	0.9998	0.031	0.062
SMX	0.031–2.0	0.0045	0.4305	0.9997	0.031	0.062

[a] Number of data point-7.

Fortification of Blanks and Preparation of Calibration Curves. Blank homogenates of buffalo meat were prepared as described above. A composite working standard containing 320 μg/mL each of TMP, SDM, SDO, and SMX was prepared from the 1 mg/mL stock solutions kept at 4 °C. From this working standard different dilutions were made to spike the homogenates. Blank homogenates of 0.5 g were spiked with working standards to obtain final concentrations 2.0, 1.0, 0.5, 0.25, 0.125, 0.062, and 0.031 μg/g of TMP, SDM, SDO, and SMX and extracted as described previously and injected into the HPLC system. Calibration curves were plotted by taking peak height to the respective concentrations. These curves were used to quantify the residues of TMP, SDM, SDO, and SMX in the buffalo meat samples analyzed.

Analytical Recovery and Precision. Analytical recoveries were determined by spiking TMP, SDM, SDO, and SMX to blank meat homogenates to yield concentrations of 0.031, 0.25, and 2.0 μg/g for TMP, and 0.031, 0.125, and 1.0 μg/g for SDM, SDO, and SMX and then analyzed. The amount of drug found by the assay method for each concentration was estimated using a linear regression equation after calibration of standard curves considering peak heights. Three determinants were made for each concentration, and the percent recovery was calculated. Both intra- and interday assay precisions were determined by analyzing three spiked concentrations of 0.031, 0.125, and 1.0 μg/g, five sets each with blank. However, intraday assay precision was determined at three occasions at least 6 h apart, whereas interday precision was determined at least 24 h apart for three successive days. The lowest concentrations of TMP and SA standards routinely used were 0.05 and 0.10 μg/g, respectively. The minimal detection limits for all drugs were 0.03 μg/g.

Limits of Detection (LOD) and Limits of Quantification (LOQ). LOD and LOQ were determined as described (*24*). A most widely acceptable definition was adopted by comparing the signal-to-noise ratio to the blank samples and for the minimum concentration of analyte spiked, which can be reliably detected. A signal-to-noise ratio of 3:1 is considered while determining LOD. Furthermore, the quantitation limit is determined by analysis of concentrations of each spiked analyte in homogenates and by establishing the minimum level at which the analyte is quantified with acceptable accuracy and precision (i.e., CV < 20%). The summarized precision data are presented in **Tables 2** and **3**.

Statistics. The recovery and precision data were evaluated with an in-house statistical software program making use of robust statistic concepts of Snedecor and Cochran (*15*).

RESULTS AND DISCUSSION

In the present study, an attempt has been made to determine simultaneous detection of residues of trimethoprim and sulfonamides by HPLC-PDA detector in buffalo meat after simple solid-phase extraction. This method does not involve large volumes of organic solvent or cumbersome procedures of cleanup, and the method is sensitive enough to determine TMP and SA levels below the MRL.

Optimization of HPLC Conditions. In this study, a PDA detector was used as a tool to optimize the wavelength in the detection of TMP and three other SA drugs. The UV spectrum of the majority of veterinary drugs lies between 200 and 400 nm (*17*), but the absorbance maximum of TMP was at 230 nm, whereas for SAs it was at 270 nm (*16*, *17*). Increasing the detection wavelength decreased the absorbance intensity of TMP. On the other hand, below the wavelength of 230 nm matrix interference was increased. However, UV maximum spectra at 265 nm with a scanning range of 240–290 nm were selected because the matrix interference was minimal below the MRL. For separation of compounds, an isocratic elution profile was used. It has been observed that a small change in the pH of the mobile phase in the vicinity of pK_a values of sulfa drugs resulted in larger changes of its retention time. Indeed, an initial attempt was made to separate the compounds with a mobile phase of 0.05 M sodium dihydrogen phosphate buffer/acetonitrile (72:28), but the mixing ratio of the above two solvents could significantly affect the analysis of TMP (*18*, *19*). When trying to separate SAs by a short chromatographic run, we observed marked ion signal weakening for TMP. A large amount of unseen polar coextractives eluted in the first part of the chromatogram was considered to be responsible to this effect. In another study, 25 mM ammonium acetate buffer (pH 5.0)/acetonitrile/methanol (65:23:12) was used as a mobile phase (*16*), but the pH of this mobile phase was slightly unstable in the same working day, which is evidenced by shifting of the chromatographic peaks from their initial retention times. This unusual effect might be due to low ionic strength coupled with buffer pH (5.0), as ammonium acetate buffer is more stable at its pK_a value of 4.8 (*19*). Finally, 50 mM ammonium acetate buffer (pH 4.6)/acetonitrile (73:27) was fixed as mobile phase with a flow rate of 0.5 mL/min. A mobile phase of this flow rate improves the efficiency of separation of compounds compared with those at a higher flow rate of 0.9 mL/min (*19*). However, retention time selectivity of the analyte was affected sharply when the pH of the mobile phase was changed, as the pK_a values of sulfa drugs are within 5.5–7.5.

