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

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Original Research Article

ABSTRACT

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

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1. INTRODUCTION

Tetracyclines antibiotics are the second most common antibiotic group that be used throughout the world [1,2]. This kind of antibiotics including oxytetracycline, chlortetracycline, tetracycline, and doxycycline. Their molecules have the same core structure (Fig. 1). Oxytetracycline, chlortetracycline, and tetracycline are produced by radioactive bacteria. Doxycycline is semi-synthetic derivatives as well as methacycline and dimethylamino tetracycline. Among these antibiotics, doxycycline is used in human clinical practice commonly [3]. Oxytetracycline and tetracycline 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].

Since tetracyclines 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 tetracyclines is 100 µg/kg for cow/goat milk, 400 µg/kg for egg, and 200 µg/kg for shrimp products. Meanwhile, the MRL of doxycycline 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 tetracyclines in the environment are still common. Therefore, the monitoring of tetracyclines in discharged wastewater is always important.

Scientist generally employs spectroscopy method to determine the tetracyclines antibiotics in most degradation studies. The spectroscopy method is neither accurate nor specific for trace tetracyclines detection in wastewater. Various other analytical protocols have been reported for the analysis of tetracyclines in all kinds of matrixes. Reverse phase high performance liquid chromatography used to employ ultra-violet [16,17], photodiode array [18] and fluorescence [19] detector. In the past few decades, mass spectrometry has been widely used in the detection of antibiotic residues. [16,20,21]. As an improvement to the 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].
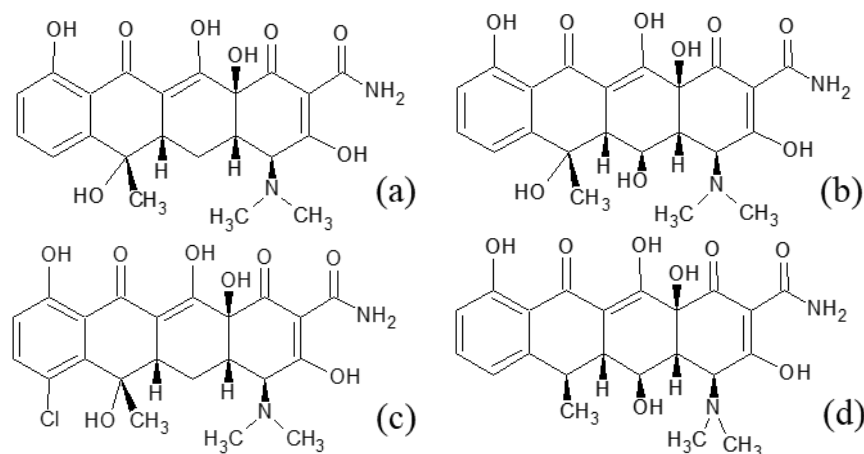

Fig. 1. Chemical structure of (a)tetracycline, (b)oxytetracycline, (c) chlortetracycline, and (d) doxycycline

In recent years, Cristea and coworkers reported an aptamer sensor to recognize the tetracycline in wastewater [24]. However, this technique is not versatile since the sensor substance is difficult to prepare. As for the determination of multi antibiotics in the water samples, there are some analytical methods reported [25]. For example, SPE-LC/MS is a more complicated method [17,26]. Cao and coworkers construct a two-dimensional fluorescent covalent organic framework nanospheres for the detection of tetracycline [27]. In their report, the sample prepare process is tedious.

Therefore, it is still interest to develop a fast and uncomplicated method to detect tetracyclines 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 tetracycline, oxytetracycline, chlortetracycline, and doxycycline. The method proposed by the ultra-high performance liquid chromatography coupled with a high resolution mass spectrometer (UHPLC/MS). Four target antibiotics were determined simultaneously. Trace oxytetracycline residue was detected in a wastewater sample that collected from local swine farm. 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 from 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 tetracyclines 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 tetracyclines was detected.

