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Research Article

A confirmatory method of tetracycline antibiotics in Chinese mitten crab (*Eriocheir sinensis*) with ultra-performance liquid chromatography tandem high-resolution mass spectrometry

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Abstract

A confirmatory method for the determination of tetracycline antibiotics (TCs) in Chinese mitten crab has been developed for the first time. The method comprises two stages: pretreatment of the samples and instrumental analysis. The samples are treated with Na2EDTA–McIlvaine buffer followed by extraction. The extract is then analyzed with ultra-performance liquid chromatography tandem high-resolution mass spectrometry (UPLC-HRMS). Four mostly used TCs, including tetracycline, oxytetracycline, chlortetracycline, and doxycycline, were detected in 10 min. The specificity was confirmed via the retention time and their high-resolution mass spectra. In the range of 50-500 μg/kg, the linear regression coefficients (R^2) for all analytes were greater than 0.9985. Recoveries at three concentration levels were between 90.3% and 106.2%. The within-laboratory reproducibility was precise. The decision limit (CC_α) and detection capability (CC_β) of the method were determined using the permitted limit (100 μg/kg) of TCs. The detection results of TCs in 12 batches of Chinese mitten crab showed that no target residues were detected. This comprehensive method is compliant with the 2002/657/EC decision. It could be applied for the determination of TCs in Chinese mitten crab samples and potentially in other aquatic products.

Introduction

Chinese mitten crab (*Eriocheir sinensis*) is a popular aquatic product in China. The cultivation area of the Chinese mitten crab has expanded from the lower reaches of the Yangtze River to cover almost the whole of China (Chen *et al*., 2007). The occurrence of antibiotic residues in Chinese mitten crab has raised food safety concerns (Fang *et al*., 2021). Antibiotics are commonly used in aquaculture to prevent and treat diseases, but their residues can pose a threat to human health if not removed properly (Chen *et al*., 2020; Bondad-Reantaso *et al*., 2023).

Tetracyclines (TCs) are a class of antibiotics commonly used in medicine and animal husbandry (Wang *et al*., 2017). There are eight forms of commercially available TCs. Four of them, including tetracycline, oxytetracycline, chlortetracycline, and doxycycline, are frequently used in food animal breeding (Gavilán *et al*., 2015; Granados-Chinchilla and Rodríguez, 2017).

When TCs are excreted into the environment through wastewater or animal feces, they can cause harm to the environment (Chopra and Roberts, 2001). The impact on the environment and ecosystem includes drug resistance, harm to aquatic organisms, drug accumulation in the human body, soil pollution, and so on (Grenni *et al*., 2018; Pashaei *et al*., 2022).

Maximum residue limits (MRL) for these four tetracyclines have been specified in various foods of animal origin, such as eggs, muscle, and milk (Commission, 2010; Zhang *et al*., 2023). Analytical techniques for tetracyclines detection in aquatic products involve various methods. High-performance liquid chromatography (HPLC) (Fritz and Zuo, 2007) and capillary electrophoresis (CE) (Kowalski, 2008) are widely used techniques for antibiotic residue detection. They usually employ ultraviolet (Granados-Chinchilla *et al*., 2012; Liu *et al*., 2013) or fluorescence (Maia *et al*., 2008) detectors to quantify tetracyclines. As an improvement to liquid chromatography, the ultra-high performance liquid chromatography (UHPLC) (Gros *et al*., 2013; Susakate *et al*., 2019) was developed. Liquid Chromatography-Mass Spectrometry (LC-MS) combines the separation power of liquid chromatography with the detection capabilities of mass spectrometry (Goto *et al*., 2005). It provides high sensitivity and selectivity for trace residue detection, allowing for accurate identification and quantification (Xu *et al*., 2016).

Other alternative methods were also reported (Mousavizadegan *et al*., 2023; Zhao and Zhao, 2023). Biosensors utilize biological components, such as enzymes or antibodies, to detect target analytes (Raykova *et al*., 2021; Hosu *et al*., 2023; She *et al*., 2024). Enzyme-linked ımmunosorbent assay (ELISA) is an immunological method that uses specific antibodies to detect tetracyclines (Wang *et al*., 2021). It offers rapid and cost-effective screening for tetracycline residues in aquatic products, although it may have limitations in terms of sensitivity and specificity.

