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Monitoring of Tetracycline Group Antibiotic Residues in Various Food Products of Animal Origin in the Turkish Market

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Abstract

The aim of this study was to detect the presence of antibiotic residues in foods of animal origin, including 42 pieces of chicken gizzard and 46 pieces of bovine kidney and 102 chicken eggs belonging to various brands. These samples were gathered from December 2020 to April 2021 in the Aegean province of Turkey. A sensitive, simple, rapid, experimentally convenient and cost effective RP-LC method with high recovery output was developed. The method was thoroughly validated for the optimized parameters and produced satisfactory results. The analysis of bovine offal by the developed RP-LC method showed the presence of oxytetracycline, tetracycline, and chlortetracycline residues in 14 (30.43%) kidney samples. Chlortetracycline was detected in 7 (16.67%) chicken gizzard samples. In addition, the analysis of chicken eggs revealed the presence of oxytetracycline and tetracycline residues in nine egg samples (8.82%). Since, the amount of antibiotic residues in these samples was below the detection limit, quantification could not be carried out. Only one (0.98%) of the 102 egg samples exceeded the MRL (267.1 mg/kg) for oxytetracycline concentration. According to the study's overall findings, it is recommended that tetracycline antibiotics should be regularly checked in a variety of foods made from animals because they were found in 32 out of 190 analysed samples. Tetracycline residues may pose dangers to human health, so it's important to conduct further research and more information should be given for both producers and consumers.

Keywords: Antibiotics, Veterinary drug residues, Food Safety, Tetracyclines, Validation, HPLC.

Introduction

Antibiotics are actively injected into animal bodies to support their protective systems during the treatment period. Antibiotics such as penicillin, gentamicin, tetracycline, danoflaxacin, neomycin, etc. are widely used to prevent and treat diseases, especially mastitis and respiratory system diseases [1]. Antibiotic residues in animal foods have become a significant threat to public health and food safety. For this reason, it has become necessary to concentrate on the amount of residue in foods [2].

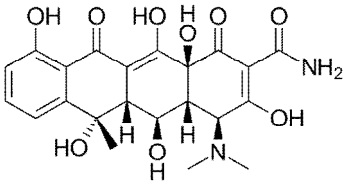
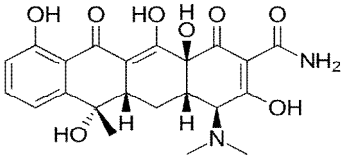
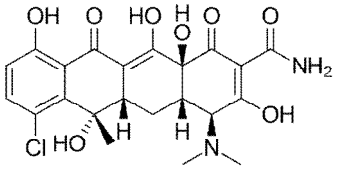
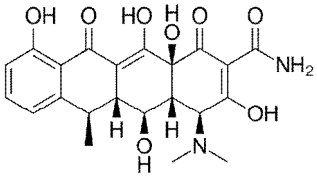
Tetracyclines (TCs) are a broad-spectrum cluster of antibiotics, and they've been employed in the treatment of microorganism infections in animals for over fifty years. However, the employment of those medicines has become a significant drawback as a result of antibiotic residues in animal

food. The adverse effects of this class of antibiotics include organ injury, allergic reactions, gastrointestinal distress, and tooth discoloration. Residues of antibiotics in animal feed are not recommended in veterinary applications. The Maximum Residue Levels (MRLs) for TCs in several foods have been set by European Regulation 2377/90 and its subsequent amendments [3]. Despite the occurrence of resistance, tetracyclines are still widely in use in animals owing to their low prices. Egg MRLs are 200 ng/g for oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC), chicken MRLs are 100 ng/g for OTC, TC, CTC, and doxycycline (DC), but the latter value is temporary and still being investigated.

