Application News

No. LCMSMS-72E

Liquid Chromatography Mass Spectrometry

Determination of Five Tetracycline Antibiotics Residues in Fish by Ultra-High-Performance Liquid Chromatography coupled with Triple Quadrupole Mass Spectrometry

This application note describes a method for the determination of five tetracycline antibiotics residues in fish using Shimadzu Ultra-High-Performance Liquid Chromatography coupled with Triple Quadrupole Mass Spectrometry. After solid phase extraction, the tetracycline antibiotics in fish were rapidly separated using Ultra Performance Liquid Chromatograph LC-30A in 4 min. The compounds were quantitatively analyzed using Triple Quadrupole Mass Spectrometer LCMS-8040. The calibration curves were plotted using external standard method. The linear range of the calibration curves of tetracycline, demethylchlortetracycline and doxycycline was 0.1-100 µg/L while for oxytetracycline and aureomycin, the range is 0.1-50 µg/L. All correlation coefficients were above 0.9977. The precision experiments were conducted at mixed standard solutions of 1 µg/L, 10 µg/L and 100 µg/L, the RSD of retention time and peak area was 0.64-0.81% and 0.70-2.90% respectively, indicating good system precision. The method detection limit was determined to be 0.035-0.057µg/L, and the limit of quantitation was 0.138-0.226 µg/L; the matrix spike recovery of fish samples ranged between 105-120%, which meets the detection requirement for tetracycline antibiotics.

Keywords: Tetracycline; Antibiotics; Triple quadrupole mass spectrometry; Fish

■ Introduction

In recent years, China's aquaculture industry experiences rapid growth. With the massive expansion of these industries, drugs are commonly used to counter the increasingly rapid and severe diseases in aquaculture. The use and residues of these drugs and the accumulation in fish and the environment pose a major concern. Tetracyclines (TCs) are a type of broad-spectrum antibiotics, which is extensively used in the treatment of human and animal infections. TCs cannot be completely absorbed by animals and a significant portion enters the food chain and environment in the form of prototypes or metabolites. These residues can indirectly affect human health. Ultra-High-Performance liquid chromatography (UHPLC) coupled with tandem mass spectrometry is an analytical technique that develops rapidly in recent years. With its high selectivity and sensitivity as well as accurate qualitative ability, it is ideal for the determination of drug residues in complex matrices.

With reference to China's national standard GB/T 21317-2007 "Detection Method of Tetracycline Veterinary Drug Residues for Food of Animal Origin - LCMS and HPLC Method", a method to determine tetracycline veterinary drug residues in aquatic products was established using Shimadzu Nexera X2 UHPLC and Triple Quadrupole Mass Spectrometer LCMS-8040. This method proves to be fast and simple technique with high selectivity and sensitivity, thus providing a reference method for related inspection personnel.

■ Experimental

1.1. Instruments

Shimadzu Nexera X2 UHPLC and Triple Quadrupole Mass Spectrometer LCMS-8040 system was used. The specific configuration included LC-30AD×2 pumps, DGU-20A5 Online Degasser, SIL-30AC Autosampler, CTO-30AC Column Oven, CBM-20A System Controller, Triple Quadrupole Mass Spectrometer LCMS-8040, and LabSolutions Ver. 5.53 Chromatography Workstation.

1.2. Analytical Conditions

<u>Liquid chromatography (LC) parameters</u>

- Chromatographic column: Shim-pack XR-ODS III 2.0 mm I.D. × 75 mm L., particle size 1.6 µm
- Mobile phase: A-0.1% formic acid solution, B-methanol
- Flow rate: 0.5 mL/min
- Column temperature: 30°C
- Injection volume: 20µL
- Elution mode: gradient elution with the initial concentration of mobile phase B at 15%; Refer to Table 1 for the detailed gradient program.