These techniques can be used individually or in combination, depending on the specific requirements of the analysis. It is important to consider factors such as sensitivity, selectivity, cost, and sample

matrix when selecting an appropriate analytical technique for TCs detection in aquatic products. According to the Chinese national food safety standard, Maximum Residue Limits for Veterinary Drugs in Food (GB 31650.2-2019), some antibiotics such as sulfamethazine, sulfadimethoxine, florfenicol, oxytetracycline, chlortetracycline, and doxycycline have maximum residue limits in crustaceans. In Commission Regulation (EU) 37/2010, the same MRL of TCs was regulated (Commission, 2010). Therefore, it is important to monitor TCs in Chinese mitten crab meat (muscle issues) to ensure food safety.

To achieve an ultrasensitive detection of oxytetracycline, one current technique is the aptasensor. Sanaz Akbarzadeh and co-workers detected oxytetracycline using an electrochemical label-free aptamer-based biosensor (Akbarzadeh *et al*., 2022). A self-powered aptasensor was established for the detection of oxytetracycline (Peng *et al*., 2021). A photoelectrochemical aptasensor based on nanocomposites has also been developed (Qiao *et al*., 2023). However, these new biosensors are not suitable for regular screening and determination of oxytetracycline in traditional laboratories.

Developing a systematic method may effectively monitor and detect the residual amount of TCs in Chinese mitten crab meat. The method may be used to help reduce pollution in fisheries and protect fishery resources. Subsequentially, it can ensure food safety and protect consumer health. Also, a confirmatory method can ensure the quality of related products exported to overseas markets.

There have been reports about TCs detection in *Penaeus monodon* (prawns) (Venkatesh *et al*., 2013), shrimp (Reddy *et al*., 2017), and other seafood products (Alanazi *et al*., 2021). However, to the best authors' knowledge, there is seldom publication on the occurrence of tetracyclines residues in Chinese mitten crab (Fang *et al*., 2021). Along with the meat of Chinese mitten crab becoming a popular food (Wang *et al*., 2016), it is imperative to develop a robust and sensitive analytical method to detect the tetracyclines residues in it.

Hereby, for the first time, we proposed an analytical method for the monitoring and measurement of TCs in Chinese mitten crab. Since the muscle is the only edible issue of Chinese mitten crab, this study aimed to provide a systematic method to determine the tetracycline, oxytetracycline, chlortetracycline, and doxycycline in Chinese mitten crab muscle. Sample pretreatment and instrumental program developed were complaints with 2002/657/EC Decision (Antignac *et al*., 2003). The developed method is rapid, sensitive, and accurate. It could be adopted for other aquatic products.

Materials and methods

Reagents

The standard of tetracycline (>99%), oxytetracycline (>98%), chlortetracycline (>80%, HPLC), doxycycline (>95%), and Ethyl acetate (≥99.5%) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The HPLC grade Methanol was purchased from Tedia Company, Inc. (Fairfield, USA). Formic acid (>98.0%) was obtained from Tokyo

Chemical Industry Co., Ltd. (Tokyo, Japan). Trichloroacetic acid (≥99), Anhydrous citric acid (≥99.5%) and disodium hydrogen phosphate were purchased from Sinopharm Chemical Reagent Co., Ltd, (China). and EDTA disodium salt dihydrate ($Na_2EDTA{\cdot}2H_2O$) was purchased from Sigma-Aldrich (MO, USA). Water was purified with a Milli-Q system (Molsheim, France).

Instruments and condition

Ultra-performance liquid chromatography tandem high-resolution mass spectrometry was performed on an UPLC system (Agilent, 1290 Infinity) coupled with a time of flight (TOF) mass spectrometer (AB Sciex, 5600^+). The C18 column is in size 50mm × 2.1mm; 1.7μm (Waters, ACQUITY UPLC BHE).

Mobile phase A was formic acid/water solution (0.1%, v/v). The mobile phase B was methanol. The total flow rate was 0.2 mL/min. The column temperature was 40°C. The sampling volume was 3 μL. The mass spectrometer worked in positive mode. The ionization voltage was +5.0 kV. The source temperature was set at 550°C. The TOF scan range was between m/z 200 and 1000.

