



## **Residue Analysis of Oxytetracycline in Milk Sample by Two Different Chromatographic Methods and Determination of Ionization Constant Values**

**Seyfi Sardoğan<sup>1</sup>, Senem Şanlı<sup>2,3\*</sup>, Barış Sardoğan<sup>4</sup> and Barış Atalay<sup>5</sup>**

<sup>1</sup>Department of Food Engineering, Faculty of Engineering, Uşak University, Uşak, Turkey.

<sup>2</sup>Department of Chemistry, Faculty of Science and Arts, Uşak University, Uşak, Turkey.

<sup>3</sup>Department of Medical Laboratory Techniques, Vocational School of Health Services,  
Uşak University, Uşak, Turkey.

<sup>4</sup>Department of Rectorate, Uşak University, Uşak, Turkey.

<sup>5</sup>İzmir Kavram Vocational College of Higher Education, İzmir, Turkey.

### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author Seyfi Sardoğan designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors Senem Şanlı and BS managed the analyses of the study. Author BA managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

In the present study, two analytical methods for the residue analysis of oxytetracycline in milk sample have been generated. In HPLC method, the analysis was performed on an X Terra RP-18 column at 25 °C with the mobile phase as methanol: water (20 : 80 )(v/v) modified to pH 5. For the second method capillary electrophoresis system was used. The analysis of oxytetracycline in milk sample could be achieved without using organic modifier in a 58 cm length capillary at a working voltage of 12 kV with 20 mM NaH<sub>2</sub>PO<sub>4</sub>-H<sub>3</sub>PO<sub>4</sub> (pH 7) by capillary electrophoresis. Tetracycline was used as internal standard in both methods. The results calculated from both methods were compared to each other. The calculated data for drugs was checked with the data predicted by the SPARC on-line pK<sub>a</sub> estimator.

\*Corresponding author: E-mail: [senem.sanli@usak.edu.tr](mailto:senem.sanli@usak.edu.tr), [senemkamacisanli@gmail.com](mailto:senemkamacisanli@gmail.com);

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## 1. INTRODUCTION

Milk is an important constituent of human diet. It is consumed by all age groups particularly children and elderly people. For this reason, the quality of milk produced and distributed is very important. In some cases milk may contain unwanted residues such as antibiotic residues, pesticides in nature, heavy metals and mycotoxins. Among these, antibiotic residues are the most important one because they cause serious problems to human health in the future.

One of the most commonly used antimicrobial drugs in animal food production is the tetracycline group [1]. Tetracycline antibiotics have a broad antimicrobial effect, especially on gram-positive bacteria, but have a weaker effect on gram-negative bacteria. In addition, these antibiotics have an effect on mycoplasmas, chlamydiae, rickettsias, spirochetes, actinomycetes, and some protozoa [2]. These drugs cannot be employed in children up to age of 6-8 years. They also may cause secondary tooth discoloration in pregnant women. Therefore, usage of these drugs is not suitable for pregnant women. Other chronic effects include nephrotoxicity, hepatotoxicity, skin hyperpigmentation in sun-exposed areas and hypersensitivity reactions.

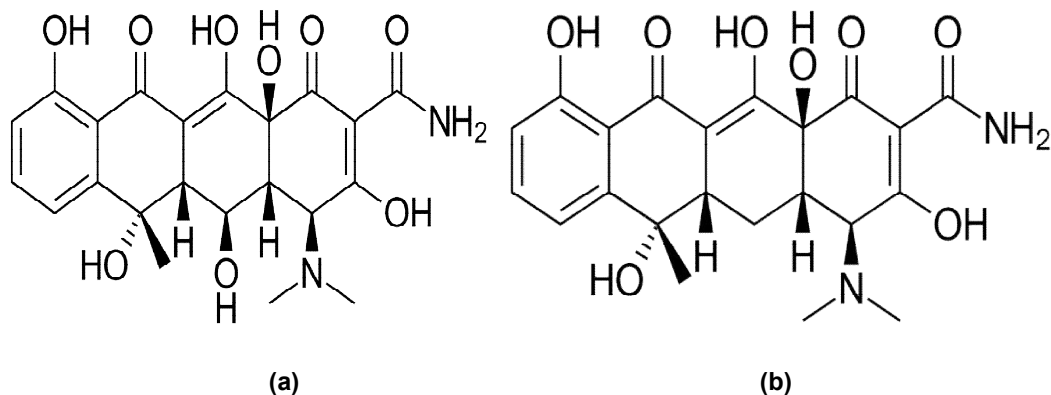
Oxytetracycline (OTC) is a drug used for especially treatment of bovine mastitis. It is also added for collective prophylaxis at therapeutic concentrations in cattle nutrition [3]. Because oxytetracycline is too much employed in veterinary area, there is a need to monitor