Table 1: Gradient Program

Time(min)	Module	Command	Value
1.50	Pumps	Pump B Conc.	95
3.00	Pumps	PumpB Conc.	95
3.01	Pumps	PumpB Conc.	15
5.00	Controller	Stop	

Mass spectrometry (MS) parameters

- Ionization mode: ESI (+)
- Ion spray voltage: 4.5 kV
- Nebulizing gas: Nitrogen 3.0 L/min
- Drying gas: Nitrogen 15 L/min
- Collision gas: Argon
- DL temperature: 250°C
- Heating module temperature: 400°C
- Scan mode: Multiple Reaction Monitoring (MRM)
- Dwell time: 30 msec
- Pause time: 3 msec
- MRM parameters: Refer to Table 2

Table 2: MRM Optimized Parameters

Compound name	Precursor ion	Product ion	Q1 Pre Basis (V)	CE (V)	Q3 Pre Basis (V)
Tetracycline	445.10	410.15* 427.25	-16 -16	-20 -14	-28 -21
Oxytetracycline	461.10	426.15* 443.15	-22 -22	-19 -12	-30 -22
Demethylchlortetracycline	465.10	448.10* 430.05	-17 -17	-17 -21	-30 -30
Aureomycin	479.05	444.10* 462.15	-23 -16	-22 -14	-30 -21
Doxycycline	445.05	428.25* 410.05	-16 -16	-19 -38	-30 -29

Note: * refers to quantifier ion

1.3. Preparation of Standard Solution and Samples

Preparation of standard solutions:

Appropriate amount of five tetracycline antibiotics (i.e. tetracycline, oxytetracycline, demethylchlortetracycline, aureomycin and doxycycline) were weighed, combined and diluted with methanol to obtain a mixed standard stock solution of 1000 mg/L for each antibiotic. The mixed standard stock solution was diluted using the initial mobile phase composition to give standard working solutions at concentration of 0.1, 0.2, 0.5, 1, 5, 10, 20, 50, 100 µg/L.

Sample pretreatment method:

The preparation, extraction and cleanup of fish samples were performed in reference to China's standard GB/T 21317-2007 "Detection Method of Tetracycline Veterinary Drug Residues for Food of Animal Origin- LC-MS and HPLC Method".

■ Results and Discussion

2.1. Q1 Scan Mass Spectra and Product Ion Scan Mass Spectra of the Antibiotics Standard Samples

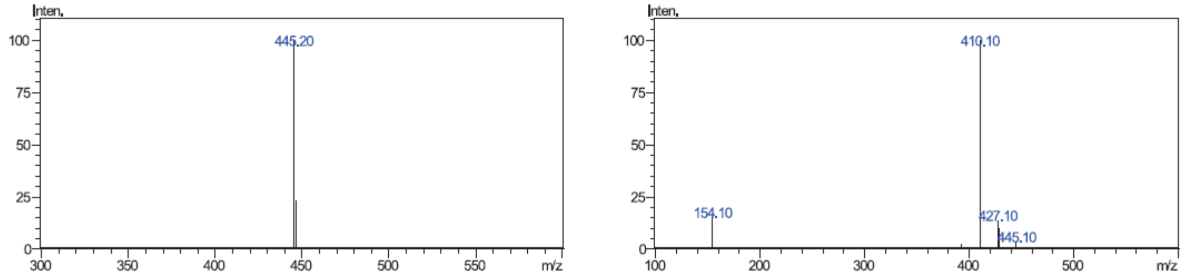

Figure 1: The Q1 scan mass spectrum (left) and product ion scan mass spectrum (CE value was -18V) of tetracycline.

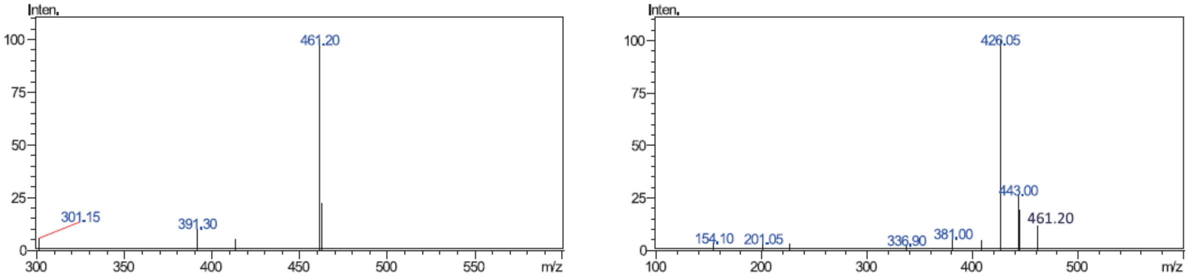

Figure 2: The Q1 scan mass spectrum (left) and product ion scan mass spectrum (CE value was -19V) of oxytetracycline.