A certain and reliable methodology for detecting tetracycline residues in animal foods is incredibly important. For this purpose, chromatographic techniques, such as high performance liquid chromatography (HPLC) with different detection modes like spectrophotometry, fluorescence and mass spectrometry [4-10], and capillary electrophoresis [11-13] have been used for the analysis of TCs. In addition, general descriptions of immunoassay and microbiological test procedures for TC screening in food products have been provided [14–15]. The lack of specificity and the lengthy incubation period necessary for microbiological testing are their biggest drawbacks [16]. Due to the very comparable structural similarity of TCs immunoassays, a misleading detection may also occur [17]. As a consequence of this, confirmatory research is necessary in order to quantify the results of screening tests performed on food products.

There is little information about the presence of TCs residues, especially in milk samples in Turkey [18-20]. However, there is a lack of information about TCs residues in chicken, bovine offal and eggs in Turkey. It appears that a useful antimicrobial residue monitoring system should be brought in place. A fast, sensitive, and economical reversed phase liquid chromatography (RP-LC) technique was built up for the analysis of OTC, TC, CTC, and DC residues. Consequently, the purpose of this study was to detect the presence of antibiotic residues in foods of animal origin, including 42 pieces of chicken gizzard, 46 pieces of bovine kidney, and 102 chicken eggs of various brands, collected in the Aegean province of Turkey between December 2020 and April 2021. The chemical structures and pK_a values of studied TCs are listed in Table 1 [21]. The developed method has been validated according to ICH rules, and recovery values were also calculated.

Table 1. **Chemical structures of the tetracycline antibiotics studied.**

Compounds	Chemical Structure
Oxytetracycline (OTC) pK_{a1} = 3.53 [21] pK_{a2} = 7.25 pK_{a3} = 9.58	 $C_{22}H_{24}N_2O_9$ MW: 460,434 g/mol Cas No: 79-57-2
Tetracycline (TC) pK_{a1} = 3.35 [21] pK_{a2} = 7.29 pK_{a3} = 9.88	 $C_{22}H_{24}N_2O_8$ MW: 444,435 g/mol Cas No: 60-54-8
Chlortetracycline (CTC) pK_{a1} = 3.25 [21] pK_{a2} = 6.72 pK_{a3} = 8.84	 $C_{22}H_{23}ClN_2O_8$ MW: 478,88 g/mol Cas No: 57-62-5
Doxycycline (OTC) pK_{a1} = 3.02 [21] pK_{a2} = 7.97 pK_{a3} = 9.15	 $C_{22}H_{24}N_2O_8$ MW: 444.44 g/mol Cas No:564-25-0

Materials and Methods

Reagents and Chemicals

Tetracycline, oxytetracyline, chlortetracycline, and doxycycline were bought from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Methanol (MeOH) and acetonitrile (ACN) of HPLC grade were obtained from Fisher Scientific (Pittsburgh, PA, USA). Phosphoric acid (H_3PO_4), hydrochloric acid (HCl), formic acid (HCOOH), trichloroacetic acid (Cl_3CCOOH, TCA), sodium hydroxide (NaOH), and EDTA were procured from Fisher Chemical (Fairlawn, NJ, USA).

Preparation of Solutions

In MeOH, 100 μg mL^{-1} stock standard solutions of TC, OTC, CTC, and DC were prepared. The mobile phase was used to dilute the working solutions to 10 μg mL^{-1}. After being diluted, these solutions were used to make a series of working standard solutions, which were then used for the daily generation of calibration curves and standard addition spikes. By injecting a solution of uracil [0.01% (v/w), in water], which was found for each combination of mobile phase and pH level, the dead time (t_o) was measured.

Instrument Description

The study was performed using an Agilent 1260 series HPLC system that includes a ternary solvent pump, an automated injection system, an in-line degasser, column heater, and a multi-wavelength detector. UV identification for the analyzed substances was done at 271 nm. The analysis was performed at a 1.2 mL min^{-1} flow rate. As the stationary phase, a Synergi 4 Hydro-RP 80A column (250 x 4.60 mm i.d. 4 μm) was employed at 25 °C. For measuring pH, a Mettler Toledo, Hanna HI 1332 Ag/AgCl combined pH electrode was used (Hanna Inst.). The water used was double-distilled and deionized through a Millipore Direct-Q 3UV; 0.22 μm water purification unit.

