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Short communication

High performance liquid chromatography determination of sulphachloropyrazine residues in broiler and turkey edible tissues

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ABSTRACT

A HPLC method to determine and quantify sulphachloropyrazine residues from broilers and turkeys is reported. This procedure permitted sulphachloropyrazine to be separated from muscle tissue, liver, kidneys and fat with skin after extraction with dichloromethane under slightly acidic conditions. The analytical methodology showed a high specificity and sensitivity and an adequate precision and accuracy with a limit of quantification of 56 ng mL^{-1}. The peak area showed a linear relationship with a concentration over the range 50–750 ng mL^{-1} for sulphachloropyrazine standard solutions. Recovery dates were also satisfactory with values between 69.7 and 77.5%.

1. Introduction

Sulphachloropyrazine (SCP) is synthetic bacteriostatic wide-spectrum antimicrobial, belonging to the group of sulphonamides that are poorly absorbed from GI tract sulphonamides. In the literature we can also find widely used in veterinary practice a similar chemical compound sulphachloropyridazine which is different from SCP in the position of the nitrogen atoms in the ring. Generally sulphonamides are broad-spectrum antimicrobials inhibiting both, Gram-positive and Gram-negative bacteria, as well as some protozoa, such as coccidia.

SCP is usually used to treat collibacteriosis [1], fowl cholera [2] and cocidiosis (*Eimeria sp.*) [3–5] infections in poultry in the daily dose of SCP 0.03% for 3–6 days [6,7].

Residues of sulphachloropyrazine may remain in edible animal tissues and then affect the human health, and this is the reason why many researchers focus on the development of a rapid, accurate, and economical time and cost methods, for the determination of this antibiotic.

Many papers have been published concerning the assay method for sulphonamides but only several about sulphachloropyrazine [8–10]. Most of the manuscripts concern sulphachloropyridazine (SCPD), the structural isomer, very similar to SCP (different chemical structure composition) (Fig. 1a and b) [11,12]. Among them, bioassay and fluorometry, which are commonly used, present lack of sensitivity and specificity, while chromatographic methods are generally preferred for their greater selectivity and simplicity. A number of instrumental techniques have been introduced for the determination of sulphonamide residues, such as thin-layer chromatography [13,14], immuno-enzymatic method [15], supercritical fluid chromatography [16], gas chromatography coupled with mass spectrometry [17,18] and liquid chromatography tandem mass spectrometry [19]. However, the most prominent place among them is occupied by high performance liquid chromatography. The HPLC method is very effective in monitoring veterinary drugs, and that technique has been reported for the determination of sulphonamides concentrations in various biological matrices [20–22]. By this method, sulphonamide residues are determined either directly after the separation on a chromatographic column using a UV detector or indirectly by applying derivatization procedures followed by measurements on a fluorescent detector [8].

The present paper describes a rapid and specific procedure for HPLC determination of the sulphachloropyrazine content in broilers and turkeys edible tissues. The EU MRL's (maximum residue limit [23]) for SCP (for all species and all tissues) is 100 ng g^{-1}. Furthermore there is very poor information in the literature about tissue concentrations of this drug in any species and therefore it is hard to predict the withdrawal time in poultry after they medicated with SCP.

2. Experimental

2.1. Animals

All experiments were carried out on 29 healthy broilers (Ross 308) and 28 turkeys (White Wide-Brest, midi type BUT-9) of both

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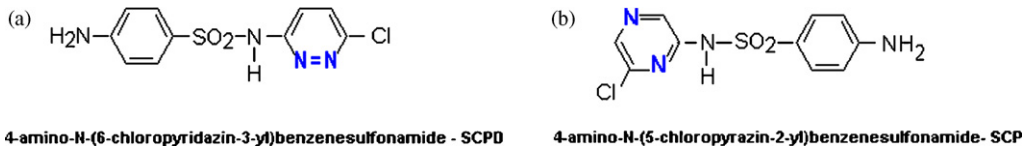


Fig. 1. Chemical structures of (a) sulphachloropyridazine (SCPD) and (b) sulphachloropyrazine.

sexes and with an initial weight of 1600–2560 g and 1720–2600 g in broilers and turkeys, respectively. Broilers were divided into 4 (A, $n=5$; B, $n=5$; C, $n=7$; D, $n=5$) and the turkeys into 5 (A, $n=4$; B, $n=4$; C, $n=4$; D, $n=7$; E, $n=4$) experimental groups. There was also one control group in both, broilers ($n=6$) and turkeys ($n=5$).

