

Investigation of the presence of some antibiotics in Raw Goat milk collected from Ankara, Kırıkkale and Çankırı provinces

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ARTICLE INFO

Article History

Received : 11.02.2022

Accepted : 11.05.2022

DOI: 10.33988/auvfd.1071743

Keywords

Ankara

Antibiotic residue

Çankırı

Goat's milk

Kırıkkale

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How to cite this article: Mahamat AT, Altıntaş L, Aluç Y (2023): Investigation of the Presence of Some Antibiotics in Raw Goat Milk Collected from Ankara, Kırıkkale and Çankırı Provinces. Ankara Univ Vet Fak Derg, 70 (3), 285-291. DOI: 10.33988/auvfd.1071743.

ABSTRACT

Antibiotics used in food producing-animals may cause residual problems in food in terms of public health. This situation can lead to serious problems in terms of human health. Raw milk is one of the foods that are likely to contain antibiotics, even in trace amounts. This study aimed to determine the residue levels of commonly used antibiotics of raw goat's milk samples offered for sale in Ankara, Çankırı and Kırıkkale. One active ingredient was selected from the five most commonly used antibiotic groups in animals and it determined the residue levels of these substances in milk. For this purpose, within one year and in two different periods, 150 raw goat milks analyzed in terms of antibiotics using HPLC method. The values for enrofloxacin, sulfamethoxazole, tylosin, penicillin G and oxytetracycline were 7.9, 9.7, 11.5, 5.4 and 7.3 minutes for retention times, 1.47, 0.8; 7.51; 2.69 and 8.89 µg/L for limit of detection (LOD) and 4.47, 2.44, 22.78, 8.16 and 26.96 µg/L for limit of quantification (LOQ), respectively. No antibiotic residues were detected in the goat milk samples. It is predicted that the higher resistance of goats to diseases compared to other milk-producing animals, and therefore the lower use of antibiotics in these animals, leads to this result. The findings obtained as a result of this study are valuable in terms of public health. It is important that no antibiotic residues are found in the analyzes.

Introduction

Milk is a biological substance produced by mammals in their mammary glands following pregnancy. It contains almost all the nutrients. In fact, the main purpose of secretion or production of the milk is to ensure the immunological adaptation of the newborn to the outside world and to meet the basic nutritional needs of the infant. Among milks, goat milk has special importance for human as it is the closest milk to human breast milk. The fact that it has much less allergic effects and trans-fat content compared to cow's milk, and its high digestibility increases its importance even more. The lower ratio of trans fatty acids compared to cow's milk also reduces the risk of heart disease (4, 6). Recently, the interest in goat milk and its products has increased with the demonstration of its beneficial aspects to human health. In addition to the use of goat milk as dairy products such as sterilized and

pasteurized drinking milk, yogurt, cheese, ice cream and dairy desserts, goat milk is involved in the manufacture of cosmetic products such as hand and bath soaps, hand and face moisturizers that can be used by atopic patients (1, 3, 24, 26). Goat milk is in the group of casein milks. Considering its composition, it has been reported that the dry matter is around 12.5% on average and this total dry matter contains on average 4% fat, 3.3% protein, 4.1% lactose and 0.8% ash (18). The composition of goat's milk differs according to the country and breed where it is grown (3, 10, 12).

The drugs used for growth promotion improved feed conversion efficiency and for the prevention and treatment of diseases in animals cause residue problems by accumulating in the tissues or organs of animals. The presence of drug residues in foodstuffs poses a significant risk to consumer health and well-being (13). Due to the

reasons described above, the use of drugs can sometimes be unavoidable. In such cases, residue levels in animal products should be kept below the levels specified by the authorities by constantly monitoring. Residues in foods exceeding the permissible amount pose a potential toxicological hazard to consumers (2, 5, 11, 22). This study aimed to determine the presence of some antibiotics in raw goat milk collected from Ankara, Kırıkkale and Çankırı provinces of Türkiye.

Materials and Methods

In this study, milk samples were collected from goat raw milk (or collection containers) of goat breeding farms in Ankara, Çankırı and Kırıkkale provinces of Türkiye twice in 2019 (March and September were preferred according to lambing time of goats). Samples were taken from five different farms in each province and five different goats from each farm. 500 mL of raw goat's milk was packed in leakproof, disposable glass containers. Samples were taken in accordance with the National Residue Monitoring Program (23). The samples were brought to the laboratory under cold chain and kept at -20°C until analysis.