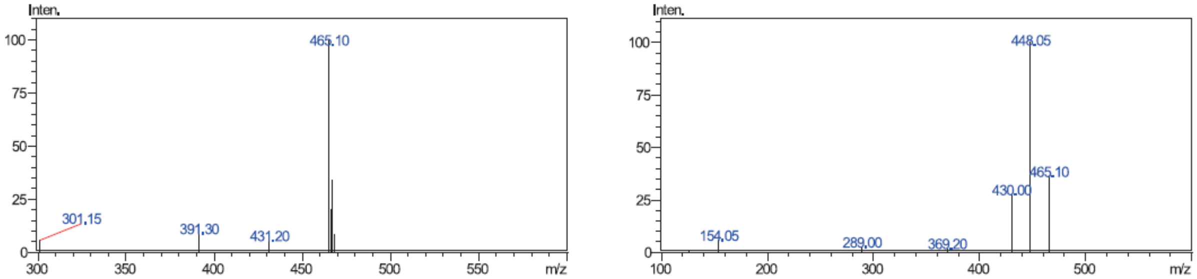

Figure 3: The Q1 scan mass spectrum (left) and product ion scan mass spectrum (CE value was -19V) of demethylchlortetracycline.

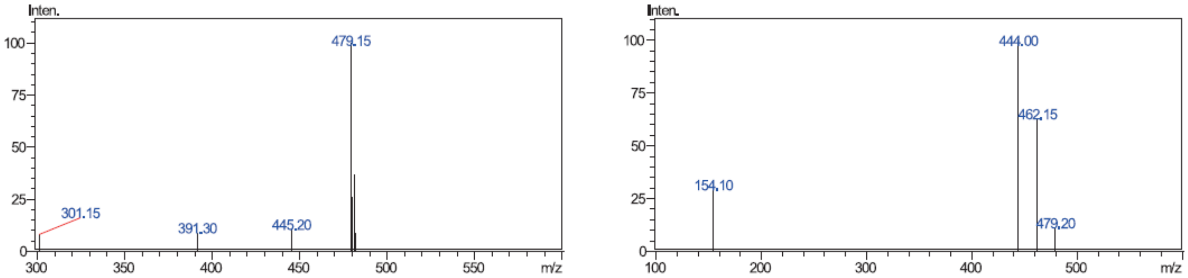

Figure 4: The Q1 scan mass spectrum (left) and product ion scan mass spectrum (CE value was -23V) of aureomycin.

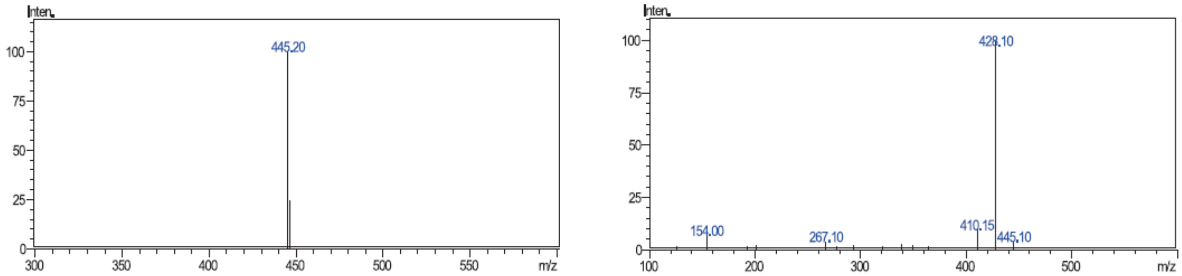

Figure 5: The Q1 scan mass spectrum (left) and product ion scan mass spectrum (CE value was -25V) of doxycycline.