Despite a slightly lower sensitivity, ammonium acetate buffer (pH 4.65) was chosen over sodium dihydrogen phosphate buffer (pH 5.0) in the mobile phase because it allowed TMP to improve

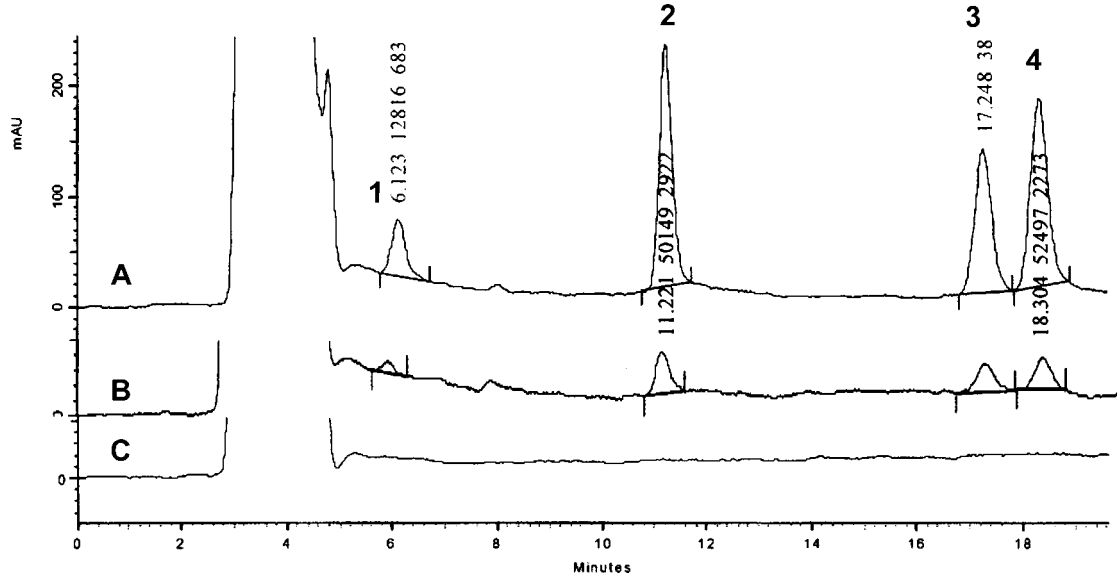

Figure 2. HPLC chromatograms of four sulfonamide antimicrobials obtained from spiked (**A** and **B**) and blank (**C**) buffalo meat homogenate. Samples were spiked at 1.0 μg/g (**A**) and 0.125 μg/g (**B**) concentrations. Peaks: 1, trimethoprime; 2, sulfadimidine; 3, sulfadoxine; 4, sulfamethoxazole.

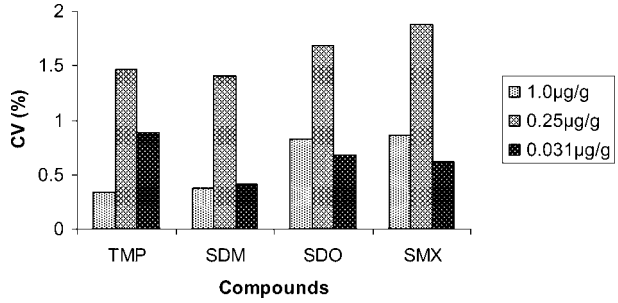

Figure 3. Changes in coefficient of variation (CV %) of retention time (RT) at different concentrations.