2.5 Method Validation

A series mixture standard solutions were prepared for linear range investigation. The concentrations of each tetracyclines 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, the 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. Tetracycline, oxytetracycline, chlortetracycline, and doxycycline did not be separated under this condition. The chromatogram before optimization were shown as Fig. 2.
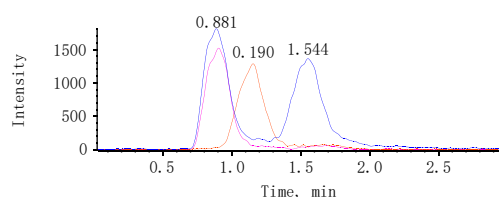

Fig. 2. Chromatogram before optimization with isocratic elution program

In this chromatogram, the tetracycline and oxytetracycline were coeluted at 0.88 min. The retention time of chlortetracycline was 1.19 min. For doxycycline, the retention time was 1.54 min. Naturally, the mass spectrum at this time cannot be used to distinguish tetracycline and oxytetracycline. Based on the isocratic results, a gradient program was iteratively optimized. The total flow rate of the 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.
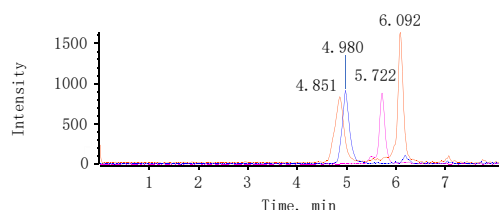

Fig. 3. Optimized chromatogram of tetracycline (4.85 min), oxytetracycline (4.98 min), chlortetracycline (5.72 min), and doxycycline (6.09 min) under gradient program

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.

The retention times of tetracycline, oxytetracycline, chlortetracycline and doxycycline were, respectively, 4.85 min, 4.98 min, 5.72 min, and 6.09 min. 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. With single standard sample and mixture samples, the retention time of four analytes were all identified. The respective peaks in mass spectra were also assigned to their proton additive ions ([M+1]$^+$). The tetracycline and doxycycline are a pair of isomers. The most abundant peak for them was m/z 445.162 (Fig. 4a and Fig. 4d). The most abundant peak for oxytetracycline was m/z 461.157 (Fig. 4b). The most abundant peak for chlortetracycline was m/z 479.123 (Fig. 4c).

3.3 Linear Range

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

3.4 Precision

In order to check the precision of this 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
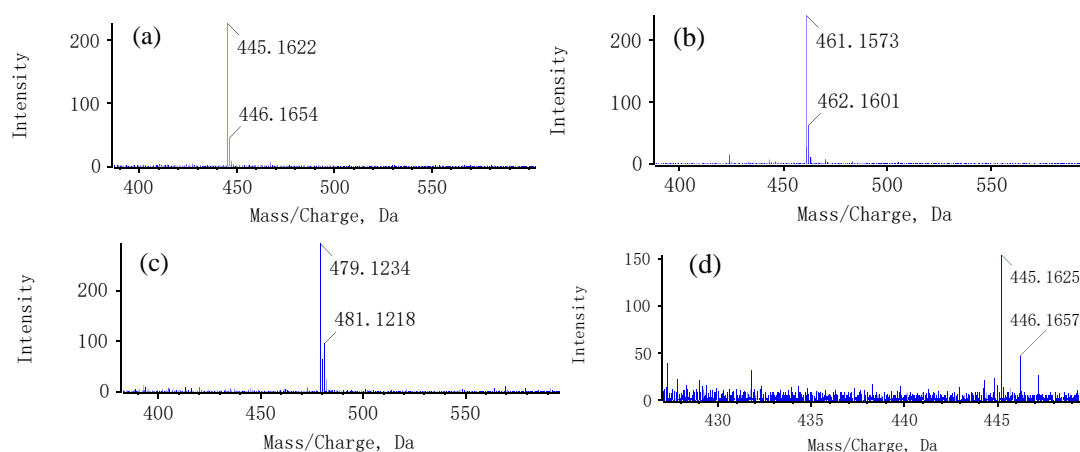
Fig. 4. The high resolution mass spectra of (a) tetracycline, (b) oxytetracycline, (c) chlortetracycline, and (d) doxycycline

Table 2. Regression analysis results for tetracycline, oxytetracycline, chlortetracycline, and doxycycline