2.2. MRM Chromatogram of the Antibiotics Standard Samples

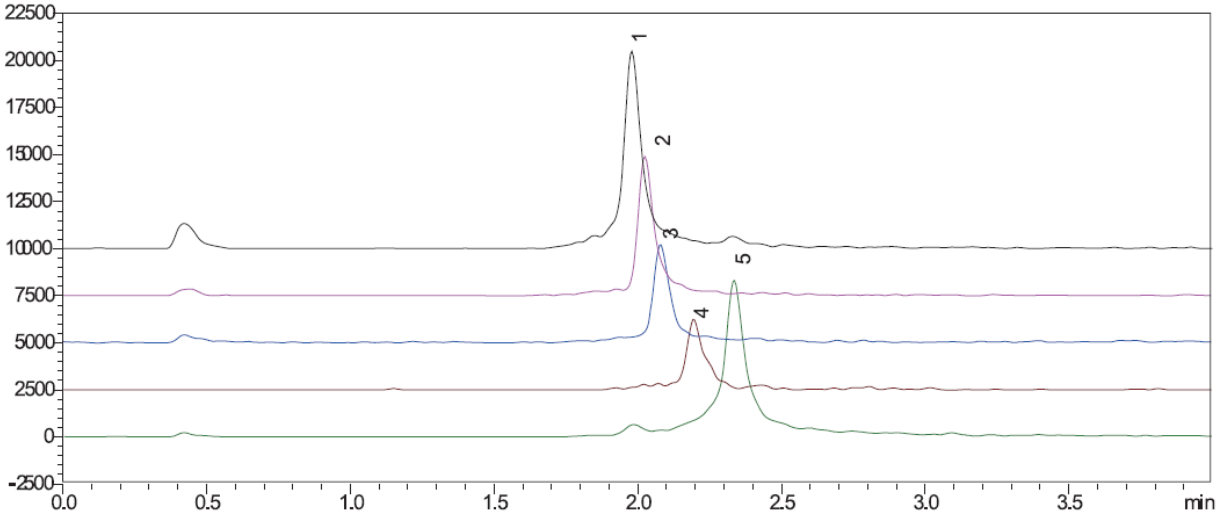

Figure 6: MRM Chromatogram of the Standard Samples (1 μg/L)
(1. Tetracycline; 2. Oxytetracycline; 3. Demethylchlortetracycline; 4. Aureomycin; 5. Doxycycline)

2.3. Calibration and Linear Range

The mixed standard working solutions were analyzed according to the parameters set in 1.2 and calibration was performed using a 9-point curve at concentrations 0.1, 0.2, 0.5, 1, 5, 10, 20, 50, 100μg/L.

External standard calibration method was used. The calibration curves, shown in Figure 7 – 11, showed good linearity; the linear equations and correlation coefficients are shown in Table 3.

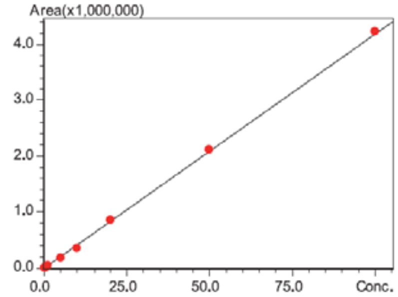

Figure 7: Calibration Curve of Tetracycline

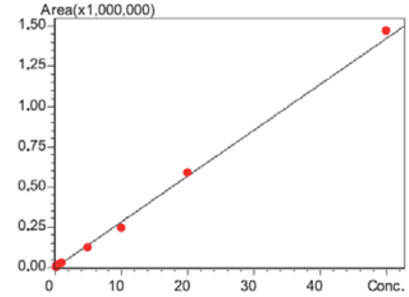

Figure 8: Calibration Curve of Oxytetracycline

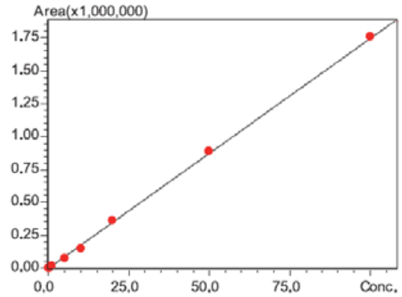

Figure 9: Calibration Curve of Demethylchlortetracycline

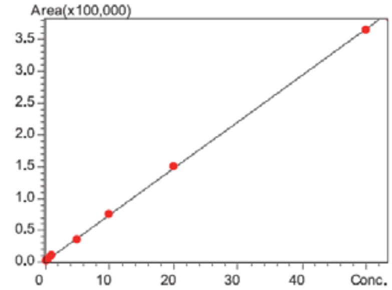

Figure 10: Calibration Curve of Aureomycin

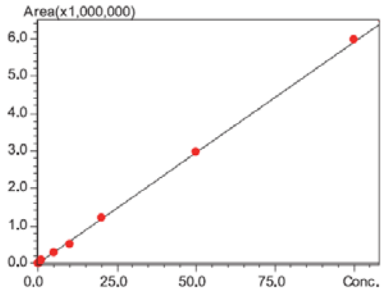

Figure 11: Calibration Curve of Doxycycline

Table 3: Calibration Curve Parameters

No.	Compound Name	Calibration curve	Linear range (µg/L)	Correlation coefficient (r)
1	Tetracycline	Y = (41813.3)X + (-1335.28)	0.1~100	0.9990
2	Oxytetracycline	Y = (28472.3)X + (-1268.34)	0.1~50	0.9977
3	Demethylchlortetracycline	Y = (17475.7)X + (-7.52695)	0.1~100	0.9989
4	Aureomycin	Y = (7312.66)X + (984.523)	0.1~50	0.9989
5	Doxycycline	Y = (59025.7)X + (1741.35)	0.1~100	0.9993

