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Screening of 49 antibiotic residues in aquatic products using modified QuEChERS sample preparation and UPLC-QToFMS analysis

Yao Gao[1], Tianwen Zhang[2], Shirong Huang[1], Xinxin Lin[1], Sisi Gong[1], Qiuhua Chen[2], Dongren Huang[2] and Min Chen[1]

[1] The School of Medical Technology and Engineering, Fujian Medical University, Fuzhou, China
[2] Fujian Fishery Resources Monitoring Center, Fuzhou, China

ABSTRACT

A precise analytical method was established for rapid screening of 49 antibiotic residues in aquatic products by ultra-high performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-QToFMS). The quick, easy, cheap, effective, rugged and safe (QuEChERS) process was refined for effective sample preparation. The homogenized samples of aquatic products were extracted with 3% acetic acid in acetonitrile, salted out with anhydrous magnesium sulfate and sodium chloride, and cleaned up by octadecylsilane (C18) and primary-secondary amine (PSA) powder. Then, the purified samples were separated on a BEH C18 column using 0.1% formic acid and methanol as mobile phases by gradient elution, detected by MS under positive Electron Spray Ionization (ESI+) mode. The linear range of matrix-matched calibration curve was 1–100 μg/L for each compound with the correlation coefficients in the range of 0.9851–0.9999. The recoveries of target antibiotics at the different spiked levels ranged from 60.2% to 117.9% except for lincomycin hydrochloride, whereas relative standard deviations (RSDs) were between 1.6% and 14.0% except for sulfaguanidine in grass Carp, Penaeus vannamei and *Scylla serrata* matrices. The limits of detection (LODs) (S/N = 3) for the analytes were 0.05–2.40 μg/kg, 0.08–2.00 μg/kg and 0.10–2.27 μg/kg and the limits of quantification (LOQs) (S/N = 10) were 0.16–8.00 μg/kg, 0.25–6.66 μg/kg and 0.32–7.56 μg/kg in grass Carp, Penaeus vannamei and *Scylla serrata*, respectively. The method was successfully applied to grass Carp, Penaeus vannamei and *Scylla serrata*, demonstrating its ability for the determination of multi-categories antibiotic residues in aquatic products.

Subjects Mass Spectrometry, Sample Handling
Keywords Antibiotics, Aquatic products, QuEChERS, UPLC-QToFMS

INTRODUCTION

Antibiotics, as a vital medicine with bactericidal or bacteriostatic effect, are widely used in modern aquaculture to prevent infectious diseases and promote growth for the increase of aquatic production (*Liu et al., 2018*; *Liu, Steele & Meng, 2017*). However, antibiotics would be a dietary risk in cultured aquatic products with abuse of antibiotics happened.

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Corresponding authors
Yao Gao, yaogao@fjmu.edu.cn
Min Chen, cmjy503@163.com

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Additional Information and Declarations can be found on page 21

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Their residues may directly enter the human body and accumulate in human organs. Therefore, they could lead to a series of adverse reactions and toxicological effects, such as allergic reactions, toxic reactions, liver damage, kidney damage, nervous system damage, and so on (*Mo et al., 2017*). More seriously, the extensive usage of antibiotics could induce antimicrobial resistance which is considered as a public health threat (*Anderson et al., 2017*). Based on both major negative effects above, regulatory limits for veterinary medicine residues are worldwide issued by many countries and organizations like Ministry of Agriculture (MOA) of China No 235 and European Union (EU) No 37/2010 (*Delatour et al., 2018*). To protect consumers, the overall situation of antibiotic residues in aquatic products that serve as a main food source in coastal areas of China has gained increasing attention from governments.

At present, the analytical methods for antibiotics in animal food mainly include liquid chromatography (LC) (*Zhou et al., 2015*), liquid chromatography tandem triple quadrupole mass spectrometry (LC-MS/MS) (*Guidi et al., 2018*) and liquid chromatography hybrid quadrupole time-of-flight mass spectrometry (LC-QToFMS) (*Ki et al., 2019*). An LC method is always equipped with fluorescence detector which has the disadvantage of lower sensitivity and poorer qualitative ability. The major shortcoming of LC-MS/MS is a limited throughput when each compound needs optimization in instrumental parameter of mass spectrometer. With the significant advances in the performance of LC-QToFMS, this platform has the outstanding merits of high resolution, high sensitivity and applicability for high throughput screening analysis in aquatic products (*Gu et al., 2019*). Owing to its excellent characteristics, hereby an ultra performance LC-QToFMS (UPLC-QToFMS) was applied for the rapid determination of multi-categories antibiotic residues at levels below their general maximum residue limits (MRLs) (2–200μg/kg) as newly set by MOA (GB 31650-2019).

