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Determination of tetracyclines in ovine milk by high-performance liquid chromatography with a coulometric electrode array system

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Abstract

A method has been developed to analyze residual tetracyclines (TCs) (oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), methacycline (MTC), doxycycline (DC)) in ovine milk, using high-performance liquid chromatography (HPLC) with a coulometric electrode array system. The samples were pretreated, using liquid–liquid extraction based on hexane. The chromatography was performed, using a C_{18} column (150 mm × 4 mm i.d. and 5 μm) with a mobile phase: sodium phosphate monobasic dihydrate (pH 2.2, 0.05 M)–acetonitrile (78:22, v/v). The flow rate of mobile phase was kept constantly at 1ml/min. The residues were monitored by an ESA electrochemical detector. Potentials of four electrodes in series were set at 400, 660, 680 and 700 mV, respectively. The first electrode was set to remove those interfering substances that may co-elute with TCs and the other three electrodes were used for quantification. The maximal potential of our detection was 700 mV. Calibration curve showed good linearity and the detection limit of TCs was 12.5, 20, 25, 10 and 25 ng/ml, respectively. Optimization of the pH of the mobile phase, the proportion of acetonitrile and the pH of the pretreatment were also performed. Recoveries of TCs from spiked samples were more than 88% and the relative standard deviations were less than 4.3%. This method was reliable, sensitive, economical and suited for routine monitoring of TC residues in ovine dairy milk.

Keywords: Milk; HPLC; Tetracyclines; Electrochemical detector

1. Introduction

Tetracyclines are an extremely important group of antibiotics having broad spectrum of activity against Gram-positive and Gram-negative bacteria, some large viruses, rickettsiae, spirochetes and mycoplasmas. They are widely used as feed additives to prevent or treat mastitis and metritis in cows. As a result, the residues of tetracyclines (TCs) may be retained in milk. The presence of these residues may cause allergic reactions in sensitive individuals [1]. Even more important, low-level doses of antibiotic in food stuffs consumed for long periods can lead to problems regarding the spread of drug-resistant micro-organisms [2]. To prevent any health problems with consumers, FAO/World Health Organization (WHO), US Food and Drug Administration (FDA), European Union (EU) and Japan (Japanese Food Sanitation Laws) have been established their maximum residue limit (MRL) in milk (0.1 μg/ml) [3–6]. To establish rigorously whether TC residues are present in milk, an analytical method for routine monitoring of TC residues in milk must be accurate, rapid, simple, economical in cost and time and capable of detecting the residues below MRLs.

To determine TC residues in milk, isolating them from milk is required first. One widely used technique for isolation is C_{18} solid-phase (SPE) column [7–10]. However, it is expensive for routine use. Moat and Harik-Khan [11] extracted samples with hydrochloric acid-acetonitrile, but this method does not deproteinate completely.

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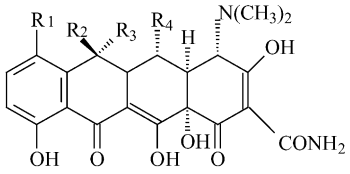
To detect TCs, the conventional and official methods of assay are the microbiological approach [12,13], these methods are not only expensive and time consuming but also poor in terms of sensitivity and specificity. Thus, there are many other methods to replace the microbiological assay. Among them, high-performance liquid chromatography (HPLC) is most widely used. Chen and Gu used UV detection at 370 nm [8]. Furasawa used photodiode array detection (DAD) [10]. However, these detections were short of selectivity and stability. McCracken et al. used HPLC method with fluorescence detection after a post-column derivatisation with aluminum ion [14] and Karno Ng used laser-based polarimetric detection [15]. These detections were more selective, but they may be time consuming.

Each tetracycline (Fig. 1) contains a phenolic substituent on position 10 and a dimethylamino substituent on position 4. In electrochemical detection (ED), the oxidation of the tetracyclins could occur through these moieties. Therefore, electrochemical detection, regarded as being high degree of selectivity, is suitable for applying to detect TCs. However, up to now, except for one report concerning the electrochemical behavior of four TCs [16] and one article about the detection of tetracycline antibiotics in pharmaceutical formulations [17], TC residues in milk have not been investigated by HPLC–ED.