Before commencing the study the birds were marked with alphabetical code (broilers—A, B, C, D and turkeys—A, B, C, D, E) which shows each terms of killing and taking of the samples.

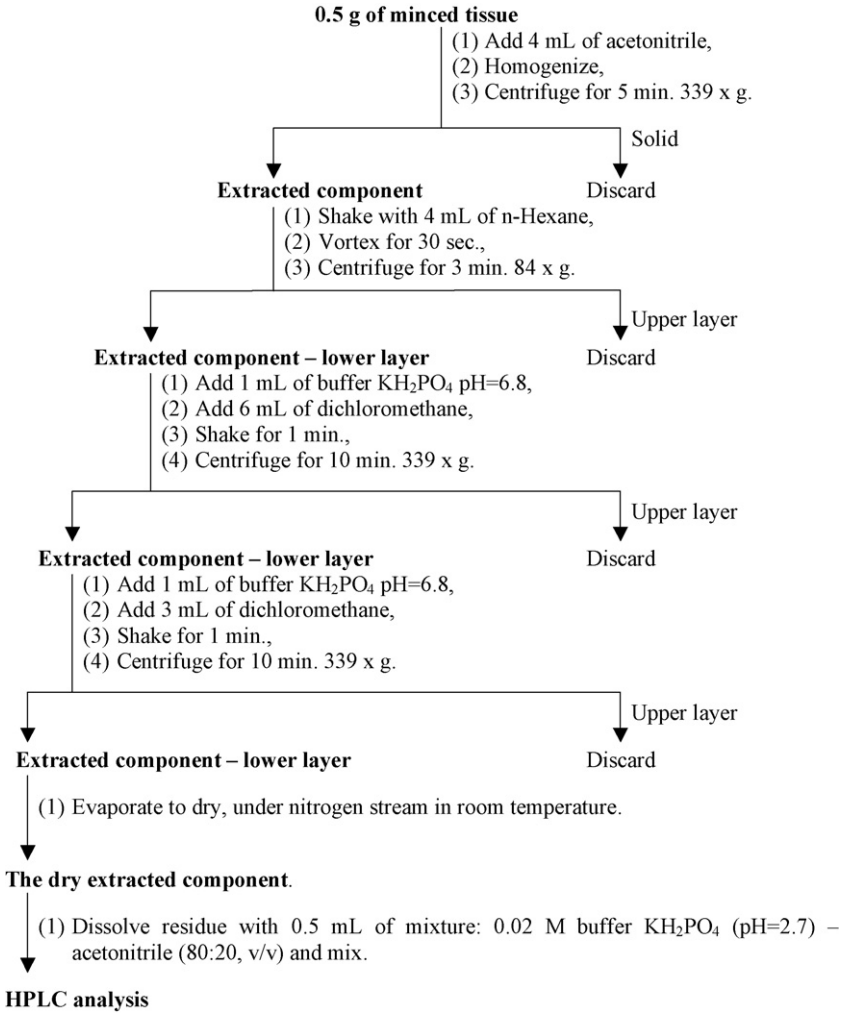
The birds were fed two times a day (at the morning, 1 h before drug administration and at the evening). Water was added *ad libitum* throughout the period of study.

Sulfatyf® was administrated (*ex tempore* water solution) orally at the same to both species, dose 50 mg kg^{-1} b.w. for 3 days continuously. The oral doses were administrated individually through a metal catheter into the crop. Control groups were given pure water by the same method of administration for three days continuously too. Next, 6 broilers and 5 turkeys from control group were humanitary slathered and the marker tissues—muscles (40 g), liver (all), kidneys (all) and skin with fat (40 g) were taken for the recovery of SCP.

The tissue samples were taken after 5, 10, 14, 16 days and 7, 12, 18, 21, 23 days of last drug application, in broilers and turkeys, respectively. Matrices were cleaned of blood and stored at −30 °C until the day of analysis by HPLC.

2.2. Chemicals and reagents

Sulfatyf® (powder 33 g sulphachloropyrazine natrium + Massa pulvis ad 100 g) was purchased from a pharmaceutical company (DZPB "Biowet" S.A., Drwalew, Poland). The drug standard Salt sodium Sulphachloropyrazine was obtained from Yangzhou Tianhe Pharmaceutical Chemical Company (Shuanggou Jiandu, China). HPLC grade acetonitrile, methanol, n-Hexane and other chemicals (potassium dihydrogen phosphate, ortho-phosphoric acid 85%, dichloromethane) were purchased from POCH chemical-company (Gliwice, Poland) at the highest purity available. The deionized



Scheme 1. Schematic diagram of solvent extraction.