Standard solution

Stock solutions of each TCs were prepared to 1.0 mg/mL. The solvent was methanol. Working standard solutions were diluted from stock solution freshly with mobile phase solution (85%A:15%B). Mixture working solutions were also prepared from stock solutions by calculated dilution.

Sample collection

The Chinese mitten crab samples were collected from the aquaculture pond. The ponds are distributed in three counties in the east of Jiangsu Province, China. The samples were stored at -20°C.

Samples preparation

Disodium EDTA-McIlvaine buffer was prepared with 9.61 g citric acid, 8.87 g disodium hydrogen phosphate, and 30.3 g $Na_2EDTA{\cdot}2H_2O$. Each of them was dissolved in water individually. Then the solutions were mixed. Finally, the buffer solution was made up to 1000 mL and adjusted to pH 4.0. For each aliquot of Chinese mitten crab muscle, 5 g of homogenized sample were weighed. These raw samples were treated with the following procedures. An aliquot sample (2 g) was added with EDTA-McIlvaine buffer (8 mL), and trichloroacetic acid (0.3 mL). The mixture was then added with ethyl acetate (6 mL). After vortex, ultrasonic bath, and centrifugation (5000 rpm, 10 min), the supernatant (2 mL) was dried with nitrogen blowing. The dried sample was finally dissolved into a 2 mL mobile phase solution (85%A: 15%B).

Fortified samples

Fortified samples were prepared by spiking target standard solutions into an antibiotic-free sample (blank Chinese mitten crab muscle, provided by Jiangsu Key Laboratory for Bio-resources of Saline, Yancheng, China). The matrix-matched sample was then treated with extraction and necessary dilution before instrumental analysis.

Results

Chromatographic optimization

The mixture standard solution (100 µk/kg of each analyte) was analyzed with an empirical full gradient LC program. The monitored ion chromatograms (MIC) of four analytes are shown in Figure 1.

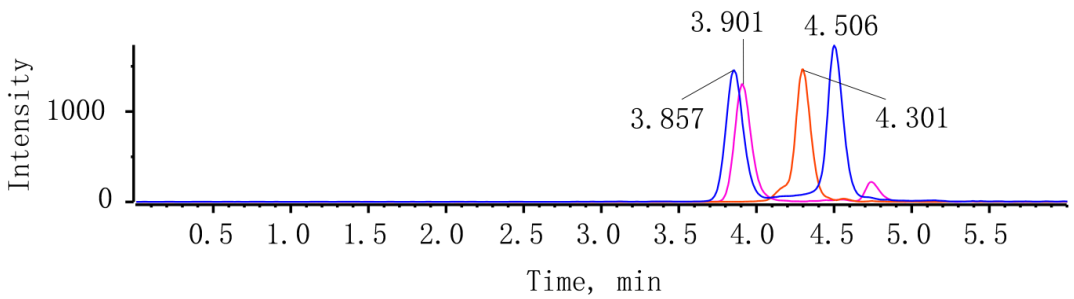

Figure 1: The monitored ion chromatogram of tetracycline, oxytetracycline, chlortetracycline, and doxycycline with the empirical LC program.

It was observed that TCs congested between 3.6 and 4.6 min. In the order of retention time, the peaks are tetracycline, oxytetracycline, chlortetracycline, and doxycycline, respectively. There is a minor retention difference between tetracycline (3.857 min) and oxytetracycline (3.901 min) under this empirical condition. Based on the empirical program, an optimized gradient program (Table 1) was iterated.

Table 1: The optimized gradient LC program of the method.

Step	Total Time (min)	Flow Rate (uL/min)	%A	%B
0	0	200	85	15
1	5	200	85	15
2	10	200	5	95
3	10.5	200	5	95
4	10.6	200	85	15
5	11.6	200	85	15

With the optimized LC program, four analytes were separated. The MIC of the mixture standard sample is shown in Figure 2. The retention times of tetracycline, oxytetracycline, chlortetracycline, and doxycycline were respectively 5.032 min, 5.519 min, 8.004 min, and 8.666 min.