This paper described a simple, sensitive and practical method for routine monitoring TC residues in milk. Samples were pretreated with hydrochloric acid–acetonitrile–hexane. This way was economical compared with SPE column and the recoveries were more than 88%. The coulomitric electrode array detection was chosen to detect TCs. This detection consists of 16 electrochemical cells arranged in series and the potential of each cell can be set independently. This enables the concurrent detection of different chemical classes, each at their optimal potential settings. So, the detection system can offer superior sensitivity over other detectors and co-eluting analytes can be resolved by making use of differences in their electrochemical behavior.



	R_1	R_2	R_3	R_4
Oxytetracycline (OTC)	H	CH_3	OH	OH
Tetracycline (TC)	H	CH_3	OH	H
Chlortetracycline (CTC)	Cl	CH_3	OH	H
Metlacycline (MTC)	Cl	$=CH_2$		OH
Doxycycline (DC)	H	CH_3	H	OH

Fig. 1. Tetracycline structures.

2. Experimental

2.1. Chemicals and reagents

Standards of OTC, TC, CTC, MTC and DC were bought from the Institute of Chinese Pharmaceutical and Biological Product Inspection (Beijing, China). Sodium phosphate monobasic dihydrate, hydrochoric acid, phosphoric acid and hexane were analytical grade from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were HPLC purity reagents from Fisher Scientific (NJ, USA). The water used in all experiments had a resistivity of 18.2 MΩ cm obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The primary stock standard solutions of OTC, TC, CTC, MTC and DC, respectively, were prepared by accurately weighing 10 mg of each compound, dissolving them in 2 ml of 0.1 M hydrochloric acid and diluting them with methanol to obtain a final solutions of 1 mg/ml. These standard solutions were stored at 4 °C for 1 week. Working mixed standard solutions of five compounds were prepared daily by mixing and diluting the stock solutions with methanol.

2.2. Apparatus

The HPLC system was an ESA chromatographic system (ESA, Chelmsford, MA, USA) equipped with two 582 pumps, an organizer chamber, a manual injector fitted with a 20 μl loop (Rheodyne 7725i, CA, USA) and a 5600A 16 channels CoulArray detector. ESA software was used for data acquisition and processing. In addition, Sartorius BS 110S electronic balance (Beijing, China). WTW inolab pH level 1 pH meter (Weiheim, Germany) and Dupont Sorvall RC 5C plus centrifuge (Newtown, CT, USA) were used in the study.

2.3. Chromatographic conditions

TCs were analyzed by reversed-phase HPLC analysis, using ODS Hypersil (150 mm × 4 mm i.d. and 5 μm particle size, Hewlett-Packard, USA) column. A guard column (Hypersil, 5 μm, Alltech Associates, USA) was used to protect the analysis column. The analyses were carried out at room temperature. The mobile phase was 0.05 M sodium phosphate monobasic dihydrate (pH 2.2)–acetonitrile (78:22, v/v). The flow rate was kept constantly at 1 ml/min. The injection volume was 20 μl. Mobile phase was prepared daily, filtered through a 0.22 μm membrane and sonicated before use. The electrodes potential were set at 400, 660, 680 and 700 mV, respectively. Typical retention time of OTC, TC, CTC, MTC and DC was obtained from final system was 1.88, 2.33, 4.88, 5.68 and 6.60 min, respectively.

In some papers, about TCs, it was mentioned that EDTA should be added to mobile phase to avoid the interferences from metals. But according to Kazemifard [17], ODS column, which was also used in my experiment, could separate tetracyclines with no need of EDTA. Moreover, different concentrations of ferrous sulphate, calcium chloride and ferric chloride, respectively, were added to the standard solutions of TCs in my experiment. The results were that the peak heights of TCs did not change significantly compared with the black standard solutions. Therefore, interferences from metals were neglectable and acceptable in my system.

2.4. Liquid–liquid extraction procedure

Ten millilitres of milk adjusted to pH 4 with 1 M hydrochloric acid, was mixed with 40 ml acetonitrile. The mixture was sonicated for 5 min and centrifuged for 10 min at 8000 × *g*. The supernatant was decanted and placed at another container. Thirty millilitres of hexane was added to extract twice. The lower layer (aqueous phase) was transferred into a 100-ml flask. The remaining organic layer was washed with 2 ml water. The aqueous phases were mixed and the mixture was evaporated to less than 10 ml under vacuum at 50 °C. The residue was diluted with methanol to 10 ml, passed through a 0.22 μm filter and 20 μl was injected in the HPLC–ED system.