Table 1
Precision date on the analysis of three increasing SCP standard concentrations (250, 500 and 750 ng mL^{-1}) on three different days.

Concentration of SCP standard (ng mL^{-1})	Mean concentration found[a] (ng mL^{-1} ±SD)					
	Day 1	RSD (%)	Day 2	RSD (%)	Day 3	RSD (%)
750	745.45 ± 5.70	0.76	750.90 ± 4.55	1.35	749.24 ± 3.39	1.98
500	508.10 ± 6.86	0.61	501.39 ± 15.12	3.00	502.96 ± 12.32	1.23
250	248.12 ± 2.98	0.45	245.86 ± 3.01	2.45	250.14 ± 4.19	1.67

[a] Three replicates.

water (18.2 MΩcm) used for preparing all the aqueous solutions was obtained by the reverse osmosis method with Milli-Q-Plus 185 system (Millipore, Molsheim, France).

2.3. Apparatus

Identification and quantification of analytes were carried out using a Varian liquid chromatography apparatus (Varian, Walnut Creek, CA, USA). It consisted of a solvent delivery pump (STAR 9002), a 50 μL volume manual injector and a variable wavelength UV–vis 9050 detector (all Varian Analitycal Instruments, USA). The samples and standards were analyzed using a Synergi 4 μ Fusion–RP (150 mm × 4.6 mm) (Phenomenex) column.

An analitycal balance (Sartorius BP 61S), Vortex (WL-1, Bio-mix, Warsaw, Poland), a centrifuge (Sigma 2-16), and Milli Q Plus 185 system (Milipore-Waters) to produce deionized water were also employed.

2.4. Chromatographic conditions

The sulfa drug under investigation is separated on a Synergi 4 μ Fusion–RP column by isocratic elution using a mobile phase that consisted of 0.02 M KH_2PO_4 pH 2.7 – ACN (80:20; v/v) at a constant flow rate of 1.7 mL min^{-1}. The standards of sulphachloropyrazine and the tissue extracts were monitored at a wavelength of λ = 270 nm. All analyses were performed at ambient temperature. The retention time of sulphachloropyrazine was about 13 min, similar to those in other studies [8]. The chromatogram run time was 15 min.

2.5. Experimental procedure

Frozen tissue samples were thawed to room temperature prior to extraction. 0.5 g of test minced tissue (muscle, liver, kidney, fat + skin) was transferred into a test-tube and mixed with 4 mL of acetonitrile. After homogenization and centrifugation for 5 min at 339 × *g*, 3.5 mL of the upper supernatant layer was transferred into a clean tube and for mixed 30 s in Vortex with 4 mL of hexane. After second centrifugation for 3 min at 84 × *g*, the upper hexane layer was discarded and to the lower layer, 1 mL of KH_2PO_4 (pH 6.8) with 6 mL of dichloromethane was added and next shaken for 1 min. After centrifugation for 10 min at 339 × *g*, the second liquid–liquid extraction with 3 mL of dichloromethane and the same amount of buffer was made. The upper layer was discarded and the lower layer was evaporated, under the stream of nitrogen in the room temperature. The dry extract was dissolved with a mixture of 0.02 M buffer KH_2PO_4 pH 2.7 – ACN (80:20; v/v) and then mixed. A 25 μL volume of elute was injected into the HPLC system.

The extraction of SCP from the broiler and turkey tissues was performed according to Scheme 1.

2.6. Standards

A stock solution (100 μg mL^{-1}) of sulphachloropyrazine sodium salt was prepared by dissolving 10 mg of compound in 100 mL of methanol. Working solutions (1000, 750, 500, 250, 150, 50 ng mL^{-1}) were prepared by appropriate serial dilution of the stock solution with a mixture: 0.02 M phosphate buffer KH_2PO_4 pH 2.70—acetonitryl (90:10; v/v). These solutions were then injected in order to obtain the calibration curve.

2.7. Accuracy/recovery

The accuracy of the method was determined, by the recovery of SCP from the all control tissue samples of broilers and turkeys (muscle, liver, kidney, skin with fat) spiked at 100 ng g^{-1}. This spiked level was prepared by adding 50 μL of standard solution of SCP (1000 ng mL^{-1}) to 0.5 g portions of the sample. The extraction of SCP was made in accordance with Section 2.5.