residual grades of this drug in milk to meet government requirements for tolerance limits. The maximum residual limit set by the EU legislation for oxytetracycline in raw cow milk is set to 0.1 mg/kg (100 ng/g). Several chromatographic techniques have been used for determination of oxytetracycline by using high-performance liquid chromatography (HPLC) with ultra-violet (UV) absorption [4-8] and capillary electrophoresis [9,10].

CE has high efficiency, rapidity, and small sample volume. Also it is possible to analyse without using organic solvent by CE. High Performance Liquid Chromatography (HPLC) detection also have significant benefit, such as rapid set-up of instrumentation, versatility and low cost. The other advantage of HPLC is that, it allows the analysis of heat-degrading and non-volatile compounds with high polarity.

The information about acid-base equilibria of tetracyclines has major pharmacological significance. The lipophilicity, solubility and permeability of these drugs are  $pK_a$  dependent, so it is significant to achieve trustworthy data about  $pK_a$  in the drug improvement process [11]. Only a few  $pK_a$  datas of tetracycline (TC) and oxytetracycline (OTC) are seen in the literature [12-14].

In this study, residue analysis of oxytetracycline in milk sample was performed by two different chromatographic methods. Also  $pK_a$  values of tetracycline and oxytetracycline (Fig. 1) in three different MeOH-water mixtures were determined by HPLC method.



**Fig. 1. Chemical structure of oxytetracycline (a) and tetracycline (b)**

## 2. EXPERIMENTAL

### 2.1 Chemicals and Reagents

Tetracycline and oxytetracycline were purchased from Sigma. Methanol was obtained from Fisher Scientific (Pittsburgh, PA, USA). Phosphoric acid, hydrochloric acid, sodium hydroxide and ethyl acetate (NaOH) were procured from Sigma (St. Louis MO, USA). Stock standard solutions of OTC and TC were made ready in MeOH at 100 µg/mL. Working solutions were diluted with the corresponding mobile phase to 10 µg/mL. The dead time ( $t_0$ ) was calculated by injecting uracil solution [0.01% (v/w), in water] which was set up for every mobile phase combination and pH. 15 mM  $\text{NaH}_2\text{PO}_4\text{-H}_3\text{PO}_4$  buffer was used for CE analysis and prepared in ultrapure water. Tetracycline was selected as the internal standard for oxtetracycline assay.

### 2.2 Apparatus

Agilent Technologies G7100A Capillary Electrophoresis Apparatus with a diode array detector was utilized for all analysis. A 55 cm x 50 µm i.d. fused silica capillary (Agilent Technologies Ext. Light Path) with an effective length of 45 cm was chosen for analysis. A Fisher Scientific AB 15 pH/ion analyzer with Fisher Scientific Acument combination pH electrode was utilized for pH measurement.

The HPLC analysis was carried out on an Agilent 1260 series HPLC system with ternary solvent pump, online degasser, automatic injection system, column heater and multi wavelength detector was used. UV identification was carried out at 220 nm for TC and OTC. Analysis was run at a flow rate of 1 mL.min<sup>-1</sup>. An Xterra RP-18 (150 x 4.60 mm i.d. x 5 µm) column was selected as stationary phase at 25°C.