3. Results and discussion

3.1. Voltammetric behavior of TCs

Proper selection of applied electrode potentials is critical for accurate measurement. In this paper, the working potentials of the detector cells were chosen through experimentation under the analytical conditions.

One microgram per milliliter standard of OTC, TC, CTC, MTC and DC was used in this experimentation. Only one electrode was selected to ensure that there were enough standards to be oxidized at each potential. The potential of the selected electrode started at 100 mV and was increased by 50 mV until 600 mV, then increased by 20 mV until 700 mV, which was the maximal potential of the detector. The relationship between the peak height (current) and potential (voltage) was shown in Fig. 2. From Fig. 2, it can be seen that OTC, TC can be oxidized at 550 mV, MTC at 450 mV and CTC, DC at 500 mV. The peak heights of all five TCs increased when the potential increased. Theoretically, the optimal potential selected for working was at the plateau, which meant that no significant increase in peak height occurred when the potential was increased. At 650 mV, the peak heights were small slightly for experiments. At 660 mV, though there was not a plateau, the peak height of each compound was high enough to detect the concentrate needed. Therefore, the following potentials were selected for electrodes: 400, 660, 680 and

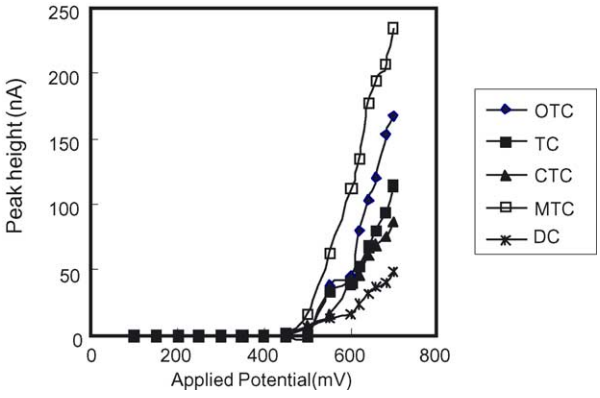


Fig. 2. Hydrodynamic voltammograms of TCs (OTC and TC 4.00 μg/ml; CTC, MTC and DC 3.00 μg/ml).

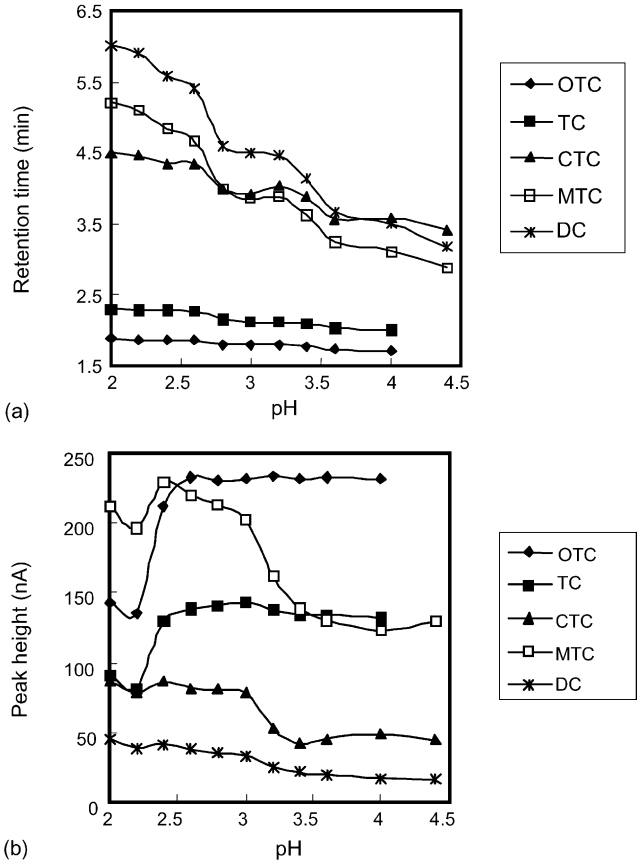


Fig. 3. Effect of pH on (a) retention time of TCs (OTC and TC 4.00 μg/ml; CTC, MTC and DC 3.00 μg/ml); (b) peak height of TCs (OTC and TC 4.00 μg/ml; CTC, MTC and DC 3.00 μg/ml).