All measurements were performed using a Shimadzu Ultra Fast Liquid Chromatograph (UFLC) (Shimadzu, Japan) system (LC-20AD, Shimadzu) equipped with a quaternary pump, a vacuum degasser, a column compartment, an auto sample, and a diode-array detector, and controlled by the LabSolutions chromatography software. The analytical column was HPLC Column, Intersil ODS4, 5 µm 4.6×250 mm. Other equipment such as pH meter (HANNA Instruments HI 2211), electronic weighing balance (Sartorius), centrifuge (NF 815), ultrasonic cleaner (Probetec) and vortex (Heidolph) were also used in this study. Standard solutions and samples prepared for analysis were injected into the instrument in 50 µL. Methanol, acetonitrile and ammonium dihydrogen phosphate solutions used as mobile phases in the method were defined to the device according to the program shown in Table 1.

Extraction: 2 mL of milk was placed into a 15 mL centrifuge tube and 5 mL of acetonitrile was added to ensure the denaturation of proteins and mixed in a vortex for 1 minute. Then, 0.25 g Sodium chloride (NaCl) was added to the tube and vortexed for 1 minute. After homogenization for 5 minutes in an ultrasonic bath, it was centrifuged at 3000 rpm for 5 minutes. Then, it was filtered through a 0.45 µm nylon filter and 50 µL was applied to the system (9, 27). The parameters of the HPLC device used for the analysis are given in Table 2.

Preparation of Standards: Main stock solutions for each active substance (Enrofloxacin, Sulfamethoxazole,

Tylosin, Penicillin G and Oxytetracycline) were prepared by dissolving the amount equivalent to 10 mg of standard substance in 10 mL of distilled water (1 mg/mL). Working solutions of 20, 40, 80, 100, 200, 400, 800, and 1000 µg/L were prepared from the main stock solution.

Validation of the Method: Accuracy, linearity and working range, selectivity, precision, limit of detection and limit of measurement were accepted as the preferred performance criteria in determining the validation of the method (5, 7, 21, 25).

Table 1. Gradient conditions.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.01	70	30
3	65	35
5	45	55
6.5	45	55
9	0	100
10	0	100
11	65	35
12.5	65	35
14	70	30

Table 2. HPLC parameters.

HPLC Parameters	
Colon	Intersil ODS4 (250 x 4.60 mm, 5 µm)
Colon temperature	40°C
Mobile phase	Mobile Phase A: Methane: acetonitrile: water (1:3:1) Mobile Phase B: NH ₄ PO ₄ 20 mM, pH: 2
Flow rate	0.8 mL/min
Wavelength	280 nm
Detector	Diode-Array Detection (DAD)
Injection volume	50 µL
Analysis time	16 min

Results

Method Validation

Accuracy: Recovery values were calculated as 96.98% for enrofloxacin, 94.08% for sulfamethoxazole, 106.95% for tylosin, 102.71% for penicillin G and 105.24% for oxytetracycline (Table 3).

Linearity and Working Range: R² values were calculated as 0.998 for enrofloxacin, 0.999 for sulfamethoxazole, 0.999 for tylosin, 0.998 for penicillin G and 0.998 for oxytetracycline (Table 4). The calibration curves obtained for each antibiotic are shown in Figure 1.

Table 3. Recovery values.

Antibiotics	Dose (µg/L)	Recovery (%)	Mean (%)
Enrofloxacin	40	92.17	96.98
	80	100.49	
	100	98.28	
Sulfamethoxazole	40	88.63	94.08
	80	91.74	
	100	102.45	
Tylosin	40	92.92	106.95
	80	91.4	
	100	104.63	
Penicillin G	40	107.57	102.71
	80	96.61	
	100	103.96	
Oxytetracycline	40	118.5	105.24
	80	99.35	
	100	97.74	

Table 4. Correlation coefficients.

Standarts	R ²	Equation
Enrofloxacin	0.998	Y=306912x+11790.2
Sulfamethoxazole	0.999	Y=523358x+18609.3
Tylosin	0.999	Y=73973x-930.373
Penicillin G	0.998	Y=100681x+24013.3
Oxytetracycline	0.998	Y=121810x-4075.2

Selectivity and Precision: Chromatograms of the blank sample (Figure 2A) and the standard loaded samples (Figure 2B) reveal the selectivity of the method. Retention times of antibiotic standards were determined as 7.9 minutes for enrofloxacin, 9.7 minutes for sulfamethoxazole, 11.5 minutes for tylosin, 5.4 minutes for penicillin G and 7.3 minutes for oxytetracycline HCl. It was also seen that no peak of any compound was detected at the same retention time. Each antibiotic was evaluated as an internal standard for the remaining active ingredients. For this reason, it was not necessary to use a different active substance for the internal standard.

Reproducibility: Reproducibility study results of the method, % recovery and %RSD values are given in Table 5.