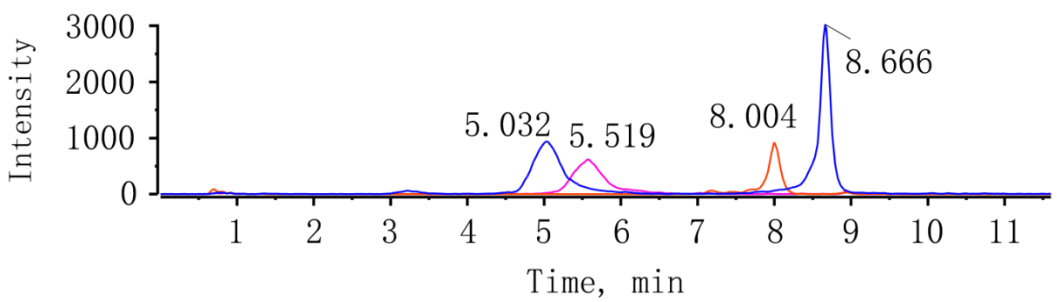

Figure 2: The monitored ion chromatogram of tetracycline, oxytetracycline, chlortetracycline, and doxycycline with an optimized LC program.

Since the retention time of each analyte was assigned, a matrix-matched mixture standard sample (100 uk/kg of each analyte) was analyzed with the optimized method. The MIC of four tetracyclines is shown in Figure 3. The mass spectra of each peak in Figure 3 were exhibited in Figure 4.

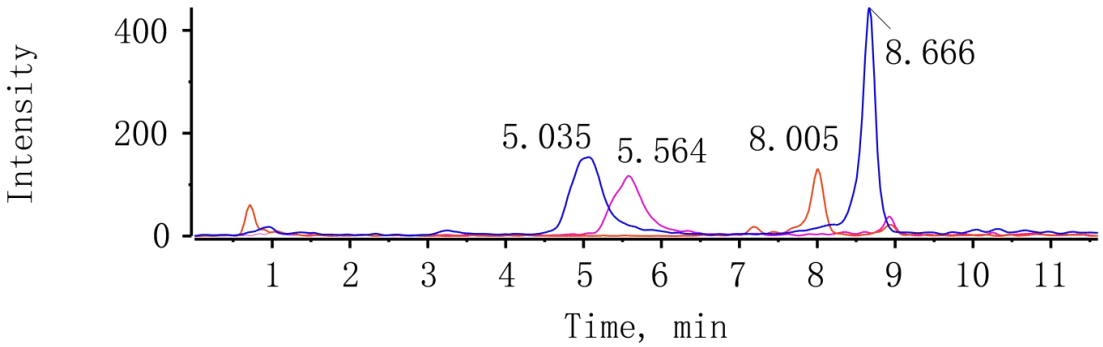

Figure 3: The monitored ion chromatogram of four TCs in matrix-matched mixture sample.