Table 1
Linearities and detection limits for TCs

Analytes	Linear equation[a]	R^2	Detection limit (ng/ml)
OTC	$H = 0.1372c + 0.0018$	0.9999	12.5
TC	$H = 0.0854c + 0.0043$	0.9995	20
CTC	$H = 0.0829c + 0.0007$	0.9995	25
MTC	$H = 0.2240c + 0.0009$	0.9999	10
DC	$H = 0.0440c + 0.0008$	0.9997	25

[a] *H*: peak height response (nA); *c*: concentration of analytes (μg/ml).

Table 2
Recovery of TCs from spiked milk samples ($n = 6$[a])

Analytes	Intra-day				Inter-day			
	Spiked (μg/ml)	Concentration found		Recovery (%)	Spiked (μg/ml)	Concentration found		Recovery (%)
		Mean	R.S.D. (%)			Mean	R.S.D. (%)	
OTC	0.10	0.092	0.6	92.2	0.10	0.093	0.7	92.5
	0.25	0.232	2.8	92.9	0.25	0.230	2.9	92.1
	0.50	0.479	3.4	95.6	0.50	0.480	3.6	96.2
TC	0.10	0.090	1.7	90.0	0.10	0.090	1.6	89.6
	0.25	0.234	1.2	93.8	0.25	0.234	0.9	93.5
	0.50	0.468	1.9	93.6	0.50	0.470	2.0	93.9
CTC	0.10	0.093	0.2	92.9	0.10	0.093	0.2	92.9
	0.25	0.222	1.2	88.6	0.25	0.221	1.2	88.4
	0.50	0.455	2.0	91.0	0.50	0.452	1.8	90.4
MTC	0.10	0.093	0.5	93.4	0.10	0.094	0.6	93.5
	0.25	0.236	1.3	94.4	0.25	0.235	1.1	94.1
	0.50	0.469	1.4	93.8	0.50	0.463	3.6	92.5
DC	0.10	0.094	1.5	94.2	0.10	0.094	1.0	93.7
	0.25	0.231	2.2	92.5	0.25	0.230	2.2	92.0
	0.50	0.456	3.0	91.1	0.50	0.459	4.3	91.8

[a] $n = 6$ means that the same sample has been pretreated by the same process on 6 consecutive days.

700 mV. The last three potentials were selected to provide adequate sensitivity for TCs determination. The first electrode was set to remove those interfering substances that may coelute with TCs and can be oxidized at low potential. This is one of the advantages of the coulometric electrode array system.

In addition, under this system, peaks can be confirmed as the same compound when two terms were satisfied at the same time. First, the retention time of the unknown peak was the same as the retention time of standard. Second, the proportion of the signal between adjacent electrodes of the peak was the same as the proportion of standard. For example, the peak height ratio of the standard of CTC between channel 2 and 3, which was automatically calculated, was 1.36. If the corresponding ratio of the unknown peak, which had the same retention time of CTC, was within 10% of 1.36, the system could deem this unknown peak as CTC. Otherwise, though the peak had the same retention time of CTC, it cannot be confirmed as CTC. This cannot be carried out using the single channel detection system, which only distinguished compounds based on the retention time. Identifying compound through comparing ratio between adjacent electrodes is another advantage of the coulometric electrode array system.

3.2. Optimization of the pH of the mobile phase

In the introduction, it is mentioned that there are two substituents that can be oxidized. So, the pH of the mobile phase can influence the formation of TCs in the solution. To analyze TCs, pH must be in acidic medium to minimize the formation of isomeric analogues, which occurs rapidly in alkaline medium [18]. Furthermore, the pH of mobile phase has effect on both the retention time and the peak height of TCs.