3. Results and discussion

3.1. Calibration, precision and recovery

The calibration graph was obtained by plotting peak area against amount and was linear over the range 50–750 ng. The equation for the calibration curve is $y = 41.31x + 489.57$ and the correlation coefficient equals 0.9997. The linear range experiments provided the necessary information to estimate the LOD and the LOQ limits based on the peak of lowest concentration in the linear range with a signal-to-noise ratio, S/N of 3.3 for limit of detection and 10 for limit of quantification. The limit of detection (LOD = $3 \cdot S_{xy}/a$, where S_{xy} is the standard deviation and a is the slope of the calibration curve) is the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified. The limit of quantification (LOQ = $10 \cdot S_{xy}/a$) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. LOD value is situated in the linearity range. The estimated values of LOD and LOQ were 18.40 ng and 55.70 ng, respectively. Moreover, the precision of the procedure was obtained from relative standard deviation (RSD%) of areas calculated for three replicate injections of three increasing SCP standard concentrations (250, 500, 750 ng). The investigations were made on three various days. The results of the assays presented in Table 1 suggest that the precision of the method, expressed as RSD, ranged from 0.5 to 3.0%.

Table 2 summarizes the average recovery of SCP from all matrices at 100 ng g^{-1} level. Satisfactory results were obtained and the recovery values were depending on tested species. The average recoveries were greater than 69.7% with coefficients of variation (CVs) between 0.4 and 1.8%. Figs. 2a and 3a show chro-

Table 2
Recovery of sulphachloropyrazine obtained from broiler and turkey tissues spiked with 100 ng g^{-1} of the drug.

Kind of spiked tissue	Recovery[b] (%) ±SD	
	Broilers	Turkeys
Muscle	74.81 (1.6) ± 1.20	72.94 (1.8) ± 1.28
Liver	69.70 (1.1) ± 0.76	72.35 (0.9) ± 0.64
Kidney	76.07 (0.4) ± 0.31	71.33 (1.7) ± 1.23
Fat with skin	77.50 (0.7) ± 0.56	74.71 (0.6) ± 0.48

[b] Data are averages. $n = 3$; coefficients of variation in parentheses (%).