	Linearity Range (ng/mL)	Regression equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
tetracycline	5-100	y = 208.58x - 182.47	0.9996	1	5
oxytetracycline	5-100	y = 238.53x – 416.00	0.9975	1	5
chlortetracycline	5-100	y = 179.30x - 260.92	0.9995	1	5
doxycycline	5-100	y = 191.06x - 172.42	0.9988	1	5

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
Tetracycline	4.05%	3.13%	0.74%	4.33%	3.59%	1.20%
oxytetracycline	3.30%	2.70%	1.46%	4.12%	3.26%	1.55%
chlortetracycline	4.06%	3.39%	1.70%	3.98%	3.89%	2.02%
doxycycline	4.81%	3.04%	1.86%	4.71%	3.35%	2.56%

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.

3.5 Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) 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 selected ion chromatogram (SIC) of them were shown in Fig. 5.

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.

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. Table 4 presents the results of the average recoveries for the four tetracyclines (n=6). It could find that with this developed method, the recoveries were ranged between 95% and 104%.
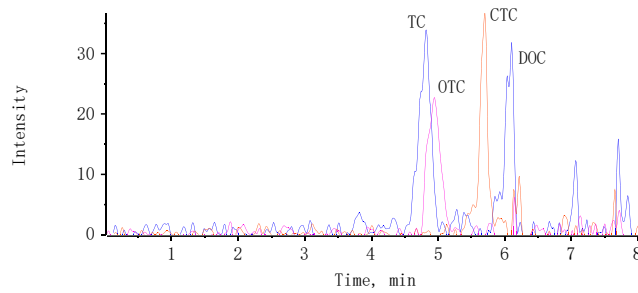
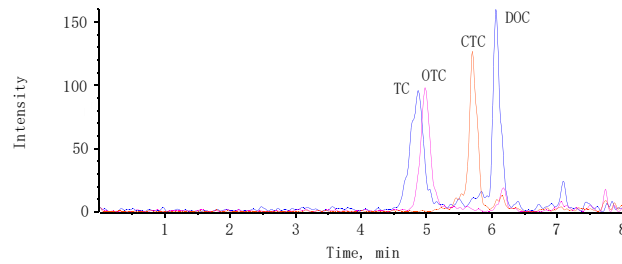**Fig. 5. The chromatogram of four tetracyclines at LOD concentration level**

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

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

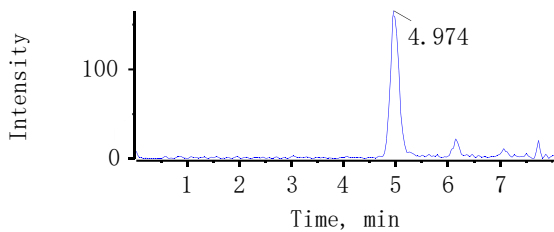|	5 ng/mL	50 ng/mL	100 ng/mL
tetracycline	98.2%	95.3%	97.9%
oxytetracycline	101.1%	98.5%	99.2%
chlortetracycline	104.0%	99.1%	99.8%
doxycycline	102.2%	98.3%	99.6%

Fig. 7. Chromatogram (SIC) of oxytetracycline detected in the wastewater sample

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 collected wastewater (before fermentation degradation treatment) was pretreated with a simple centrifugation and filtrating. In sample, the oxytetracycline was detected. Other 3 tetracyclines were not detected. The selected ion chromatogram (SIC) of oxytetracycline was shown in Fig. 7.

The concentration of oxytetracycline in sample was determined to be 15 ng/mL (n=6). In the wastewater after fermentation degradation, the oxytetracycline was not detected. The detected trace oxytetracycline in collected sample implies that this optimized method for tetracyclines detection is useful in real-world scenarios.