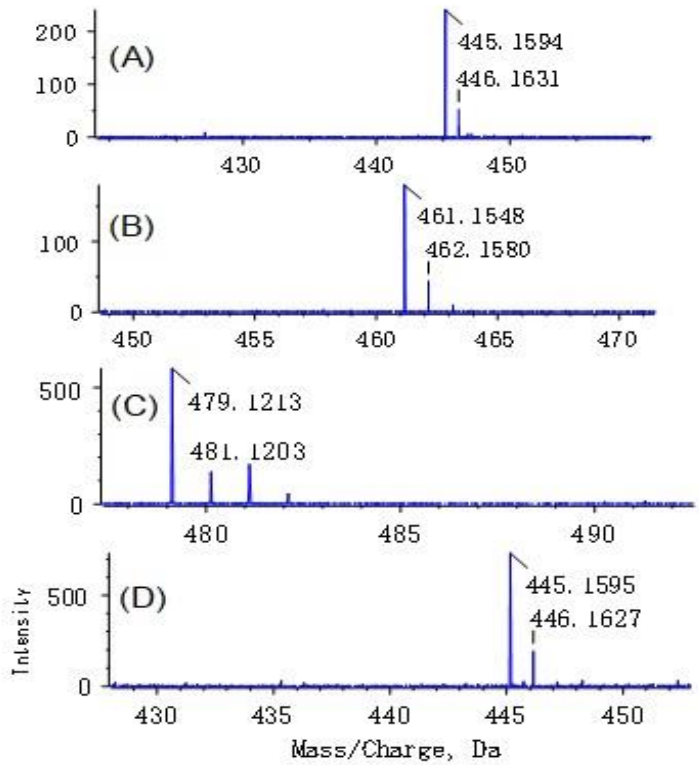

Figure 4: High-resolution mass spectra of (A) tetracycline@5.03 min, (B) oxytetracycline@5.56 min, (C) chlortetracycline@8.00 min, and (D) doxycycline@8.66 min of the matrix-matched mixture standard sample.

Method investigation

The linearity of the method was evaluated with the calibration curve. For linearity range and calibration curve investigation, a batch of matrix-matched fortified calibration standards was prepared. The concentration levels of analytes in the fortified sample were 0, 50, 100, 150, 200,

250, and 500 µg/kg. Each sample was analyzed in triplicate. The calibration curve was constructed by plotting the peak area versus the spiked concentration of each compound. The peak area was calculated in its monitored ion chromatogram (MIC). The linearity results are listed in Table 2.

The fortified samples at concentration levels of 50, 100, and 150 µg/kg were prepared, extracted, and analyzed with two operators separately. Two sets of samples were both analyzed with six replicates. Peak area results were combined (n=12) to calculate the standard deviation (SD) and coefficient of variation. The results are listed in Table 3.

Table 2: Linearity results of four analytes.

	Range (µg/kg)	Regression equation	R2
tetracycline	50-500	y = 57.975x - 576.28	0.9985
oxytetracycline	50-500	y = 36.62x - 129.65	0.9996
chlortetracycline	50-500	y = 23.735x - 385.07	0.9991
doxycycline	50-500	y = 71.701x - 780.98	0.9998

Table 3: Precision of the method (within-laboratory reproducibility).

Concentration (ng/g)	analyte	average of Peak Area	SD	CV
50	tetracycline	2362.211	198.1734	8.39%
	oxytetracycline	1666.918	150.1679	9.00%
	chlortetracycline	862.7839	57.58176	6.67%
	doxycycline	2493.55	154.7221	6.20%
100	tetracycline	5012.915	292.5155	5.84%
	oxytetracycline	3385.831	155.1107	4.58%
	chlortetracycline	2041.121	96.67856	4.74%
	doxycycline	6314.92	350.4597	5.55%
150	tetracycline	8154.197	288.8771	3.54%
	oxytetracycline	5309.004	235.2553	4.43%
	chlortetracycline	3034.597	115.0617	3.79%
	doxycycline	9943.397	363.409	3.65%

According to the performance criteria and other requirements for analytical methods in the European Commission decision (2002/657/EC), the decision limit (CCα) was established. The corresponding concentration at the permitted limit (100 ug/kg) plus 1.64 times the standard deviation (SD) of the within-laboratory reproducibility (n=20) makes the decision limit (α = 5%). The results are shown in Table 4. The concentration at CCα plus 1.64 times the standard deviation (SD) of the within-laboratory reproducibility (n=20) yielded the corresponding detection capability CC_β (β=5%), also listed in Table 4.

Table 4: Decision limit (CCα) and Detection capability (CCβ).

	CCα (µg/kg)	CC_β (µg/kg)
tetracycline	110.8114	121.6227
oxytetracycline	106.8085	113.6169
chlortetracycline	108.37	116.74
doxycycline	110.6804	121.3608

Ruggedness Applicability/ stability

The ruggedness of the method was evaluated with the mean of concentrations, SD, and CV based on two matrix conditions. In the first condition, four matrix-matched blank samples were prepared. Each sample was spiked with one single standard at 100 μg/kg. In the second condition, one matrix-matched blank sample was spiked with a mixture of 4 analytes. Four TCs analytes were all 100 μg/kg in this multi-fortified sample. Each sample was analyzed in triplicate. The results were accepted (CV≤7.88%), as listed in Table 5.