The limit of Detection (LOD) and Limit of Quantification (LOQ): LOD and LOQ results; 1.47 µg/L and 4.47 µg/L for enrofloxacin, respectively; 0.8 µg/L and 2.44 µg/L for sulfamethoxazole; 7.51 µg/L and 22.78 µg/L for tylosin; 2.69 µg/L and 8.16 µg/L for penicillin G, and 8.89 µg/L and 26.96 µg/L for oxytetracycline, respectively, are demonstrated in Table 6.

Determination of Antibiotic Presence in Goat Milk: After validation parameters of the method were made, 150 goat milk samples collected from Ankara, Çankırı and Kırıkkale provinces were analyzed. According to the results of the analysis, no antibiotic presence was detected in the samples.

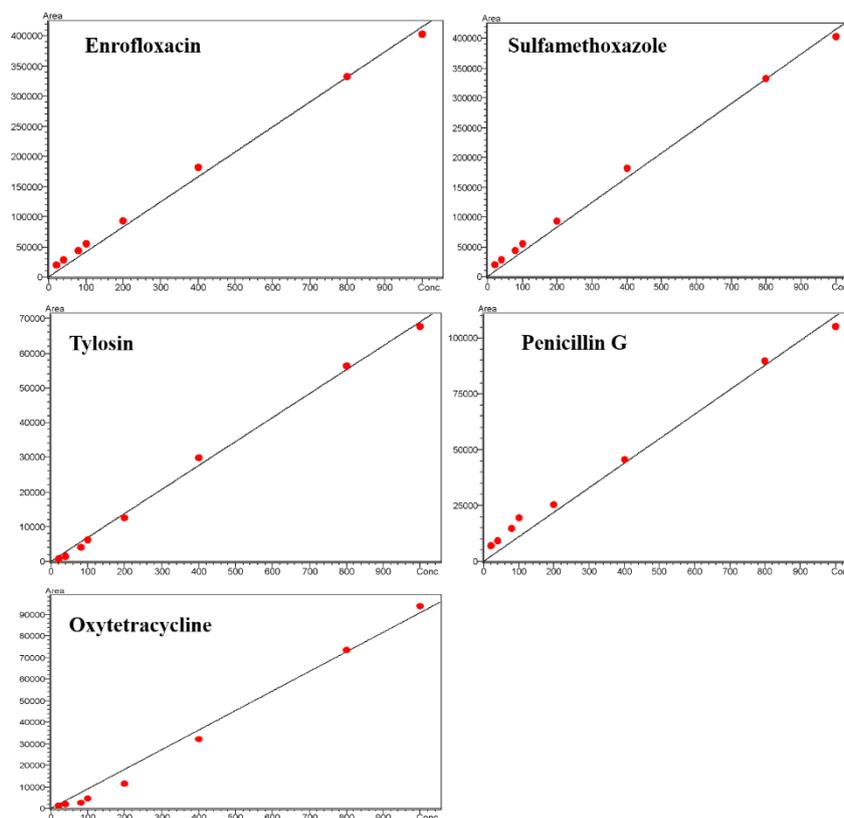
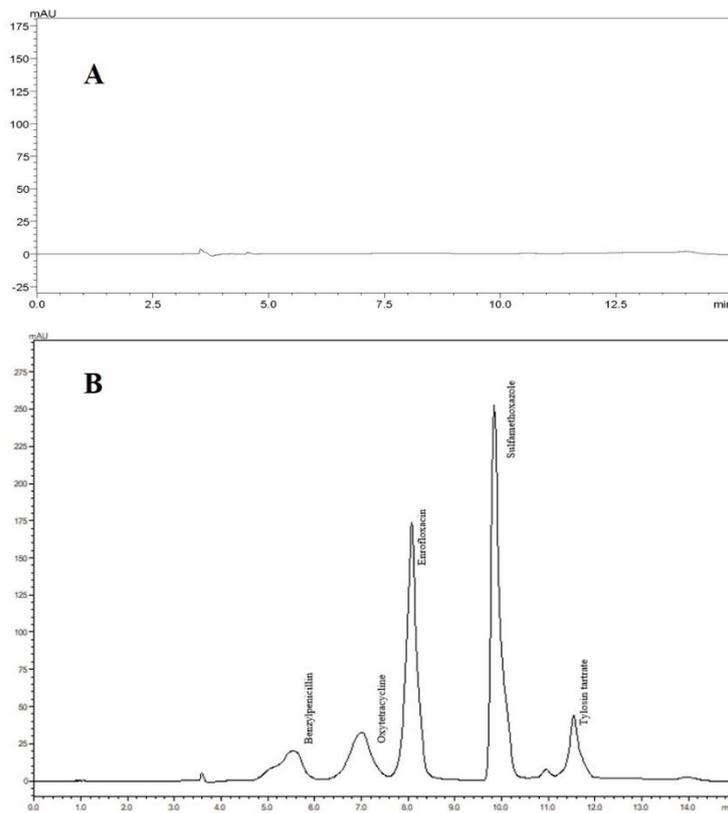
**Figure 1.** Calibration curves for tested antibiotics.