2.4. Precision Experiment

The prepared mixed standard working solutions at various concentrations were analyzed successively six times to evaluate instrument precision. The repeatability results of retention time and peak area are shown in Table 4. The results indicated that the RSD of retention time and peak area of standards at different concentrations was 0.64-0.81% and 0.70-2.90% respectively, indicating good instrument precision.

2.5. Detection Limit

The mixed standard working solution at concentration of 0.1 µg/L were analyzed (n = 7). From the results, the standard deviation (S) of each of the antibiotic was determined after eliminating outliers. The method detection limit (MDL) is defined as 3.14 times of S, while the lower limit of quantitation (LOQ) is calculated to be 4 times of MDL. The method performance results were determined and shown in Table 5.

2.6. Spike and Recovery Experiment

A mixed standard working solution at concentration of 0.1 µg/L was spiked to the fish sample and prepared according to the sample preparation method stated in 1.3. The chromatogram of blank fish matrix and matrix spike sample are shown in Figures 12 and 13 respectively, and the matrix spike sample displayed a good recovery at the limit of quantitation.

Table 4: Repeatability Results of Retention Time and Peak Area (n=6)

Sample name	RSD% (1 µg/L)		RSD% (10 µg/L)		RSD% (100 µg/L)	
	R.T.	Area	R.T.	Area	R.T.	Area
Tetracycline	0.81	2.1	0.65	1.6	0.74	0.70
Oxytetracycline	0.79	1.5	0.65	1.6	0.73	0.89
Demethylchlortetracycline	0.81	2.0	0.64	1.8	0.73	0.84
Aureomycin	0.79	2.4	0.65	1.6	0.73	1.16
Doxycycline	0.81	2.9	0.65	2.0	0.73	1.40

Table 5: Method Detection Limit and Lower Limit of Quantitation of Tetracycline Antibiotics

No.	Compound Name	Standard deviation (SD)	Method detection limit (µg/L)	Lower limit of quantitation (µg/L)
1	Tetracycline	0.011	0.035	0.14
2	Oxytetracycline	0.013	0.041	0.16
3	Demethylchlortetracycline	0.012	0.038	0.15
4	Aureomycin	0.018	0.057	0.23
5	Doxycycline	0.014	0.044	0.18