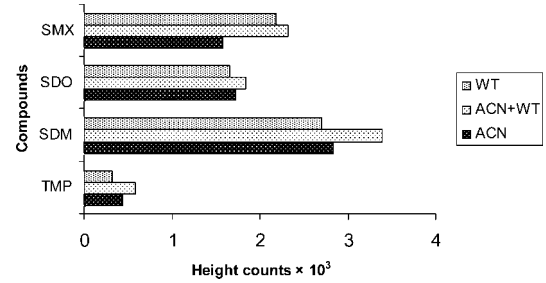

Figure 4. Effects of different solvents on the extraction efficiency of sulfonamide drugs.

chromatographic separation. The investigated SAs are ampholytes with weakly basic or acid characteristics: the weakly basic characteristics arise from the nitrogen of aniline substituent, designated to protonation for chromatographic detection, whereas the acidic characteristics arise from the N–H linkage of the sulfoamide group. Because of marked acidic properties, chromatographic separation was performed in ion suppression by adding acetic acid to the mobile phase. Under good separation conditions, which are adopted in this work, there is little matrix effect, and interestingly this behavior is nearly similar to that observed when SAs from extracts of poultry blood serum and animal tissues are injected (*16*, *23*). It has been observed that at the pH of 4.65, TMP was eluted significantly earlier than other drugs with a retention time of 5.8 min. SDM and SDO followed TMP in the elution profile with retention times of approximately 11 and 17 min, respectively. SMX eluted last with a retention time of 18.4 min. **Figure 2** represents a chromatogram of a sample spiked with four sulfonamides at 0.125 μg/g (near the MRL concentration). The total ion current peaks obtained for each compound were well resolved from each other, and the sample matrix makes a minimal contribution to the chromatographic background. TMP, SDM, SDO, and SMX remained well resolved from each other when the pH of the mobile phase was near 4.6. Hence, a mobile phase of 50 mM ammonium acetate/acetonitrile was satisfactory for the separation of all four drugs. The coefficients of variation (CV %) at retention time (RT) for all drugs at 0.03 μg/g were found to be 1.09, 0.28, 0.68, and 0.62, respectively (**Figure 3**); they did not vary significantly.

Sample Extraction and Cleanup. The well-documented solvent acetonitrile was found to be suitable for the extraction of SA compounds. Acetonitrile has been used for the extraction of SA and TMP residues by other authors as well (*11*, *17*, *19*, *23*). This solvent was found to have advantages over a number of organic solvents such as methanol, acetone, ethyl acetate, acetonitrile/metaphosphoric acid, and methanol/metaphosphoric acid in terms of deproteinization (removal of >99% of protein and fat), recovery, chromatographic interference, and emulsion formation (*19*). Similarly, other researchers also reported that acetonitrile is the best solvent for extraction because it is easy to evaporate and the lipid/oil interference could readily be removed from the extract solution by introducing *n*-hexane (*11*). The only disadvantage of this solvent was its binding with highly polar compounds from the sample matrices, which ultimately come first in the reverse phase chromatography (*17*). To avoid the unwanted effect of *n*-hexane, acetonitrile, acetonitrile/water (1:1), and water were chosen for extraction of the SA compounds with the corresponding pH values of 9.0, 8.0 and 7.0, respectively. Higher extraction efficiency for TMP and all SAs was obtained (**Figure 4**) when an acetonitrile/water mixture (1:1) was used (pH 8.0), and an obvious decrease was found when either acetonitrile (pH 9.0, pH determined with digital pH meter) or water (pH 7.0) only was used. As the pK_a values of all SA drugs are within the range of 5.5–7.5, they might be completely extracted either by one log reduction or by increasing the solvent pH. However, at a high pH of 9.0 (only acetonitrile extraction) more polar compounds bound with the extraction solution and it came first in the chromatogram, whereas pH 7.0 (water only extraction) was not sufficient to extract the TMP compounds

from the spiked samples. Therefore, a two-step extraction, that is, acetonitrile followed by acetonitrile/water (1:1), method was adopted for effective recovery of the drugs from the sample matrices.