## 3. PROCEDURE

### 3.1 $pK_a$ Analysis

For  $pK_a$  analysis, as mobile phase MeOH–water at 20, 25 and 30% (v/v), containing 20 mM  $\text{NaH}_2\text{PO}_4$  were used. Injection of TC and OTC standard solutions were performed between mobile phase pH 2.5-6. At 1.0 mL/min flow rate was chosen and 20 µL of TC and OTC were injected to HPLC system. For calculation of retention time, three injection of drugs for every mobile phase and pH were done.  $k = (t_R - t_0) / t_0$

equation was used for calculation of retention factor data. The dead time ( $t_0$ ) was calculated by injecting uracil standard for every mobile phase and pH. The  $pK_a$  values of TC and OTC were obtained by using NLREG program [15].

### 3.2 Sample Preparation Procedure

Milk samples were taken 12 hours after the animals injected with oxytetracycline drug. One milliliter of milk was measured into a conical flask, and 3 mL of phosphate buffer (pH = 3.4), 1 mL of acetonitrile and 1 mL 5 M NaOH were added; the flask was whirled later every addition to solution the contents. In this way, fat and protein were precipitated in milk. After 5 min, 1 mL sample was taken and filtered. At the end, internal standard was added, milk samples were injected to capillary electrophoresis and HPLC system, respectively.

## 4. RESULTS AND DISCUSSION

### 4.1 Determination of $pK_a$ Values

The tetracyclines contain different ionizable functional groups. These group antibiotics show amphoteric behavior because of acidic substituents and the basic dimethylamino group. The first dissociation constant ( $pK_{a1}$ ) is associated with tricarbonyl group (Fig. 1). The retention factor of both these compounds and the  $pK_{a1}$  value were determined by using Xterra RP-18 (150 x 4.60 mm i.d. x 5 µm) column.

Several methods for  $pK_a$  analysis have been used such as spectrophotometry, potentiometry, and etc. Among these techniques HPLC has been widely used for practical and accurate  $pK_a$  determination. In this study from these reason, HPLC was chosen for  $pK_a$  analysis of TC and OTC. In Table 1, calculated  $pK_a$  data with the values predicted by SPARC [16] are given. All of the data were compatible. In Fig. 2, data pairs of  $k/pH$  for tetracycline in 20% (v/v) MeOH-water is indicated and the correlation among the experimental capacity factors of the drugs investigated over the whole experimental pH interval was well. The results showed that  $pK_a$  values increased slightly by increasing percentage of methanol.

### 4.2 Analysis of Milk Sample

About 1% animal origin products in the USA and in Europe contain antibiotic residues in very low concentrations. The cause for the incidence of

the antibiotic residues in milk is in 92% due to their administration in mastitis therapy. In most countries, the most frequently detected antibiotics are  $\beta$ -lactam antibiotics while tetracyclines are detected in milk rarely only [1].

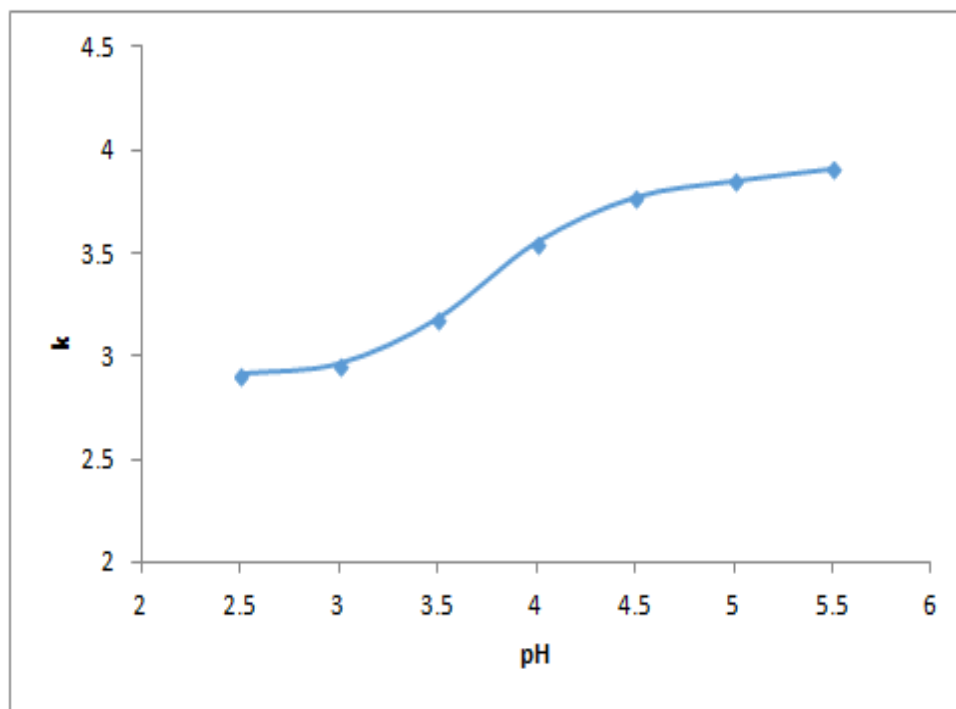
In this study, two different chromatographic techniques were chosen for the analysis of oxytetracycline from milk samples. HPLC separation was obtained using X Terra RP-18 (150 x 4.60 mm i.d. x 5  $\mu$ m) column at 25 °C, with a mobile methanol-water (20:80, v/v) phosphate buffer pH 5.0 at flow rate 1 mL/min. Under these conditions, analysis time was about nine minutes with symmetrical peaks. A chromatogram of OTC and TC standards at 220 nm was given in Fig. 3.