A mixer with 100-300 mL flasks and a vortex (IKA Ms 3 Basic Vortex) were used depending on the sample. Additional glassware and required equipment include 50 mL conical falcon tubes and graduated cylinders. The used glassware was detergent cleaned and rinsed with 0.01 M HCl and pure water.

Sample Collection

From December 2020 to April 2021, a total of 88 offal samples and 102 chicken eggs belonging to various brands were collected in Aegean province, Turkey. The samples were bought from major supermarkets and local grocery stores and meat galleries, including butchers and chicken stores, and carried to the laboratory after sampling.

Sample Extraction and Clean-Up

Meat and chicken tissue samples

For meat and chicken tissue samples, 42 pieces of chicken offal (gizzard) and 46 pieces of bovine offal (kidney) were purchased and stored in plastic containers at 4 ^oC in the dark until used within four days in this study. Tissue was cut into small pieces with a side of 1 cm or less. Approximately 8 g of offal sample (or larger or smaller as desired) was weighed exactly into a 300 mL mixing glass. The mixture was stired for two minutes or until no visible pieces of tissue remained. After that, it was placed in a falcon tube and 15 mL of methanol was added, and vortex treatment was applied for 5 minutes. Thus, protein precipitation was achieved. Then, 4 mL of 1% formic acid was added and vortex treatment was applied for 3 minutes. Finally, 400 μL of

0.01 M EDTA was added and vortex treatment was applied for 2 minutes. The resulting mixture was centrifuged at 2000 rpm for 10 minutes. After the centrifugation process, the supernatant part was removed with the help of an injector and injected into the HPLC device by passing it through a 0.22 µm filter.

Eggs

A batch of 6 of the eggs belonging to each brand was taken, blended, and homogenized. 4 grams of the resulting homogeneous mixture was taken and placed in a falcon tube and left in a dark place for 15 minutes. Vortex treatment was applied for 10 minutes by adding 8 mL of 5% TCA to it. After that, 15 minutes were spent for centrifuging the mixture at 4000 rpm. After centrifugation, a 0.22 µm filter was used to separate the supernatant fraction, which was then injected into the device.

Results and Discussion

Method Development and Validation

The presented HPLC technique offers a facile method for the simultaneous determination of TC, OTC, CTC, and DC in chicken and beef tissues, as well as in egg samples by diode array detection. The investigated tetracycline compounds were effectively determined simultaneously by using the chosen column as the stationary phase. It is a C-18 bonded phase and end-capped column with non-polar groups to supply extreme retention of polar and hydrophobic compounds in high aqueous mobile phases with 19% carbon loading. The high silica area (475 m^2g^{-1}) of the 4 µm surface combined with the coverage of the dense bonded phase permits the high interaction between the analyte and the bonded phases. The obtained results are so persistent in the C-18 phase, they are well suited for separating the compounds studied.

To specify the best conditions for the chromatographic separation of TCs, the flow rate, pH of the buffer, temperature, and buffer concentration parameters were investigated. For separation, a 15–30 mM concentration was studied. A 25 mM phosphate buffer concentration was selected because of the best separation of the studied TCs. In terms of the mobile phase pH value, values in the range of pH 2.5–3.0 were tested. The optimum peak resolution was seen at pH 2.8. The applied flow rate was examined in the range of 0.8, 1.0, 1.2, 1.5, and 1.7 mL/min. Sharper peaks and shorter retention times were observed with increasing flow rates. Since the minimum retention time is necessary for LC, 1.2 mL/min was selected for the optimum results due to the back pressure. The temperature was tested in the range of 25 to 35 ^oC as the temperature was increased by 10 ^oC and worked at 35 ^oC. Here, with the increase in temperature, distortions in peak shapes and an in crease in retention time were observed. The column temperature was fixed to 25 ^oC. The ACN concentration in the mobile phase was changed from 30 to 20% (*v/v*), and 20% (*v/v*) ACN was chosen as the optimum condition due to the peak shape and analysis time.