Table 5. The reproducibility of the method.

Antibiotic	Day	Dose (µg/L)	Repetitions	Mean (%)	Recovery (%)	Standard deviation	RSD (%)	Relative Error (%)
Enrofloxacin	1	40	3	36.52	91.3	0.29	0.79	8.7
		80	3	79.64	99.55	0.65	0.81	0.06
		100	3	97.95	97.95	0.27	0.27	2.05
	2	40	3	35.93	89.82	0.67	1.86	10.17
		80	3	75	93.75	1.22	1.62	6.25
		100	3	97.74	97.74	0.55	0.56	2.26
	3	40	3	38.29	95.72	1.46	3.81	4.27
		80	3	76.04	95.05	0.73	0.96	4.95
		100	3	102.25	102.25	2.75	2.68	-2.25
Sulfamethoxazole	1	40	3	35.35	88.37	0.26	0.73	11.63
		80	3	73.39	91.73	0.002	0.002	8.26
		100	3	102.43	102.43	0.03	0.02	-2.43
	2	40	3	34.92	87.3	0.42	1.2	12.7
		80	3	72.15	90.18	0.04	0.05	9.81
		100	3	102.43	102.43	0.04	0.03	-2.43
	3	40	3	35.05	87.65	0.24	0.68	12.37
		80	3	72.15	90.18	0.04	0.33	9.81
		100	3	102.74	102.74	0.30	0.29	-2.74
Tylosin	1	40	3	37.5	93.75	0.62	1.65	6.25
		80	3	71.83	89.78	1.11	1.54	10.21
		100	3	105.77	105.77	1.18	1.11	-5.77
	2	40	3	39	97.5	2.97	7.61	2.5
		80	3	78.77	98.46	3.27	4.15	1.53
		100	3	105.03	105.03	2.36	2.24	-5.03
	3	40	3	37.45	93.62	0.29	0.77	6.37
		80	3	77.84	97.3	3.21	4.12	2.7
		100	3	104.92	104.92	0.61	0.58	-4.92
Penicillin G	1	40	3	43.66	109.15	0.54	1.23	-9.15
		80	3	76.9	96.12	2.01	2.61	3.87
		100	3	104.64	104.64	1.54	1.47	-4.64
	2	40	3	43.23	108.07	1.22	2.82	-8.07
		80	3	74.33	92.91	1.30	1.74	7.08
		100	3	106.09	106.09	2	1.88	-6.09
	3	40	3	41.24	103.1	1.16	2.81	-3.1
		80	3	82.89	103.6	1.92	2.31	-3.6
		100	3	111.35	111.35	1.56	1.4	-11.35
Oxytetracycline	1	40	3	45.75	114.37	2.43	5.31	-14.37
		80	3	81.30	101.62	2.04	2.5	-1.62
		100	3	98.38	98.38	1.10	1.11	1.62
	2	40	3	43.99	109.97	2.62	5.95	-9.97
		80	3	77.92	97.4	1.62	2.07	2.6
		100	3	99.84	99.84	2.71	2.71	0.16
	3	40	3	42.95	107.37	1.26	2.93	-7.37
		80	3	76.70	95.87	1.21	1.57	4.12
		100	3	96.03	96.03	0.32	0.33	3.97

Table 6. LOD and LOQ values.

Antibiotic	LOD ($\mu\text{g/L}$)	LOQ ($\mu\text{g/L}$)
Enrofloxacin	1.47	4.47
Sulfamethoxazole	0.8	2.44
Tylosin	7.51	22.78
Penicillin G	2.69	8.16
Oxytetracycline	8.89	26.96

**Figure 2.** Chromatograms of blank (A) and antibiotic standards (B).

Discussion and Conclusion

Medicines used for various purposes in animals may leave residues in animal products due to erroneous and misguided use, thereby creating negative consequences for human health and resulting in economic losses. In Türkiye, drugs and chemical substances, including antibacterial drugs in animal products are monitored by the "National Residue Monitoring Program," launched by the Ministry of Agriculture and Forestry.

