Original Research Article

Simultaneous Analysis of Trace Tetracyclines in Wastewater with Ultra-High Performance Liquid Chromatography Coupled with Mass Spectrometry

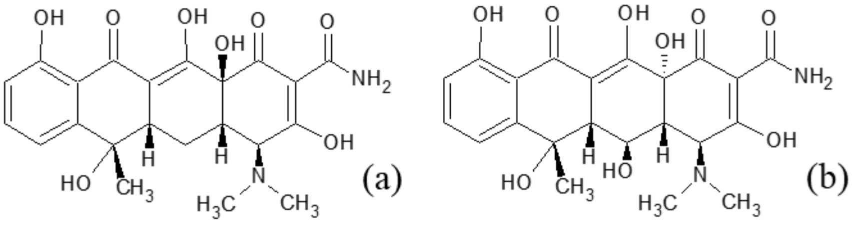
ABSTRACT

A comprehensive method was developed for determination of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DOC) in wastewater. Based on ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC–MS), the developed method presents several advantages such as high throughput, simple sample pretreatment. Specificity was confirmed with both retention time and accurate m/z value of four antibiotics. Good linearity was achieved in the range 5-100 ng/mL for target compounds. Repeatability of method was proved along with satisfied intra-day and inter-day precision. The LOD and LOQ was low enough at several ng/mL. Recoveries of three concentration levels were between 95% and 104%. The concrete sample collected in local swine farm was determined to containing 15 ng/mL OTC. The developed method provides a reliable and robust tool that can be used for routine analysis of TCs in aqueous samples.

Keywords: trace analysis; antibiotics; tetracycline; wastewater; UHPLC-MS

1. INTRODUCTION

Tetracyclines (TCs) are the second most common antibiotic group that be used throughout the world[1, 2]. This kind of antibiotics including oxytetracycline (OTC), chlortetracycline (CTC), tetracycline (TC), and doxycycline (DOC). Their molecules have the same core structure (Fig. 1). OTC, CTC, and TC are produced by radioactive bacteria. DOC is semi-synthetic derivatives as well as methacycline and dimethylamino tetracycline. Among these antibiotics, DOC is used in human clinical practice commonly [3]. OTC and TC are used in veterinary drugs. Tetracycline antibiotics are mainly applied for diseases caused by mycoplasma, chlamydia, rickettsia, and spirochete infection. The bactericidal mechanism is to inhibit bacterial protein synthesis. In more cases, tetracyclines are used to treat infections in poultry, husbandry and even aquaculture. In aquaculture, tetracycline is added directly to feed or water to control the infection [4].



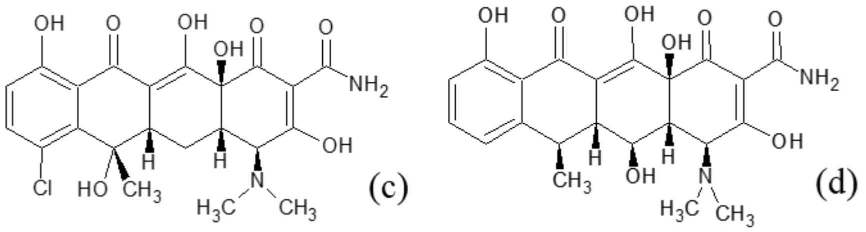


Fig. 1. Chemical structure of (a) tetracycline (TC), (b)oxytetracycline (OTC), (c) chlortetracycline (CTC), and (d) doxycycline (DOC).

Since tetracycline antibiotics are not easily decomposed, the residues of them have been recognized as a new class of pollutants[5]. Due to the high concentration in original dose usually, tetracyclines residues in water and surface soil have strong biological toxicity. Naturally, it has a great impact on human health and ecological environment. In China, the maximum residue limit (MRL) for veterinary drugs in foods is regulated by National food safety standards [6]. In Chinese regulation, the MRL of total TCs is 100 μg/kg for cow/goat milk, 400 μg/kg for egg, and 200 μg/kg for shrimp products. Meanwhile, the MRL of DOC is 10 μg/kg in egg.