HPLC separation was obtained using Synergy 4µ Hydro-RP 80A (250 × 4.60 mm id × 4 µm) column at 25℃, with a mobile phase acetonitrile-water (20:80, v/v), pH 2.8 (phosphate buffer) and flow rate of 1.2 mL/min. Under these circumstances, the analysis time increased to almost nine minutes with symmetrical peaks, and the retention times were 3.16 ± 0.05, 3.92 ± 0.08, 6.44 ± 0.06, and 7.64 ± 0.09 min for OTC, TC, CTC, and DC, respectively. A representative chromatogram for the examination of TCs standards is shown in Fig. 1 at a wavelength of 271 nm.

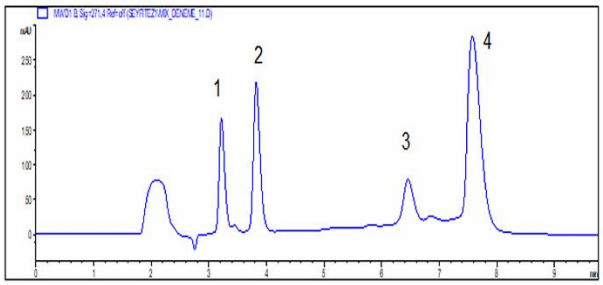


Figure 1. **The chromatogram of standard mixture of studied compounds 1) OTC 5 µg mL^{-1}, 2) TC 5 µg mL^{-1}, 3) CTC 50 µg mL^{-1}, 4) DC 20 µg mL^{-1} monitored at 271 nm absorbance under optimum conditions.**

The detector response linearity was tested using OTC and TC standard solutions ranging from 0.1 to 20 µg mL^{-1}, chlortetracycline standard solutions ranging from 1 to 80 µg mL^{-1}, and doxycycline standard solutions ranging from 0.5 to 20 µg mL^{-1}, respectively (Table 2). Plotting concentration versus peak area from the chromatograms of the standard samples allowed for the construction of calibration curves.

Table 2. **Statistical evaluation of the calibration data of OTC, TC, CTC and DC by RP-LC.**

Compounds	OTC	TC	CTC	DC
Linearity range/ µg mL^{-1} (n = 7)	0.10 – 20.0	0.10 – 20.0	1.0 – 80.0	0.50 – 20.0
Slope	238.516	374.857	17.785	215.874
Intercept	-48.171	-94.127	-27.997	-91.531
Correlation coefficient (r^2)	0.9994	0.9991	0.9991	0.9994
SE of slope	2.580	5.311	0.284	2.713
SE of intercept	22.452	46.225	10.688	25.508
Limit of detection (LOD) / µg/mL	0.0050	0.0041	0.1163	0.0076
Limit of quantification (LOQ) / µg/mL	0.0151	0.0125	0.3525	0.0229
Retention time (min) (n = 16)	3.16	3.92	6.44	7.64
RSD% of retention time	1.58	2.04	0.932	1.18

Tetracycline standards were prepared every day, and estimations of the concentrations of the analytes in the samples were extrapolated from the graphs. The precision of the approach is demonstrated by the low slope and intercept standard error (SE) values. The standard deviation of the response (s) and the slope (m) of the related calibration curve were utilized in the following formulae to calculate the LOD and LOQ values [22, 23].

$$LOD = 3.\frac{3s}{m}; LOQ = \frac{10s}{m} \quad (1)$$

Recovery studies were determined from egg, chicken, and beef offal samples for the accuracy and precision of this method. The recovery values were determined by spiking the previously studied samples with the appropriate amounts of OTC, TC, CTC, and DC at the time of homogenization. The results of the recovery analysis are given in Table 3. Table 3 suggests that the recovery calculated from these antibiotics ranges from 82.31% to 96.22%.