Table 5: Ruggedness (CV) under the conditions of single- and multi-analytes exist.

analyte	Mean Concentration (μg/kg)	SD	CV (n=6)
tetracycline	96.16	6.52	6.78%
oxytetracycline	96.07	4.10	4.27%
chlortetracycline	101.44	8.00	7.88%
doxycycline	96.22	6.55	6.81%

Comparison with the existing method

To provide a confirmatory method, we investigated the decision limit (CCα) and detection capability (CCβ) instead of the limit of detection (LOD) and limit of quantitation (LOQ). Although the CCα depends on the regulatory limit (100 ug/kg), the actual LOD was as low as 10 μg/kg. This value is better than the results of a relatively recent report (Alanazi *et al*., 2021), where the LOD of the method was 15μg/kg. Other validation parameters are all better than their report, including LOQ, precision, accuracy and recovery.

Occurrence of TCs in Chinese mitten crab

The developed method was used to analyze 12 batches of Chinese mitten crab collected from three counties around the authors' residence. For all samples, no tetracycline antibiotics (tetracycline, oxytetracycline, chlortetracycline, and doxycycline) were detected. It shows that tetracycline antibiotics are well-controlled in this region.

Discussion

Since the matrix of Chinese mitten crab meat is complicated, it is important to separate analytes from each other as well as other substances in a chromatogram. The specificity of each target compound was confirmed by both the retention time and the accurate molecular ion m/z value. It is obvious that the retention times of four tetracyclines are constant in both standard mixture sample and the matrix-matched mixture sample. There was no obvious interference substance for every analyte. The m/z value of each compound was accurate, as shown in the mass spectrum at the corresponding retention time (Fig. 4). The calibration curve and the linear regression coefficients (R^2) for all compounds exceeded 99.8% in the range of 50-500 μg/kg.

Since no certified reference material (CRM) is available, the trueness of measurements was assessed through recovery. 18 blank samples were fortified with the analytes at 3 concentration levels of 50, 100, and 150 μg/kg. For each level, six parallel samples were analyzed. The

concentration results were calculated with the calibration curve. The coefficient of variation (CV) and average recovery were calculated accordingly. The recovery was determined by the measured content over the fortification level. The average recovery results are listed in Table 3. For all analytes at every concentration level, the recoveries of this method are better than the guideline ranges (80% - 110%).

The precision of quantitative methods was assessed with repeated analysis under within-laboratory reproducibility conditions. Since the coefficients of variation of 4 analytes at 3 concentration levels are all less than 9%, the method developed was determined to be precise (<15%).

The term decision limit (CCα) in the European Commission decision (2002/657/EC) corresponds to the limit of detection (LOD) in general analytical methodology. According to Commission Regulation (EU) 37/2010, the maximum residue limits (MRLs) of tetracycline, oxytetracycline, and chlortetracycline are all 100 μg/kg for muscle issues. The MRL of doxycycline is 100 μg/kg in Regulation (EEC) 2377/90, although it is no longer in force. Therefore, the CCα of the method was established in the case of the analyte with an established permitted limit.

The term detection capability (CC_β) corresponds to the limit of quantification (LOQ) in general analytical methodology. CC_β was also established in the case of substances for which a permitted limit has been established as well.

Conclusions

This confirmatory method has been developed in confirmation of Decision 2002/657/EC. Tetracycline, oxytetracycline, chlortetracycline, and doxycycline were separated within 10 minutes. Good specificity, linearity, recovery, precision, and robustness were achieved for the Chinese mitten crab sample. In the 50-500 μg/kg range, the linear regression coefficients (R^2) for all analytes were greater than 0.9985. Recoveries at three concentration levels were between 90.3% and 106.2%. The coefficients of variation of within-laboratory reproducibility were all less than 9.00% for analytes at 3 concentration levels, which is far less than the value (15%) regulated in the 2002/657/EC Decision. The decision limit (CCα) and detection capability (CCβ) of the method were both calculated. The calculation was based on the permitted limit for all tetracyclines. The real samples, 12 batches of Chinese mitten crab that were collected in the east of Jiangsu Province in China, showed no observed residues. This comprehensive method could be confidently applied to the determination of TCs in Chinese mitten crab samples.

Conflicts of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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