In recent years, many methods have been proposed to eliminate tetracycline in water environment [7], such as electrochemical oxidation [8], microbial degradation [9], photocatalytic method[10], adsorption method[11, 12], advanced oxidation process[13] and so on[14, 15]. Despite of above researches, the real application of these techniques is limited. The residues of TCs in the environment are still common. Therefore, the monitoring of TCs in discharged wastewater is always important.

It could be found that the scientist generally employing spectroscopy method to determine the TCs in most degradation studies. The spectroscopy method is neither accurate nor specific for trace TCs in wastewater. Various other analytical protocols have been reported for the analysis of TCs in all kinds of matrixes. Reverse phase high performance liquid chromatography (RP-HPLC) coupled with UV[16, 17], PDA[18] and fluorescence [19] are traditional techniques. In the past few decades, mass spectrometry has been widely used in the detection of antibiotic residues. [16, 20, 21]. As an improvement to liquid chromatography, the ultra-high performance liquid chromatography (UHPLC) [22] is developed and been widely used. The better separation ability f of this technique is based on the evolution of packing materials and progress of the instrument [23]. As for the determination of multi antibiotics in water samples, there are analytical methods reported [24]. For example, SPE-LC/MS is a more complicated method [17, 25]. Although SPE-LC can detect antibiotics in the low ng/L range, it need SPE column and related instrument. The sample prepare process is more tedious. Therefore, it is still interest to develop a fast and uncomplicated method to detect TCs in wastewater, especially in wastewater that discharged from livestock and poultry farm.

This study describes a simple and comprehensive analysis method to analysis four mostly used tetracyclines including TC, OTC, CTC, and DOC by ultra-high performance liquid chromatography coupled with a high resolution mass spectrometer (UHPLC/MS). Four target antibiotics were determined simultaneously. Trace OTC residue in a local swine farm wastewater was detected. The method could be applied to other similar antibiotics detection as well.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

The standard materials of tetracycline (>99%), oxytetracycline (>98%), chlortetracycline (>80%, HPLC), and doxycycline (>95%) were purchased from Aladdin Biochemical Technology Co.,Ltd. (Shanghai, China). Acetonitrile and methanol (HPLC grade) were purchased from Tedia Company, Inc. (Fairfield, USA). Formic acid (>98.0%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Pure water was obtained form a Milli-Q system (Molsheim, France).

2.2. Instruments and condition

Ultra-high performance liquid chromatography was conducted on an instrument system consisted of Agilent Technologies 1290 Infinity binary pump, autosampler, column thermostat (Germany). The column employed is an ACQUITY UPLC BHE C18 column with size of 50mm × 2.1mm, 1.7μm (Waters Co., Singapore). Mass spectrometer is mode in triple TOF 5600 $^+$ (AB Sciex, Singapore).

The flow rate of chromatographic experiments was 0.2 mL/min. Mobile phase A was formic acid/water solution (0.1%, v/v) while the mobile phase B was methanol. The sampling volume for all samples was 3 μL. The column temperature was maintained at 38 °C.

The mass spectrometer worked in positive mode. The electro spray ion source temperature was set at 550 °C. The ionization voltage was 5.5 kV. The TOF scan range was between 200 and 1000 dalton.

2.3. Standards solution

Stock solutions of each TCs were prepared to 1.0 mg/mL according to calculated molecular weight. The solvent is a mixture of methanol and water (90:10, v/v). Stock solutions were kept at -20°C and isolated from light. Working standard solutions were diluted from stock solution freshly. The mixture solutions were also prepared from stock solution.

2.4. Concrete sample pretreatment

The wastewater was collected in a local swine farm. The collections were conducted before and after the fermentation treatment. The collected sample was then treated with centrifugation and filtration. No other sample pretreatment was carried out. The wastewater after fermentation was also used as blank sample matrix since no target TCs was detected.