Table 3. **Recovery of studied compounds from egg, chicken and beef offal samples.**

Compounds	Percent Recovered					
	Egg	***RSD %***	***Chicken Gizzard***	***RSD %***	***Bovine Kidney***	***RSD %***
OTC	86.96	2.16	89.06	4.16	92.99	4.25
TC	92.34	1.64	96.22	5.21	89.63	6.55
CTC	90.88	1.97	94.82	2.38	90.90	3.73
DC	82.31	1.05	95.74	2.43	93.74	7.90

As an example, chromatograms of bovine kidney, chicken gizzard, and egg samples were given in Fig. 2.

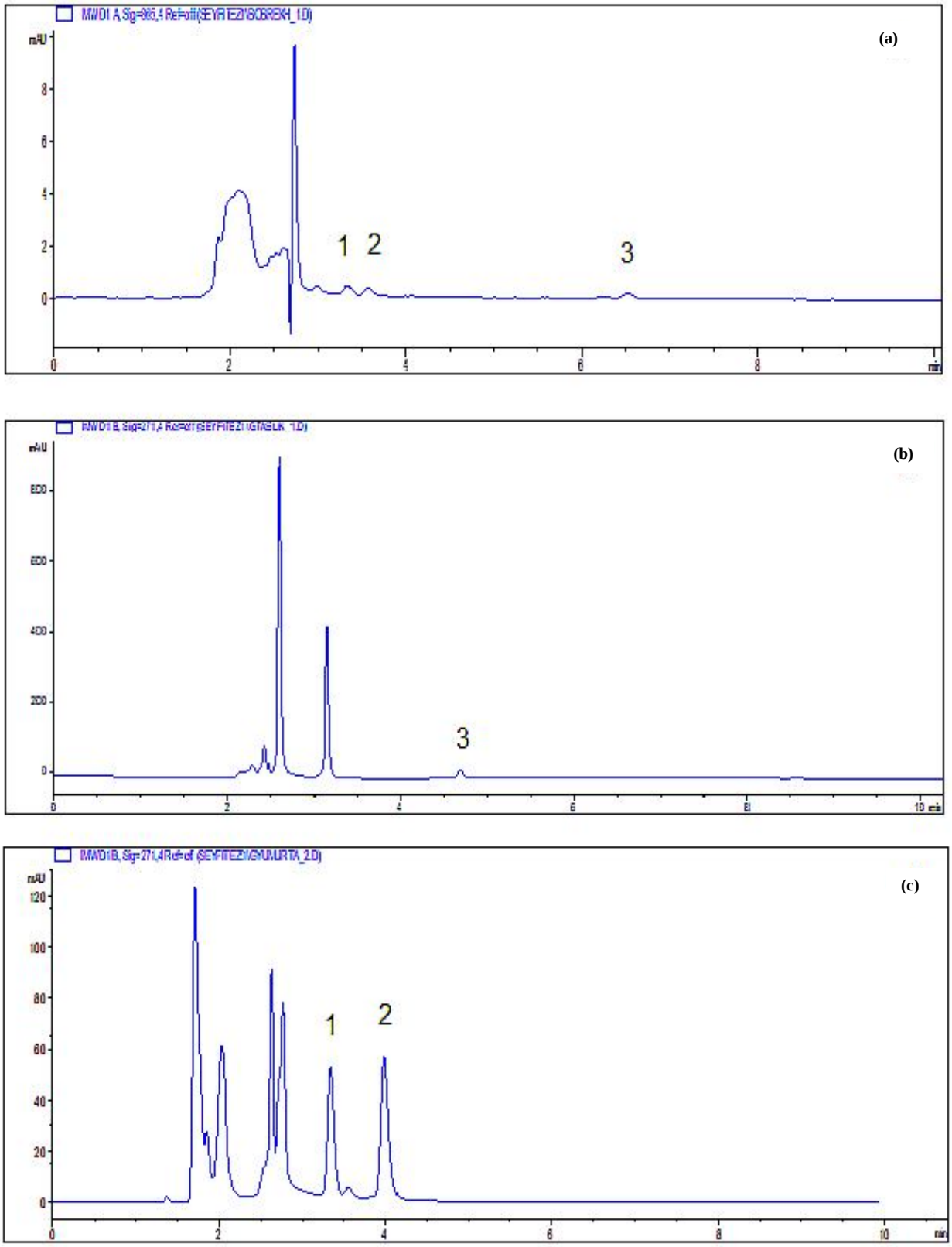


Figure 2. **Chromatograms of the TC compounds in studied samples. Optimum conditions and number of studied compounds are the same as Fig. 1. (a) Bovine kidney, (b) Chicken gizzard, (c) Egg**

The proposed method offers better recovery and greater sensitivity. It is clear from the calculated recovery data of all tetracyclines in various samples, which are within the AOAC acceptable range for trace analysis; 60-115% [24], and the values of OTC, TC, CTC, and DC of meat and egg samples, which were provided in accordance with the Codex Alimentarius Commission and European Union regulation 2002/657/EC. Additionally, relative standard deviation (RSD) values calculated in this work were less than 10%, which complies with the codex alimentarius commission. According to a statement, if the RSD data are sufficient, the method may be regarded as verified.

After RP-LC analysis (Table 4), OTC and TC were found in nine of the egg samples. According to Turkish legislation on veterinary drug residues, oxytetracycline and tetracycline given for maximum residue in chicken eggs (MRL) of 200 µg/kg. A value above the MRL value (267 µg/kg oxytetracycline) was detected in only 1 (0.98%) sample [25]. The levels of TC (LOD 0.004 µg/kg) ranged from 89.4 to 122.3 µg/kg was determined in two samples.

***Table 4*. Levels of antibiotic residues detected by the RP-HPLC technique.**

Number of samples analysed	RP-HPLC results			
	OTC	***TC***	***CTC***	***DC***
102[a]	7[d] (< 0.004 -0.06; 8.82%)		-	
	1 (139.0; 0.98%) 1 (267.1; 0.98%)	1 (122.3; 0.98%) 1 (89.4; 0.98%)		-
42[b]	-	-	7 (< 0.12; 16.67%)	-
46[c]	14 (< 0.004 – 0.12; 30.43%)			

Notes: [a] Number of egg samples collected from various brands.