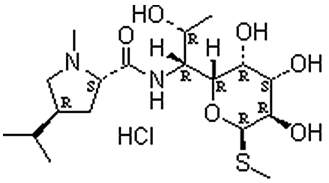
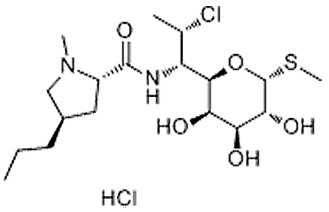
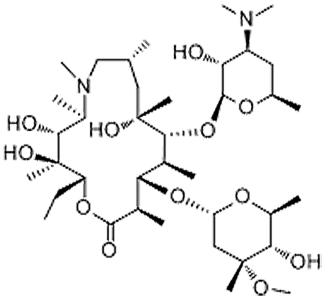
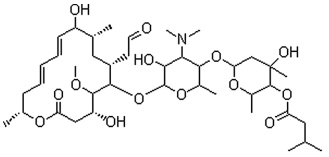
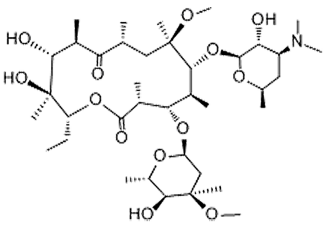
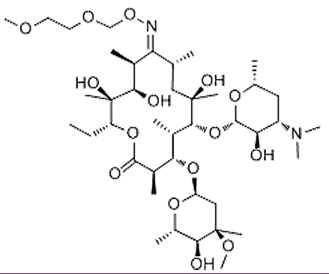
The quick, easy, cheap, effective, rugged and safe (QuEChERS) method introduced to improve extraction efficiency and to elevate method reliability in a great variety of samples, has been significantly developed and successfully applied in the residues analytical field (*Garcia & Gotah, 2017*; *Serra-Compte et al., 2017*). To our knowledge, previous researchers always focused on one sample type or a single class of veterinary drugs. *Villar-Pulido et al. (2011)* established a fast QuEChERS-LC-ToFMS method to detect 13 drug residues in shrimps. *Zhang et al. (2016)* used a QuEChERS procedure without solid-phase extraction step for rapid quantification of 90 kinds of veterinary drugs in royal jell. In this study, several kinds of aquatic products were continuously analyzed where efficiently extract multi-residues from the complex matrices is the most tough and trouble step. Therefore, development of a rapid, sensitive and simultaneous analytical method aiming at antibiotic residues at trace levels in aquatic products is urgent.

MATERIALS AND METHODS

Chemicals and solutions

A total of 49 antibiotics selected for the study contains four families including lincosamides (two), macrolides (nine), quinolones (16) and sulfonamides (22) (Table 1). Forty-nine

Table 1 CAS number, molecular formula, molecular weight, RT, characteristic ions and structural formula of 49 antibiotics.

Antibiotic	CAS	Molecular formula	Molecular weight	RT (min)	Precursor ion (m/z)	Product ions (m/z)	Structural formula
Lincomycin hydrochloride	859-18-7	$C_{18}H_{35}ClN_2O_6S$	443.00	8.17	407.2213	126.1281,359.2176	
Clindamycin hydrochloride	21462-39-5	$C_{18}H_{33}ClN_2O_5S$	461.44	11.76	425.1877	158.1179,590.3893	
Azithromycin	83905-01-5	$C_{38}H_{72}N_2O_{12}$	748.99	10.86	749.5153	158.1180,591.4227	
Leucomycin	1392-21-8	$C_{40}H_{67}NO_{14}$	785.96	13.19	786.4618	109.0657,174.1132, 558.3282	
Clarithromycin	81103-11-9	$C_{38}H_{69}NO_{13}$	747.96	13.65	748.4853	158.1180,590.3899	
Roxithromycin	80214-83-1	$C_{41}H_{76}N_2O_{15}$	837.05	13.77	837.5327	158.1185,679.4380	