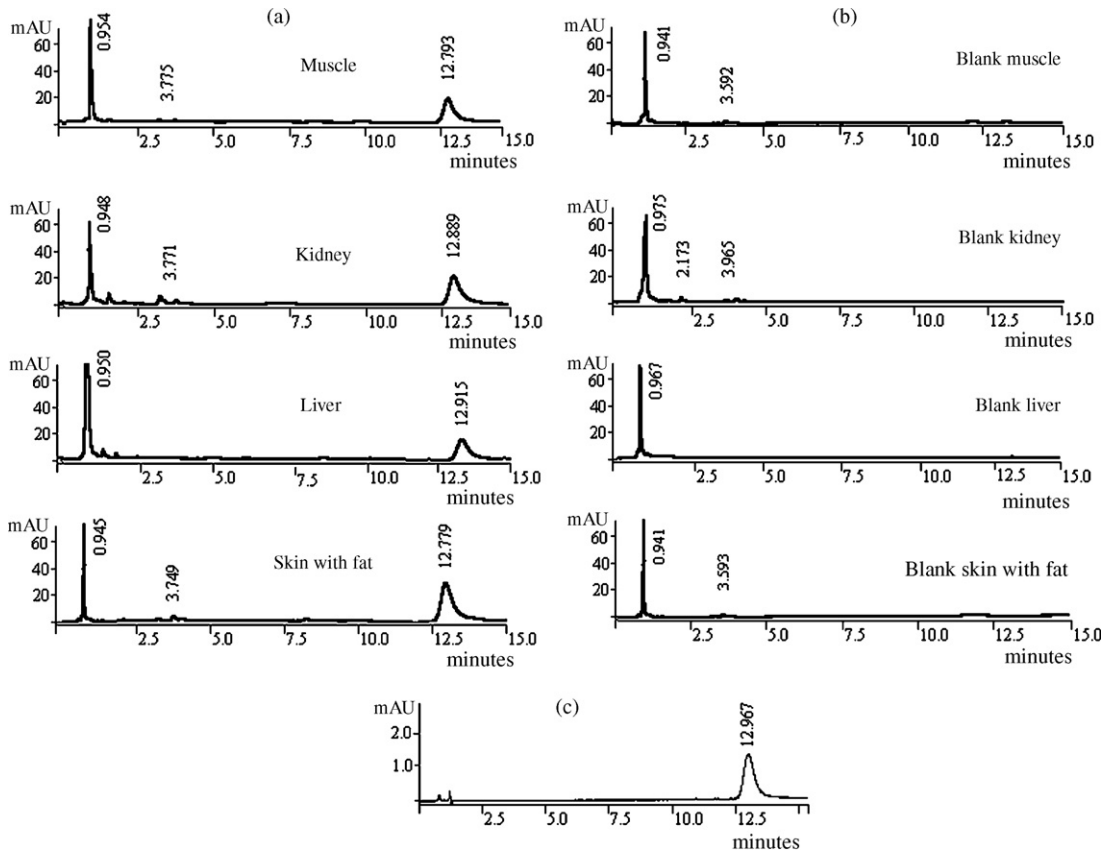


Fig. 2. Representative HPLC chromatograms obtained from (a) broiler edible tissue samples (muscle, kidney, liver, fat with skin) spiked with 100 ng g^{-1} of sulphachloropyrazine sodium salt, (b) blank tissue samples and (c) working standard solution of sulphachloropyrazine sodium salt (250 ng mL^{-1}).

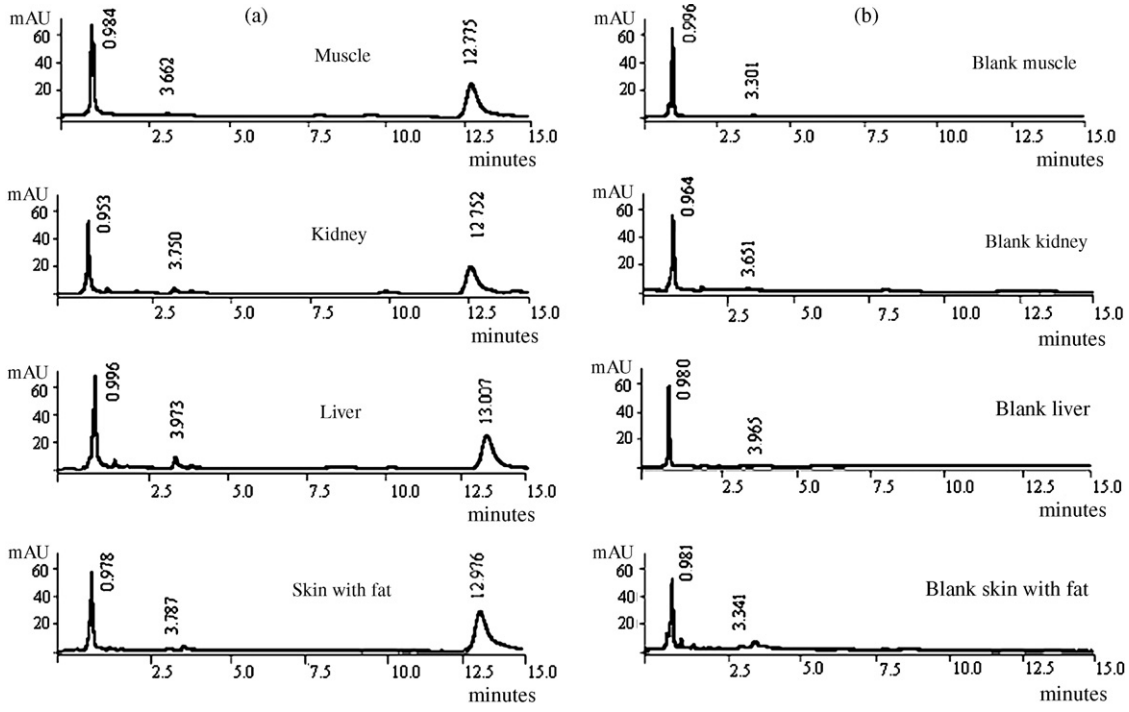


Fig. 3. Representative HPLC chromatograms obtained from (a) turkey edible tissue samples (muscle, kidney, liver, fat with skin) spiked with 100 ng g^{-1} of sulphachloropyrazine sodium salt, (b) blank tissue samples.

matograms obtained from broiler and turkey tissues containing sulphachloropyrazine.