The matrix interference can be removed satisfactorily by adequate cleanup of the crude extract before any chromatographic determination (*11*). In our study, a Bond-Elute C_{18} cartridge column was used for cleanup of the sample extract. Furthermore, use of a precolumn (guard column) was made as an additional effort to remove remaining matrix interferences from the sample after acetonitrile and acetonitrile/water extraction (*18*). As SAs are ampholytic (with slightly acidic or basic characteristics) in nature, addition of acetonitrile and Milli-Q water (1:1) in a second extraction increased polarity and thereby recovery of compounds. Recently, matrix-solid-phase dispersion techniques with hot water as extractant were also reported (*3*, *20*–*22*). The sample extraction procedure attempted in this study was rapid and simple, and no complex extraction and cleanup procedures were involved.

Validation of Analytical Methodology. The analytical method was validated by evaluating percent recovery, precision, linear dynamic range, sensitivity, LODs, and LOQs of the analytes. In recovery and precision studies, the efficiency of acetonitrile and water in extracting SAs from meat homogenate was assessed at three spike levels of 0.03, 0.125, and 1.0 μg/g. These levels correspond, respectively to one-third of the maximum residue limit (MRL), near the MRL (0.10 μg/g), and 10 times the MRL set by the CAC and EU for SAs, whereas in the same tissue, levels for TMP were 0.031, 0.25, and 2.0 μg/g, which correspond to half the MRL, 5 times the MRL, and 40 times the MRL, respectively. At each concentration five measurements were performed considering peak height integration (*19*, *25*, *26*), and data are given in the statistical summary (**Table 1**). The average recoveries of all SAs were in the range of 93.76–96.64% with CVs ranging between 3.5 and 11.6% at the MRL level (0.10 μg/g). However, absolute recoveries for TMP, SDM, SDO, and SMX were 73.81, 87.76, 83.94, and 90.66%, respectively, at this concentration. The average recovery value for TMP was 77.47% with a CV of 22% at 0.03 μg/g concentration.

The reproducibility of the developed method was determined by the intra- and interday assay precisions. The intra- and interday precisions (CV %) were investigated at low, medium, and high concentrations, respectively, according to calibration curve ranges. The results of intra- and interday assay precisions for the multiresidue method of TMP and three other sulfa drugs in tissue sample are shown in **Table 2**. Blank determinants were performed to ensure that no interference from background peak could be observed on the chromatogram. A total of 15 independent determinations were conducted for each concentration that was spiked into tissue samples. Results from **Table 2** indicate that the method was sensitive and reproducible. However, variable CVs were obtained for both precisions for all compounds. The extent of this unwelcome effect is related to both concentrations and affinities of protonated sulfa drugs for coextracted and coeluted matrix components (*19*).The linearity (r^2) values for TMP, SDM, SDO, and SMX were 0.9989, 0.9999, 0.9998, and 0.9997, respectively (**Table 3**). The LOD and LOQ for TMP, SDM, SDO, and SMX are presented in **Table 3**. It is evident from the results that LODs and LOQs of the method were well below the MRLs set by the EU, CAC, or U.S. FDA for residues of TMP and sulfa drugs in bovine tissue sample, and the analytical method is apt and sensitive enough to carry out residue analysis for TMP, SDM, SDO, and SMX in buffalo meat.

However, results of the survey samples, with regard to LD and SS revealed that no sample contained residues of either TMP or SAs.

Conclusions. HPLC coupled with photodiode array detection and acetonitrile/water as the extraction medium was successfully employed for the simple and rapid determination of TMP and SA antibacterial residues in meat. In comparison to the pretreatment methods mentioned previously, the proposed HPLC method is environmentally friendly and inexpensive and easily performed. In addition, simultaneous analysis was accomplished with high sensitivity. Therefore the proposed method will be useful and practical in future residue monitoring of TMP, SDM, SDO, and SMX in meat.

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