For capillary method, several pH values, ejection time and separation voltage were examined for

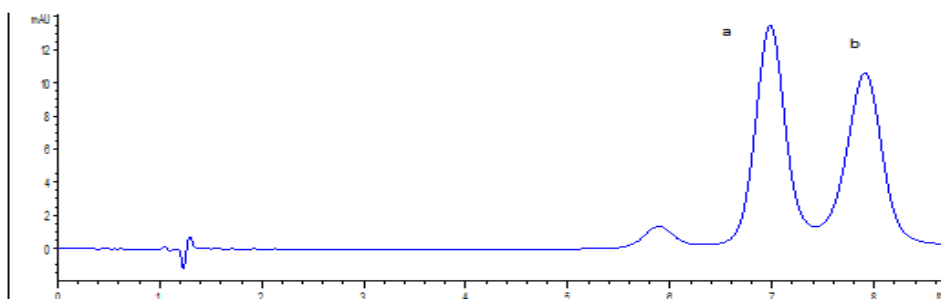
getting the better peak shape, peak current and sharp response. 15 mM  $\text{NaH}_2\text{PO}_4$ -  $\text{H}_3\text{PO}_4$  was used as the buffer for capillary system. pH 7.0 was selected as a optimum pH according to peak shape and resolution. Voltage of 12 kV was chosen for reasonable analysis time with best peak efficiency. Injection time is also an important parameter for CE. Three different injection time was investigated (3, 5 and 10 s). While increasing injection time, peak area is increased, however peak broadening is observed. Therefore 0.5 psi injection pressure for period of 5 s was chosen to get better sensitivity without peak broadening. The optimum was achieved at applied voltage 12 kV, with an analysis time of within 30 min. The resulted electropherogram under this optimum operation condition is shown in Fig. 4. The analysis could be performed without using organic solvent.