The relationship between pH and the retention time, pH and the peak height was shown in Fig. 3 a and b, respectively. From Fig. 3a, it can be seen that the retentions time of TCs decreased with increasing pH. The retention times of OTC and TC were so early that their peaks were not separated from the peak of solutions at pH 4.4. From Fig. 3b, the peaks of TCs changed greatly from pH 2.0 to 2.2. Because, pH 2.0 is the lowest value my column could bear and the longevity of the column could be effected heavily if experiments were operated under this condition. Therefore, pH 2.2, at which

Table 3
Comparing HPLC–ED method with DAD method

Analyte	Spiked 0.10 μg/ml		Spiked 0.50 μg/ml	
	HPLC–ED method (%)	DAD method (%)	HPLC–ED method (%)	DAD method (%)
OTC	92.2	91.6	95.6	91.2
TC	90.0	90.8	93.6	92.8
CTC	92.9	80.8	91.0	87.3
MTC	93.4	–	93.8	–
DC	94.2	81.2	91.1	85.2

(–): MTC was not contained in his experiment.

the retention time could be short and all compounds could be separated from each other, was chosen.

3.3. Optimization of the pH of pretreatment

Adjusting the pH of samples not only ionizes TC compounds but also has effect of deproteinization. Samples were processed at pH 2, 3, 4, 5 and 6, respectively, and most protein was found to deposit between pH 4 and 5. In this pH range, most protein was removed from samples, which was favorable for the following analysis. Because, the TC molecules exist in the fully protonated forms as a singly charged cation [19] which is favorable for stability in acidic pH, the pH of pretreatment was chosen at 4.

3.4. Calibration range, linearity and limit of detection

Under optimized experimentation conditions, all five TCs showed good linearities between the concentrations and peak heights. The concentrations examined were 1, 0.5, 0.25, 0.1, 0.08 and 0.05 μg/ml for TCs. Therefore, concentration range of TCs we studied was 0.05–1 μg/ml. The peak heights of the second channel were applied to calibrate curves. The correlation coefficients (R^2) of calibration curves were between 0.9995 and 0.9999. These curves were used to calculate the unknown concentrations in the samples. The detection limits of TCs, defined as the signals three times the noise level, were also studied. All the results were listed in Table 1.

3.5. Recovery test

The average recoveries of TCs from milk samples at three different spiking levels (0.10, 0.25 and 0.50 μg/ml each compound) were summarized in Table 2. From the table, we can see that the average recoveries were more than 88% and the relative standard deviations (R.S.D.) were less than 4.3%. The results of intra- and inter-day were very similar which demonstrated good reproducibility and accuracy within the concentration range selected. The recoveries of 0.10 and 0.50 levels were compared with those in the method of processing milk with SPE column and detection by DAD [10] (Table 3). One of the chromatograms of recovery test was shown in Fig. 4. From (a) and (c) it can be seen that the first electrode does not oxide TCs compounds. It removed interfering substances that can be oxidized at low potential. It can also be seen that there were very high peaks below 2 min. It was because the samples and standards were diluted by methanol not by the mobile phase, so these big peaks below 2 min were the peaks of solution and not from interfering substances. From (b), it can be seen that there were not interfering substances at the retention time of TCs.

3.6. Real samples analysis

Six different labels of milk products were studied under optimized experimentation conditions. Two of them were not