4. CONCLUSION

A comprehensive method was developed for determination of tetracycline, oxytetracycline, chlortetracycline, and doxycycline 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. The method was proved to be specific, sensitive, precise, and accurate. 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 all analytes were between 95% and 104% at low, medium, and high concentrations. This study was to developed a robust method for livestock and poultry wastewater analysis. The concrete sample collected in a local swine farm was analyzed with the developed method. The oxytetracycline 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 for routine analysis of tetracycline, oxytetracycline, chlortetracycline, and doxycycline in real-world scenarios. In future, the method can be adopted to detect tetracyclines in aquatic product.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Wang X, Jia J, Wang Y. Combination of photocatalysis with hydrodynamic cavitation for degradation of tetracycline. Chem. Eng. J. 2017;315:274–282.
    Available:https://doi.org/10.1016/j.cej.2017.01.011
2. Gu C, Karthikeyan KG. Interaction of tetracycline with aluminum and iron hydrous oxides. Environ. Sci. Technol. 2005;39(8):2660–2667.
    Available:https://doi.org/10.1021/es048603o
3. Chopra I, Roberts M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 2001;65(2):232–260.
    Available:https://doi.org/10.1128/MMBR.65.2.232-260.2001

4. Granados-Chinchilla F, Rodríguez C. Tetracyclines in food and feedingstuffs: from regulation to analytical methods, bacterial resistance, and environmental and health implications. J. Anal. Methods Chem. 2017;2017:1–24.
    Available:https://doi.org/10.1155/2017/1315497
5. López-Peñalver JJ, Sánchez-Polo M, Gómez-Pacheco CV, Rivera-Utrilla J. Photodegradation of tetracyclines in aqueous solution by using UV and UV/H2O2 oxidation processes. J. Chem. Technol. Biotechnol. 2010;85(10):1325–1333.
    Available:https://doi.org/10.1002/jctb.2435
6. Zhang H, Zhang M, Li L, Dong W, Ren Q, Xu F, Wang Y, Xu T, Liu J. Rapid limit test of eight quinolone residues in food based on TLC-SERS, a new limit test method. Molecules. 2023;28(18):6473.
    Available:https://doi.org/10.3390/molecules28186473
7. Scaria J, Anupama KV, Nidheesh PV. Tetracyclines in the environment: An overview on the occurrence, fate, toxicity, detection, removal methods, and sludge management. Sci. Total Environ. 2021;771:145291.
    Available:https://doi.org/10.1016/j.scitotenv.2021.145291
8. Jiang B, Liu F, Pan Y, Tan Y, Shuang C, Li A. Preparation of graphite-UiO-66(Zr)/Ti electrode for efficient electrochemical oxidation of tetracycline in water. PLOS ONE. 2022;17(8):e0271075.
    Available:https://doi.org/10.1371/journal.pone.0271075
9. Chen X, Ke Y, Zhu Y, Xu M, Chen C, Xie S. Enrichment of tetracycline-degrading bacterial consortia: microbial community succession and degradation characteristics and mechanism. J. Hazard. Mater. 2023;448:130984.
    Available:https://doi.org/10.1016/j.jhazmat.2023.130984
10. Wu S, Hu H, Lin Y, Zhang J Hu YH. Visible light photocatalytic degradation of tetracycline over TiO2. Chem. Eng. J. 2020;382:122842.
    Available:https://doi.org/10.1016/j.cej.2019.122842
11. Aliyu M, Abdullah AH, Tahir MIBM. Adsorption tetracycline from aqueous solution using a novel polymeric adsorbent derived from the rubber waste. J. Taiwan Inst. Chem. Eng. 2022;136:104333.

Available:https://doi.org/10.1016/j.jtice.202
2.104333

12. Gao Y, Li Y, Zhang L, Huang H, Hu J, Shah SM, Su X. Adsorption and removal of tetracycline antibiotics from aqueous solution by graphene oxide. J. Colloid Interface Sci. 2012;368(1):540–546. Available:https://doi.org/10.1016/j.jcis.2011.11.015

13. Wang JS, Yi XH, Xu X, Ji H, Alanazi AM, Wang CC, Zhao C, Kaneti YV, Wang P, Liu W, Yamauchi Y. Eliminating tetracycline antibiotics matrix via photoactivated sulfate radical-based advanced oxidation process over the immobilized MIL-88A: Batch and continuous experiments. Chem. Eng. J. 2022;431:133213. Available:https://doi.org/10.1016/j.cej.2021.133213

14. Akbari MZ, Xu Y, Lu Z, Peng L. Review of antibiotics treatment by advance oxidation processes. Environ. Adv. 2021;5:100111. Available:https://doi.org/10.1016/j.envadv.2021.100111