**Table 1. The  $pK_a$  data of TC and OTC estimated by SPARC and calculated by HPLC method**

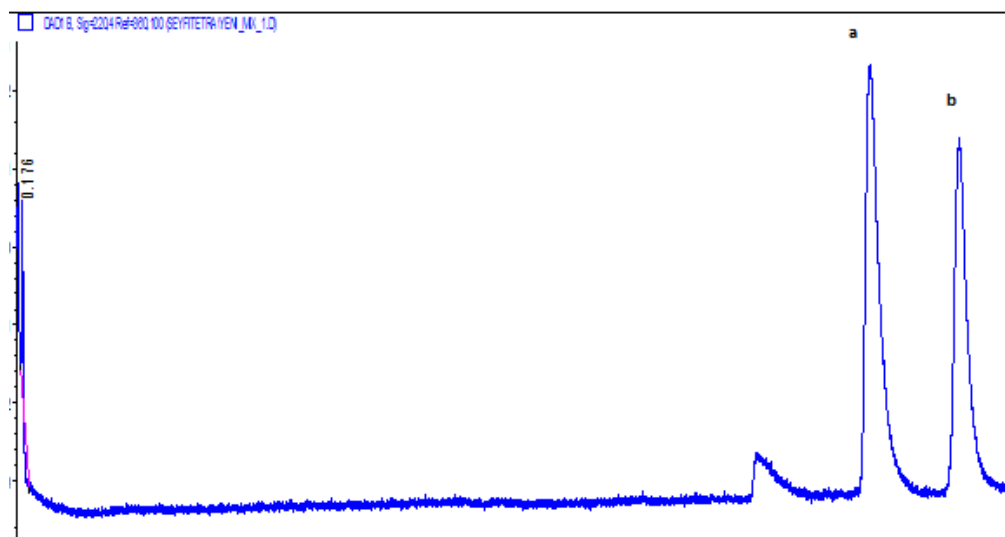
Drugs	SPARC	NLREG		
		20 % (v/v) MeOH	25 % (v/v) MeOH	30 % (v/v) MeOH
Tetracycline	3.01	3.78 $\pm$ 0.06	3.84 $\pm$ 0.04	3.93 $\pm$ 0.08
Oxytetracycline	3.79	3.24 $\pm$ 0.17	3.35 $\pm$ 0.12	3.51 $\pm$ 0.14



**Fig. 2. Plot of chromatographic retention factor,  $k$ , vs. the pH of mobile phase of tetracycline in 20 % (v/v) MeOH-water mixture**



**Fig. 3. Chromatogram of standard mixture of OTC and TC, mobile phase containing 4.0  $\mu\text{g mL}^{-1}$  TC (a, IS) and 8.0  $\mu\text{g mL}^{-1}$  OTC (b).**



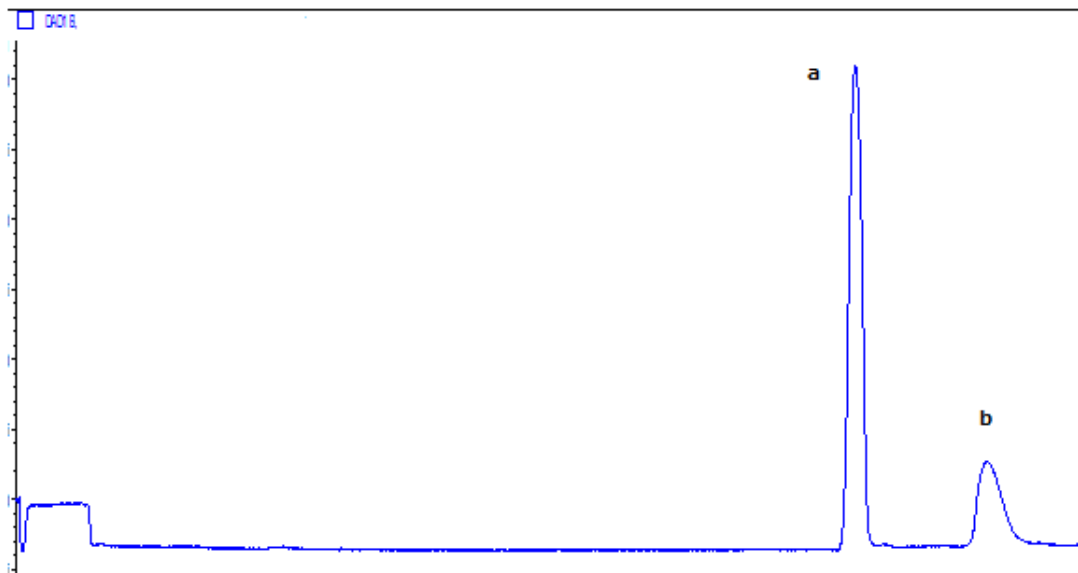
**Fig. 4. Representative electropherogram obtained from a standard mixture of TC and OTC under optimum conditions, containing 10  $\mu\text{g mL}^{-1}$  TC (a) and 10  $\mu\text{g mL}^{-1}$  OTC (b). Running buffer 15 mM  $\text{NaH}_2\text{PO}_4$ -  $\text{H}_3\text{PO}_4$  buffer solution (pH 7.0); uncoated fused silica capillary 55 cm (45 cm to detector) 50  $\mu\text{m}$  i.d.; applied voltage 26 kV; detection UV absorbance at 220 nm; pressure injection, 0.5 psi for 5 s**