2.5. Method validation

A series mixture standard solutions were prepared for linear range investigation. The concentrations of each TCs were 1, 5, 25, 50, 75, and 100 ng/mL, respectively. Single standard working solutions were prepared at the same concentration. Precision investigation was conducted at three concentration levels 5, 50 and 100 ng/g. The sample was analyzed three times in one day to calculate the intra-day precision of the method. For inter-day precision, the sample was analyzed in consecutive three day. To obtain the recoveries of the proposed method at different concentration level, the blank wastewater matrix was spiked of standards at 5, 50 and 100 ng/g.

3. RESULTS AND DISCUSSION

3.1. Optimization of Chromatographic program

In order to obtain a short chromatographic time, a mixture solution of four standards was used as model sample. The concentration of each analyte was 50.0 ng/mL. Employing 0.1% formic acid water as mobile phase A and pure methanol as mobile phase B, an isocratic chromatography (A:B = 50%:50%, v/v) was conducted. TC, OTC, CTC, and DOC did not be separated under this condition. The chromatogram of them were shown as Fig. 2.

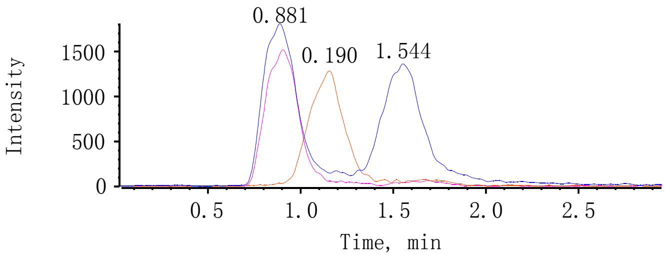


Fig. 2. Chromatogram of TC (0.88 min), OTC (0.88 min), CTC (1.19 min), and DOC (1.54 min) under isocratic condition.

In this chromatogram, the TC and OTC were coeluted at 0.88 min. Naturally, the mass spectrum at this time cannot be used to distinguish the TC and OTC. Based on the isocratic results, a gradient program was iteratively developed. The total flow rate of optimized method was 0.2 mL/min. The elution program started with 15% (v/v) phase B. Kept for 3 mins, the phase B increased linearly to 95% within 4 minutes. Then the phase B returned to the initial condition (15%) to equilibrium the column for 1 minute. The gradient program was abbreviated in table 1.

Table 1. Optimized gradient program

Time (min)	Phase A (%)	Phase B (%)
0	85	15
3	85	15
7	5	95
7.1	85	15
8.1	85	15

With the optimized method, one typical chromatogram (selected ion chromatogram, SIC) of four analytes were shown in Fig. 3.

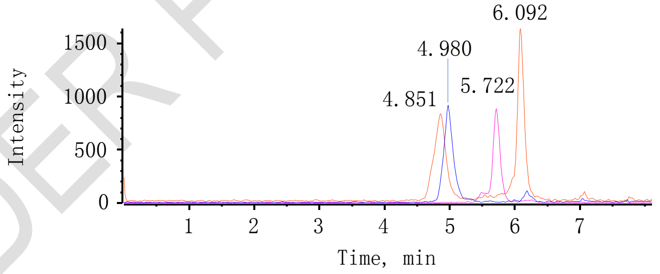


Fig. 3. Optimized chromatogram of TC (4.85 min), OTC (4.98 min), CTC (5.72 min), and DOC (6.09 min) under gradient program.

The retention times for TC, OTC, CTC and DOC were 4.85, 4.98, 5.72, and 6.09 min, respectively. With this chromatographic resolution, the four analytes can be confirmed with their respective mass spectra at each retention time.

3.2. Specificity

The specificity of the developed UHPLC-MS method for target compounds was validated with two approaches. One is the accurate mass to charge ratio (m/z) of the target analytes. Another is retention time of each of them. With single standard sample and mixture samples, the retention time of four analytes were all identified. The peaks in mass spectra of each compound were also assigned to their proton additive ions ($[M+1]^+$). The most abundant peak for TC and DOC was m/z

445.162 (Fig. 4a and Fig. 4d). The TC and DOC are a pair of isomers. The most abundant peak for OTC was m/z 461.157 (Fig. 4b). The most abundant peak for CTC was m/z 479.123 (Fig. 4c).