[b] Number of chicken gizzard samples
[c] Number of bovine kidney samples
[d] Number of positive samples (antibiotic levels, µg kg^{-1}; percentage).

Other samples had residual levels that were lower than the global standards established by the European Union and the Turkey-allowed limits. 32 bovine kidney samples, 35 gizzard samples, and the remaining 93 egg samples tested negative for RP-LC detection. In the production of eggs and meat, the massive and incorrect application of TCs and the misguided following of withdrawal periods may result in the presence of their residues in food products. The risks to human health from the maximum residual TC ranges remain even though they were below the limit.

Conclusion

Egg, chicken gizzard, and bovine kidney samples collected from supermarkets, local grocery stores, and meat galleries, including butcher shops and poultry shops, in the Aegean province contain traces of TC. Although the majority of these levels fall below the thresholds established by the European Union and Turkish law, their presence can still be viewed as posing a threat to consumer health. This is due to their potential to trigger allergic reactions or help to breed bacteria that are resistant to antibiotics, both of which have emerged as major issues in the treatment of infectious diseases that affect humans. Therefore, comprehensive surveillance plans for the management of veterinary drug residues in animals and their products must be established by national authorities.

In addition, the RP-LC method was used due to its accuracy, straightforward, quick, convenient, cost-effective, and high recovery throughput. The method was completely validated, with satisfactory results for each of the examined method validation parameters. The developed method promises to be applicable to the identification and analysis of frequently found TCs in other offal

samples such as spleen, liver, and chicken breast as well.

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Declaration of interest

The authors declare no conflict of interest.

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