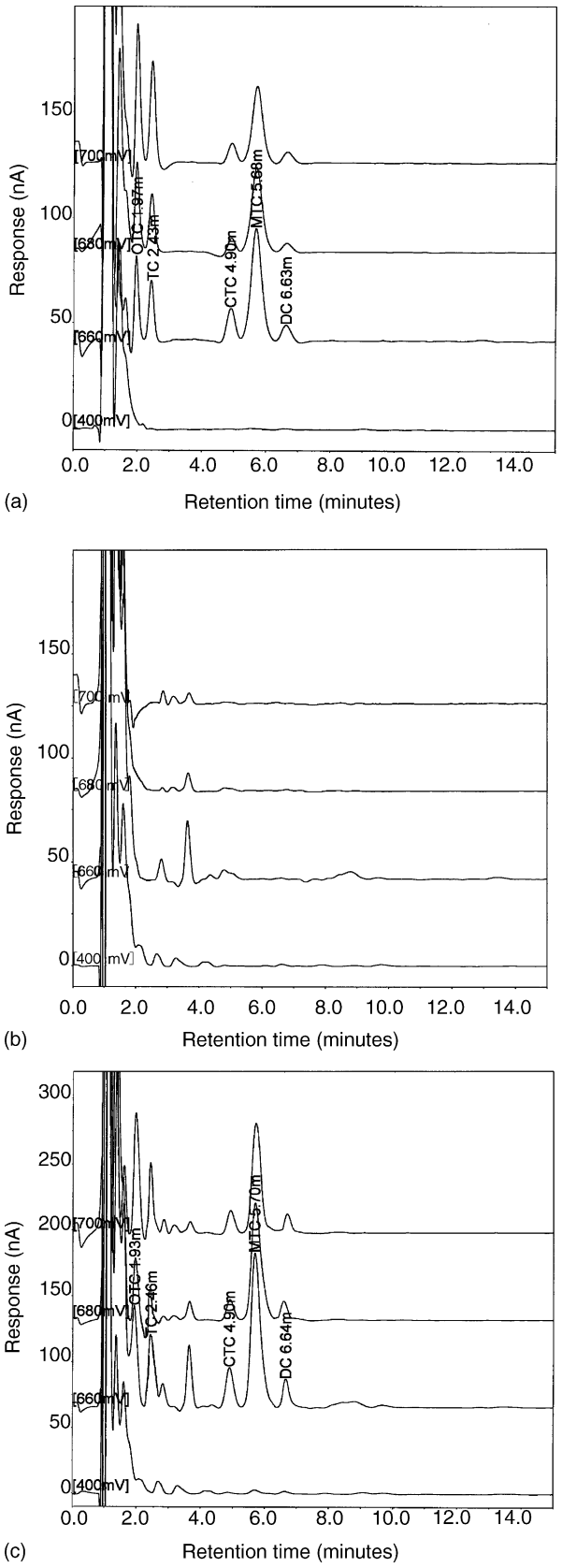


Fig. 4. HPLC chromatograms for (a) standard of TCs (0.10 μg/ml of each TC); (b) blank milk sample and (c) spiked (0.25 μg/ml of each TC) milk sample.

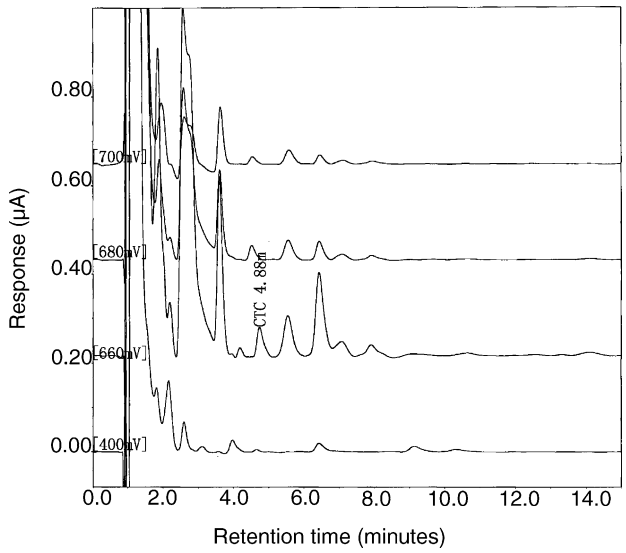


Fig. 5. The HPLC chromatography of a real sample.

certified, one had TC residue and the other had CTC. The chromatography of the later was shown (Fig. 5).

From Fig. 5, it can be seen obviously that the sample contain CTC and the concentrate of CTC was 5.4 μg/ml, which was fifty times more than MRL (0.1 μg/ml). Of course, this kind of milk was very harmful to people's health. So, it is necessary to monitor the products of milk.

4. Conclusions

A method has been developed for determining TC residues in ovine milk. The samples were pretreated with an extraction method, which was different from common methods. The target compounds were detected with an HPLC–ED system. The whole method is simple, accurate, selective and can detect the concentration of TC residues in ovine milk below MRLs. The recovery tests showed that this method was reliable. In addition, it took less than 30 min to pretreat samples and less than 15 min to analyze samples. All reagents and materials used are economical and easy to obtain. Therefore, the method is suitable for the routine monitoring of TC residues in ovine milk.

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