In order to verify the selectivity of the method we analyzed all blank tissue samples from two species. No interference was detected in the region of interest where the analyte was eluted as is shown in the blank sample chromatograms (Figs. 2b and 3b).

The stability of SCP was determined in two different ways: in solvent (working solutions) and in tissue samples containing SCP. The working solutions were analyzed every week and the instrumental responses were compared with peak areas obtained on the day of solution preparation. No degradation phenomenon was observed during a storage of 4 months at 4 °C.

The stability of sulphachloropyrazine in tissue samples was determined from blank matrices spiked at MRL level. Samples were frozen at −30 °C and analyzed after 7, 14 and 21 days, evaluating the amounts of sulphachloropyrazine present. SCP was stable throughout the freezing process. No differences were observed between the amount of sulphachloropyrazine spiked and that observed in the samples stored during different periods of time.

3.2. Extraction

A critical aspect of drug residue analysis is the sample extraction purification steps required to isolate sulphonamide residues from broiler and turkey biological matrices. Sulphonamides have similar chemical and physicochemical properties. These compounds are not very soluble in non-polar solvents, but have good solubility in polar solvents. Traditionally the extraction of sulphonamide from meat, milk and eggs has been done with organic solvents such as: chloroform, methylene chloride, acetone, acetonitrile and ethyl acetate [9,20,22]. Clean-up methods reported in the literature use many different techniques: SPE cartridges filled with different stationary phases, e.g., silica gel, cationic exchanger [24], C18 [20], clean-up procedure with ultracentrifuge [25]. Sample clean-up procedures also include liquid–liquid purification to reduce or eliminate interferences. Based on the extraction method for the determination of sulphonamides in animal tissues, acetonitrile was selected to precipitate proteins and hexane was employed to extract out lipids [21,26,27]. We tried another clean-up step i.e. ethanol, ethyl acetate, 5% solution of trichloroacetic acid and 1 M solution of hydrochloric acid but this method permitted too many compounds that interfered with the chromatography of the analyte. Results showed that deproteinization of the sample with acetonitrile followed by hexane washing of the aqueous acetonitrile extract could achieve significant purification. Subsequent extraction of sulphachloropyrazine was continued as a series of liquid–liquid partition clean-up procedures with the aid of dichloromethane and phosphate buffer (pH 6.8). A double extraction improved better recovery of SCP from animal tissues.

3.3. HPLC optimization

Methanol and acetonitrile as organic solvents in the mobile phase were studied in order to find better selectivity and resolution, using phosphate buffer (pH 2.7) in the mobile phase. The injections resulted in peak broadening when methanol was used, whereas in acetonitrile did not. Therefore acetonitrile was chosen as organic solvent in the composition of the mobile phase. We also checked the percentage of acetonitrile (10 up to 50%) in the mobile phase. Obviously, as acetonitrile concentration increased the analysis time decreased, as expected, but the interesting peak was broad and wide. A 20% ACN was chosen as suitable, according to the peak shape and run time as well (Fig. 2c). For that reason, the pH of the mobile phase played an important role in the chromatographic separation. The optimum pH of the buffer was 2.70, at which sulphachloropyrazine gave excellent retention. Similar composition of the mobile phase employed previously by our group for the determination of sulfadiazine combined with trimethoprim [28]. The satisfactory retention time of sulphachloropyrazine was achieved when the flow-rate of mobile phase was 1.7 mL min^{-1}. Separation of sulphonamides is usually performed with silica-based reversed phased columns, mainly C18, C8 or C4, but in some cases ion-pair column is also used [11]. In our study we used Synergi 4 μ Fusion–RP 80A (150 mm × 4.6 mm) (Phenomenex) column which was recommended to separation of SAs.

4. Conclusion

Several spectroscopic techniques, such as UV absorption, FLD [17,18], DAD [15,20] or MS [21] are used for detection of different sulphonamides in LC. UV detection is often carried out at 270–280 nm, in some cases at 255 nm [11] and 254 nm [8,23]. Based on the literature, 270 nm was selected as a wavelength for the separation of SCP in poultry tissue samples [28,29].

After optimization of the mobile phase parameters, the wavelength of detection and the flow rate, sulphachloropyrazine was detected with satisfactory recovery. Sulphachloropyrazine peak showed a good shape and no interferences with other peaks (impurities), what indicates a high selectivity and sensitivity of this method. This makes it valuable and adequate in many applications, particularly in veterinary medicine studies.

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