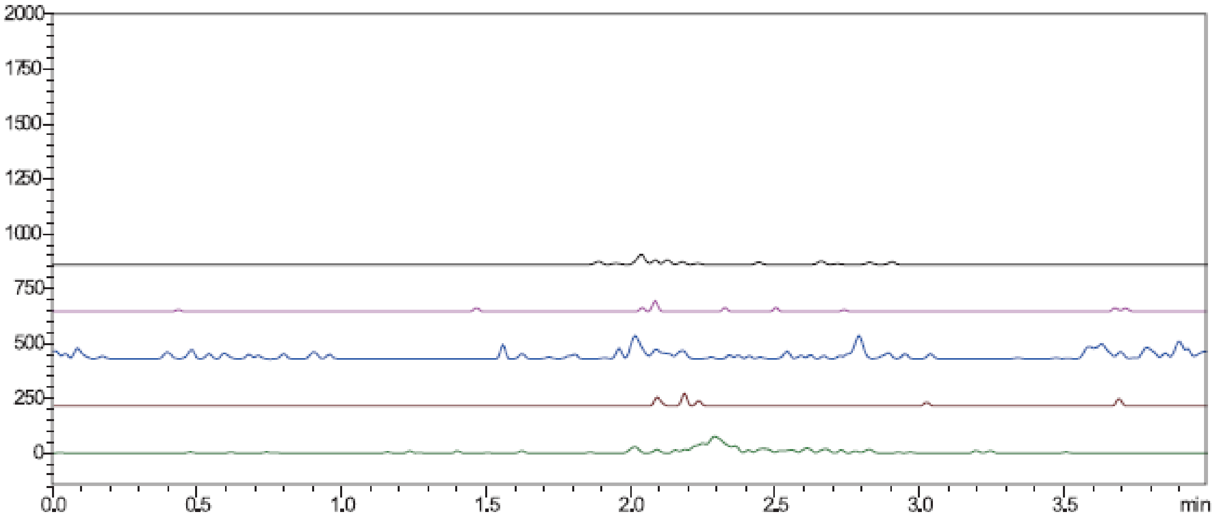

Figure 12. MRM Chromatogram of Fish Sample

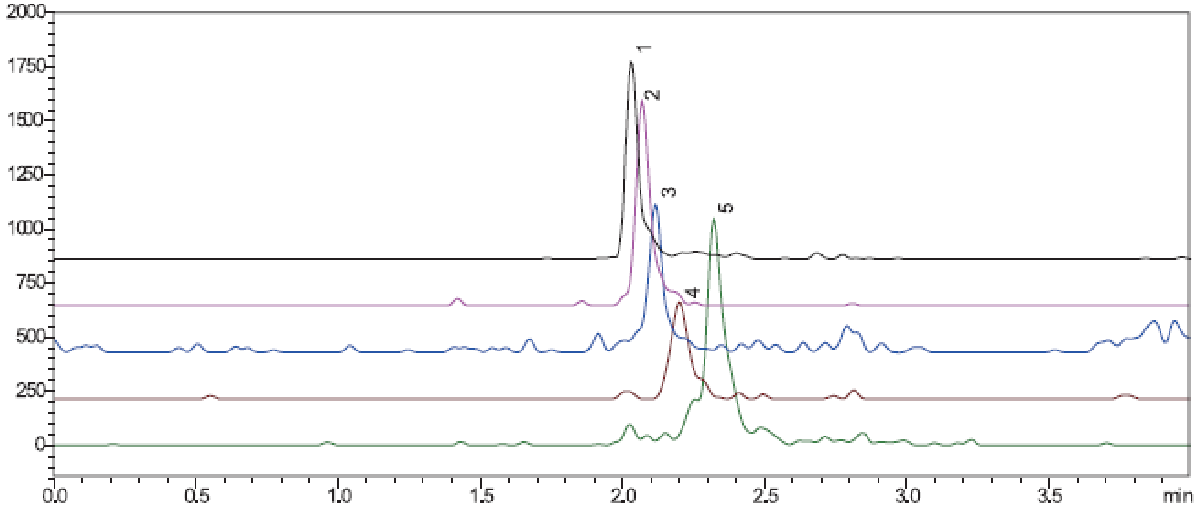

Figure 13: MRM Chromatogram of 0.1 µg/L Matrix Spike Fish Sample
(1. Tetracycline; 2. Oxytetracycline; 3. Demethylchlortetracycline; 4. Aureomycin; 5. Doxycycline)

Table 6: Matrix Spike and Recovery Results of Tetracycline Antibiotics

No.	**Compound Name**	**Measured concentration of Fish Sample 1 (µg/kg)**	**Measured concentration of Fish Sample 2 (µg/kg)**	**Average recovery (%)**
1	Tetracycline	0.10	0.11	105
2	Oxytetracycline	0.13	0.11	120
3	Demethylchlortetracycline	0.11	0.11	110
4	Aureomycin	0.11	0.09	100
5	Doxycycline	0.12	0.11	115

■ Conclusion

A method for the determination of tetracycline antibiotics residues in fish using Shimadzu Ultra-High-Performance Liquid Chromatograph LC-30A and Triple Quadrupole Mass Spectrometer LCMS-8040 was established. This method can separate and analyze five tetracyclines antibiotics in 4 minutes.

The linear range of the calibration curves of tetracycline, demethylchlortetracycline and doxycycline was 0.1-100 µg/L; while the range is 0.1-50 µg/L for oxytetracycline and aureomycin; all correlation coefficients of these tetracyclines were above 0.9977. The precision was determined by analyzing (n = 6) of the mixed standard solutions at concentrations of 1 µg/L, 10 µg/L and 100 µg/L, the RSD% of retention time and peak area was 0.64-0.81% and 0.70-2.90% respectively, indicating good instrument precision. The method detection limit was in the range of 0.035-0.057µg/L, and the limit of quantitation was 0.138-0.226 µg/L; the method spike and recovery ranged between 105-120%.

The described instrument and method demonstrated high sensitivity, good repeatability and high-throughput, this method is suitable for the detection of tetracycline, dxytetracycline, demethylchlortetracycline, aureomycin and doxycycline in fish.

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