15. Wang, J.; Zhuan, R. Degradation of antibiotics by advanced oxidation processes: An overview. Sci. Total Environ. 2020;701:135023. Available:https://doi.org/10.1016/j.scitotenv.2019.135023

16. Liu Y, Yang H, Yang S, Hu Q, Cheng H, Liu H, Qiu Y. High-performance liquid chromatography using pressurized liquid extraction for the determination of seven tetracyclines in egg, fish and shrimp. J. Chromatogr. B. 2013;917–918, 11–17. Available:https://doi.org/10.1016/j.jchromb.2012.12.036

17. Granados-Chinchilla F, Sánchez J, García F, Rodríguez C. A novel green chemistry method for nonaqueous extraction and high-performance liquid chromatography detection of first-, second-, and third-generation tetracyclines, 4-epitetracycline, and tylosin in animal feeds. J. Agric. Food Chem. 2012;60(29):7121–7128. Available:https://doi.org/10.1021/jf301403f

18. Fritz J, Zuo Y. Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography. Food Chem. 2007;105(3):1297–1301. Available:https://doi.org/10.1016/j.foodchem.2007.03.047

19. Maia PP, Rath S, Reyes FGR. Determination of oxytetracycline in tomatoes by HPLC using fluorescence detection. Food Chem. 2008;109(1):212–218. Available:https://doi.org/10.1016/j.foodchem.2007.12.019

20. Xu JJ, An M, Yang R, Tan Z, Hao J, Cao J, Peng LQ, Cao W. Determination of tetracycline antibiotic residues in honey and milk by miniaturized solid phase extraction using chitosan-modified graphitized multiwalled carbon nanotubes. J. Agric. Food Chem. 2016;64(12):2647–2654. Available:https://doi.org/10.1021/acs.jafc.6b00748

21. Goto T, Ito Y, Yamada S, Matsumoto H, Oka H. High-throughput analysis of tetracycline and penicillin antibiotics in animal tissues using electrospray tandem mass spectrometry with selected reaction monitoring transition. J. Chromatogr. A. 2005;1100(2):193–199. Available:https://doi.org/10.1016/j.chroma.2005.09.056

22. Gros M, Rodríguez-Mozaz S, Barceló D. Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J. Chromatogr. A. 2013;1292:173–188. Available:https://doi.org/10.1016/j.chroma.2012.12.072

23. Susakate S, Poapolathep S, Chokejaroenrat C, Tanhan P, Hajslova J, Giorgi M, Saimek K, Zhang Z, Poapolathep A. Multiclass analysis of antimicrobial drugs in shrimp muscle by ultra high performance liquid chromatography-tandem mass spectrometry. J. Food Drug Anal. 2019;27(1):118–134. Available:https://doi.org/10.1016/j.jfda.2018.06.003

24. Hosu O, Melinte G, Ștefan G, Casian M, Cristea C. Towards selective tetracycline recognition in wastewater based on gold nanovoids@aptamer sensing. Electrochimica Acta. 2023;460:142556. Available:https://doi.org/10.1016/j.electacta.2023.142556

25. Goeury K, Vo Duy S, Munoz G, Prévost M, Sauvé S. Assessment of automated off-line solid-phase extraction LC-MS/MS to Monitor EPA priority endocrine disruptors in tap water, surface water, and wastewater. Talanta. 2022;241:123216.

Available:https://doi.org/10.1016/j.talanta.2022.123216

26. Pailler JY, Krein A, Pfister L, Hoffmann L, Guignard C. Solid phase extraction coupled to liquid chromatography-tandem mass spectrometry analysis of sulfonamides, tetracyclines, analgesics and hormones in surface water and wastewater in luxembourg. Sci. Total Environ. 2009;407(16):4736–4743.

Available:https://doi.org/10.1016/j.scitotenv.2009.04.042

27. She WZ, Li CH, Li RS, Ling J, Cao Q. Construction of two-dimensional fluorescent covalent organic framework nanospheres for the detection and removal of tetracycline. Sep. Purif. Technol. 2024;330:125294.

Available:https://doi.org/10.1016/j.seppur.2023.125294.