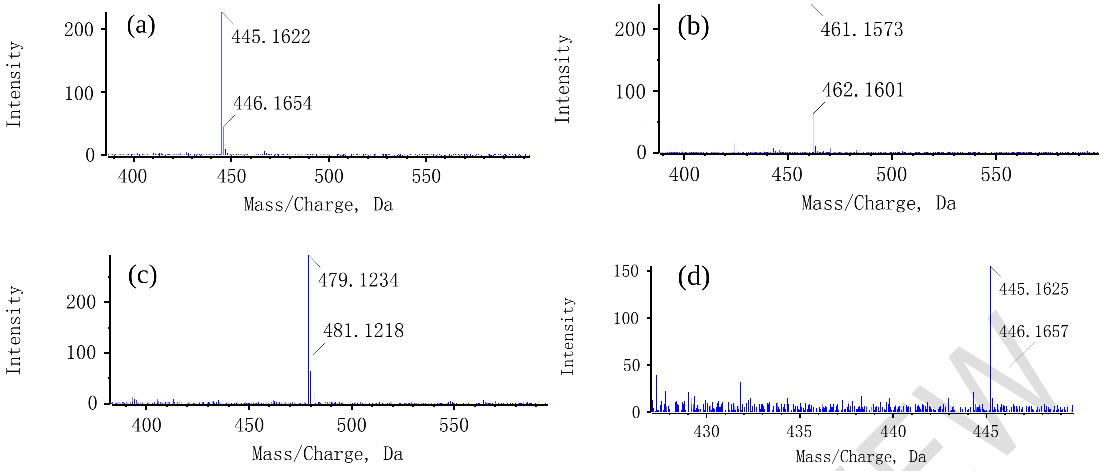


Fig. 4. The high resolution mass spectra of (a)TC, (b)OTC, (c)CTC, and (d)DOC

3.3. Linear range

Linearity was studied by using standard materials in a consideration concentration range (from 1 to 100 ng/mL). The results showed that even at trace levels (several ng / L), the four TCs could be satisfactorily quantified. The linear regression coefficients are all better than 99% in the studied range. The regression equations fitted in linear range of every compounds were listed in table 2.

Table 2. Regression analysis results for TC, OTC, CTC, and DOC.

TCs	Linearity Range (ng/mL)	Regression equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
TC	5-100	y = 208.58x - 182.47	0.9996	1	5
OTC	5-100	y = 238.53x – 416.00	0.9975	1	5
CTC	5-100	y = 179.30x - 260.92	0.9995	1	5
DOC	5-100	y = 191.06x - 172.42	0.9988	1	5

3.4. Precision

In order to check the precision of method, 3 concentration levels of four analytes were prepared. The low concentrations for 4 compounds were all 5 ng/mL. The medium concentrations were all 50 ng/mL while the high concentrations were all 100 ng/mL. Intra-day and inter-day precisions were studied by analysis of every model sample in triplicate. The relative standard deviation (RSD n=3) was calculated accordingly. The results are shown in table 3. The RSD were all less than 5%. It shows that the developed method was precise.

Table 3. Intra-day and inter-day precision (RSD n=3).

	Intra-day			Inter-day		
	5 ng/mL	50 ng/mL	100 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
TC	4.05%	3.13%	0.74%	4.33%	3.59%	1.20%
OTC	3.30%	2.70%	1.46%	4.12%	3.26%	1.55%
CTC	4.06%	3.39%	1.70%	3.98%	3.89%	2.02%
DOC	4.81%	3.04%	1.86%	4.71%	3.35%	2.56%

3.5. **LOD and LOQ**

The LOD and LOQ values were determined based on the signal to noise ratio of 3:1 and 10:1 respectively.

The LOD for 4 compounds were determined to be 1 ng/mL. The typical SIC of them were shown in Fig. 5.