For both the methods, the calibration graph was constructed by linear least squares regression. Tetracycline was used for internal standard at 4  $\mu\text{g/mL}$  for HPLC and 10  $\mu\text{g/mL}$  for CE. The linearity was calculated by plotting the peak area ratio of oxytetracycline to tetracycline vs. concentration of the drug. The developed HPLC

and CE methods data were reported in Table 2. The methods showed good linearity based on a correlation coefficient  $> 0.999$  for TC. The LOD and LOQ were calculated as  $\text{LOD} = 3.3s m^{-1}$  and  $\text{LOQ} = 10.0 s m^{-1}$  where  $s$  is the standard deviation of response and  $m$  is the slope of the calibration curve [17].

**Table 2. Statistical evaluation of the calibration graph of OTC by HPLC and CE**

	OTC (HPLC)	OTC (CE)
Linearity range ( $\mu\text{g/mL}$ )	1-12 ( $n = 5$ )	2-50 ( $n = 5$ )
Slope	0.145	0.068
Intercept	-0.076	0.032
Correlation coefficient ( $r$ )	0.999	0.999
Detection limit (LOD) ( $\mu\text{g/mL}$ )	0.002	0.049
Quantitation limit (LOQ) ( $\mu\text{g/mL}$ )	0.007	0.148



**Fig. 5. Electropherogram of milk sample spiked with  $10 \mu\text{g mL}^{-1}$  TC (a) and OTC (b)**

The methods were finally used in the determination of oxytetracycline in milk sample which was taken 12 hours from the animal after the injection of oxytetracycline. After the analysis of milk samples, oxytetracycline amounts were calculated as  $179 \mu\text{g/L}$  by CE and  $158 \mu\text{g/L}$  by HPLC. It was seen that the values obtained by these two chromatographic techniques have been found very close to each other. As an example electrochromatogram of milk sample (spiked with tetracycline) was given in Fig. 5. The recoveries were obtained by spiking milk  $100 \mu\text{g/L}$  OTC and  $97.3\%$  and  $96.8\%$  recovery values were calculated by HPLC and CE, respectively ( $n=5$ ).

In the literature, there is no method for determination of OTC by two different comparable chromatographic methods in milk sample. The HPLC method was developed for determination of OTC in milk sample by Priyanka et al. [6]; the LOD and LOQ values were reported as  $48 \mu\text{g/kg}$  and  $98 \mu\text{g/kg}$  respectively with longer analysis time. Biswas et al. [18] reported for the LOD values of tetracyclines as  $0.031 \mu\text{g/g}$ .

Tetracycline antibiotics determination in milk with CE (UV detection) by Mu et al. [19] They applied their method to the assay of five different commercial milk samples and all the tests gave negative results. LOD value was calculated for OTC as a  $0.0745 \mu\text{g/mL}$ .

The HPLC method described above provides a combination of faster analysis time and improved limits of detection for OTC. Also by CE methods OTC could be detected without using organic modifier with low limit of detection value.

## 5. CONCLUSION

The calculation of  $pK_a$  datas of TC and OTC in MeOH-water mixture were done for the first time by liquid chromatography with this study. The lipophilicity, solubility and permeability of drugs are  $pK_a$  dependent. The  $pK_a$  value can also affect drug-receptor binding. Therefore  $pK_a$  value of TC and OTC were determined and reported. Also in literature, there is no method for the residue analysis of OTC in milk sample by capillary electrophoresis method. Also by CE analysis just water was used for solvent. This is important for Green Chemistry. For milk sample, results which calculated by CE and HPLC have been found close to each other.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## ACKNOWLEDGEMENT

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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