One of the most important steps in performing drug analysis in matrices with complex structures (such as milk) is the process of extraction. Liquid-liquid extraction is actually a versatile sample preparation technique specified in many analytic methods. This technique has the disadvantages of being tedious for incorporating several stages and taking too much time to perform. Also, it was reported that too many poisonous and expensive chemicals are used in this process, and it has the potential

to lead to environmental pollution (17, 18, 20). In this study, the chemical substances used were fewer and these chemicals were used in lower quantities compared to other methods, and therefore, this may be seen as an advantage for this study.

In the present study, the presence of antibiotic residues in goat milk was examined and its significance for public health was assessed. Additionally, the data related to the method employed in this study and analysis results were compared to similar methods and results.

Oruç and Sonal (16) investigated residues of oxytetracycline, penicillin G and sulfadimidine in 25 raw milk samples in Bursa using the HPLC method and reported no antibiotic residues in the samples. The results of this study were similar to the data of our study.

Nina et al. (15) used microbial tests and immunoassay method in the preliminary survey of 1,259 raw milk samples collected over a period of three years in

Croatia to identify the presence of certain antibiotics including chloramphenicol and reported the antibiotic residue in 37 samples. In the same study, the validation of the positive samples using the HPLC method demonstrated that only three samples contained residues above the permitted limit values (2 µg/kg penicillin, 19 µg/kg amoxicillin and 1.671 µg/kg tetracycline).

Navratilova et al. (14) studied 150 raw cow milk samples collected from the South Moravia and Vysočina regions of Czechia using the HPLC method and reported fluoroquinolone residues in 87.3% of the samples. The difference between the results of this study and the current study may have resulted from the fact that the samples analyzed were collected from different countries and in different periods.

Boultif (8) used the ELISA and HPLC methods to look for any residue of oxytetracycline and penicillin G in 120 milk samples in Algeria and reported oxytetracycline residues in 22 milk samples. The difference between the results of this study and the current study may have resulted from the fact that the samples analyzed were collected from different countries and in different periods.

In the study, the residual presence of enrofloxacin, sulfamethoxazole, tylosin, penicillin G and oxytetracycline in raw goat milk collected from Ankara, Kırıkkale and Çankırı provinces was investigated by HPLC method. No antibiotic residue could be detected in 150 milk samples. Considering that goats are more resistant to diseases than other animals whose milk is used, and therefore antibiotic use in goats is considered as at a lower level than other animals, it is predicted that this result has been achieved.

In the current study, the adaptation and validation of the test method for determining enrofloxacin, sulfamethoxazole, tylosin, penicillin G, and oxytetracycline in raw goat milk was performed. The method validated in the study was found to be fast, easy, practical and reliable for the analysis of the samples, and the chemical substances used were kept at a minimum, ensuring an analysis of more samples at a shorter time and with lower costs, which were considered the advantages of the method selected. Likewise, the ability to analyze five different active substances with a simple application following a single process of extraction highlights the usability of the method. In this regard, this method may be useful in ensuring that analysis for residue monitoring can be performed rapidly, and this may allow it to be used in a more widespread manner.

When the reasons for drug residues in animal source foods are examined, the failure to comply with the waiting period before slaughtering generally stands out. Therefore, it is critical to inform the breeders who use drugs on animals of this requirement. Likewise, awareness

raising activities targeting veterinary physicians, animal breeders, facilities producing or selling veterinary drugs, public organizations and institutions and consumers are important as well. In this context, rational drug use, compliance with principles of preventive medicine and, in particular, use of prescription drugs is obligatory.

Considering the results of this study, it can be said that it is good news for public health that no residue from the antibiotics in question could be determined in the goat milks collected from the specified regions. It was concluded that the national residue monitoring programs, the activities in which the importance of lack of residues is stressed for breeders, the antibiotic awareness week, and the efforts and programs implemented as part of the One Health Approach were effective in obtaining these results. It should be remembered that such activities and programs should be maintained in the future as the residue problem is a matter that continues to be relevant at all times and that has an international dimension. Even though such analyses are performed within the framework of the National Residue Monitoring Program, the number of analyses performed on samples of other types of milk than cow milk, such as goat milk, water buffalo milk and other milks produced less compared to cow milk, but enjoying increased popularity should be supported. This study was conducted on a sizable number of goat milk samples and did not find any risk factor for public health in them in terms of antibiotics examined.

Acknowledgements

This study was derived from the PhD thesis of the first author.

Financial support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

LA and ATM conceived and planned the experiments. ATM and YA carried out the experiments. LA, ATM and YA planned and carried out the simulations. LA and ATM contributed to sample preparation. LA, ATM and YA contributed to the interpretation of the results. ATM took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study did not present any ethical concerns.

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