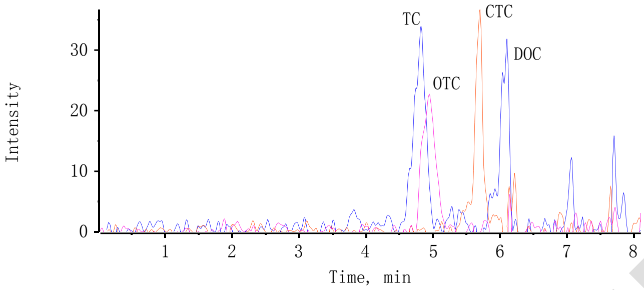


Fig. 5. The chromatogram of four TCs at LOD concentration level

The LOQ for them were determined to be 5 ng/mL. The value was also the lowest concentration in linear range. The typical chromatogram at LOQ concentration level were shown in Fig 6.

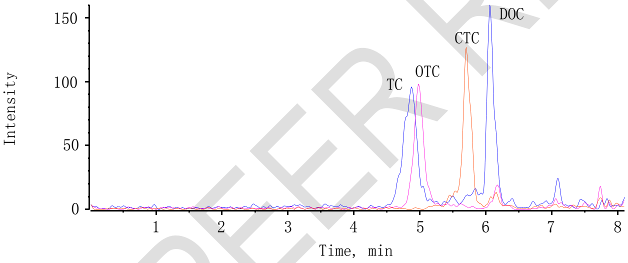


Fig. 6. The chromatogram of four TCs at LOQ concentration level

3.6. Recovery

Trueness of the method was calculated in terms of recovery. Recoveries were determined by using spiked samples (blank wastewater) at 5, 50 and 100 ng/g concentration levels. The results of average recoveries of four TCs are given in table 4 (n=6). It could find that with this developed method, the recoveries were ranged between 95% and 104%.

Table 4. Average recoveries at low, medium, and high concentration

	5 ng/mL	50 ng/mL	100 ng/mL
TC	98.2%	95.3%	97.9%
OTC	101.1%	98.5%	99.2%
CTC	104.0%	99.1%	99.8%
DOC	102.2%	98.3%	99.6%

3.7. Concrete sample

The concrete sample was collected in a local swine farm. In this farm, the wastewater was treated with fermentation degradation before discharge. The fresh wastewater (before fermentation degradation treatment) was pretreated with simple centrifugation and filtrating. In sample, the OTC was detected. Other 3 TCs were not detected. The SIC of sample was shown in Fig. 7.

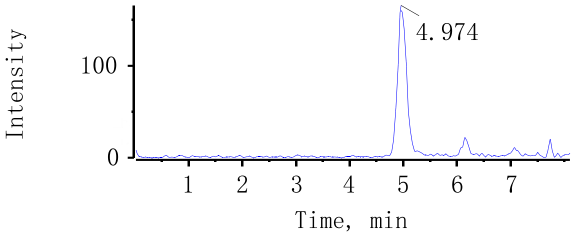


Fig. 7. Chromatogram (SIC) of OTC detected in wastewater.

The concentration of OTC in sample was determined to be 15 ng/mL (n=6). In the wastewater after fermentation degradation, the OTC was not detected.

4. Conclusion

A comprehensive method was developed for determination of TC, OTC, CTC, and DOC in wastewater. Based on UHPLC–MS technique, the developed method presents several advantages such as high throughput, simple sample pretreatment. The chromatography was accomplished in 8.1 minutes. Specificity was confirmed with both retention time and accurate m/z value. For all analytes, good linearity was achieved in the range 5-100 ng/mL. Repeatability of method was proved along with intra-day and inter-day precision. The LOD of method was as low as 1 ng/mL. The LOQ was 5 ng/mL. Recoveries of low, medium, and high concentration of all analytes were between 95% and 104%. This study was to developed a robust method for livestock and poultry wastewater analysis. The concrete sample collected in local swine farm was analyzed with the developed method. The OTC was detected in it and the concentration was determined to be 15 ng/mL. It proved that the developed method provides a reliable and robust tool. The tool can be valuable to support studies dealing with the occurrence of antibiotic resistance in aquatic environment. Indeed, this method can be used for routine analysis of TC, OTC, CTC, and DOC in aqueous samples.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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