(Continued)

Table 1 (continued)

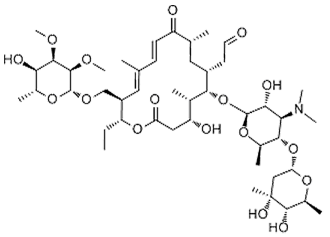
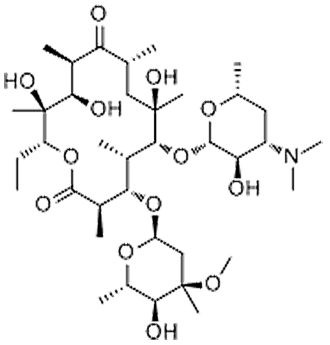
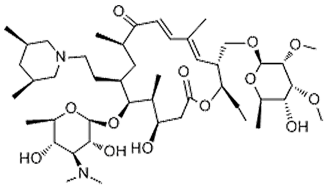
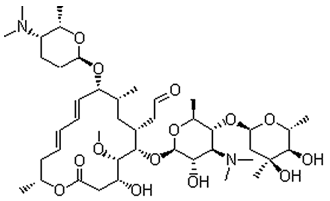
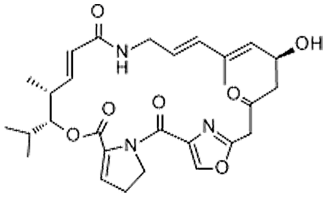
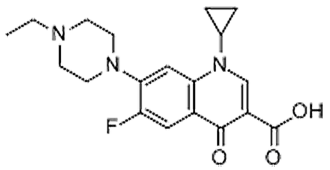
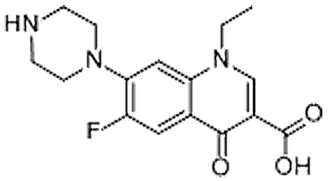
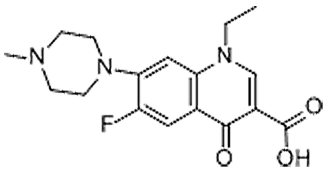
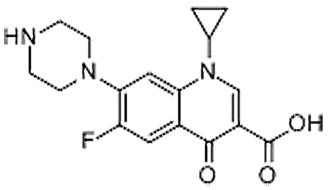
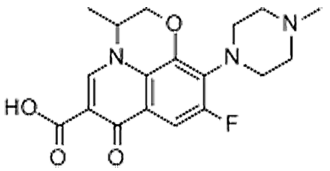
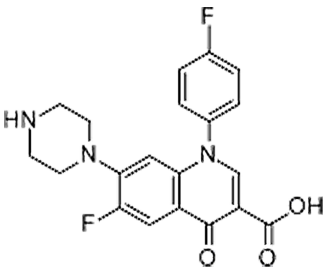
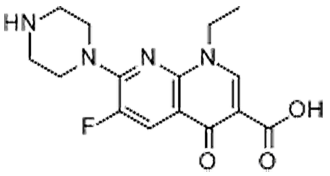
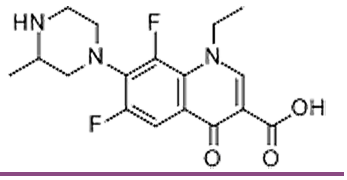
Antibiotic	CAS	Molecular formula	Molecular weight	RT (min)	Precursor ion (m/z)	Product ions (m/z)	Structural formula
Tylosin	1401-69-0	$C_{46}H_{77}NO_{17}$	916.10	12.62	916.527	174.1131,772.4469	
Erythromycin	114-07-8	$C_{37}H_{67}NO_{13}$	733.93	12.83	734.4663	158.1181,576.3743	
Tilmicosin	108050-54-0	$C_{46}H_{80}N_2O_{13}$	869.15	11.43	869.5726	174.1134,696.4655	
Spiramycin	8025-81-8	$C_{43}H_{74}N_2O_{14}$	843.06	10.46	843.5208	174.1128,540.3170	
Virginiamycin M1	21411-53-0	$C_{28}H_{35}N_3O_7$	525.59	13.34	526.2552	337.1193,508.2453	
Enrofloxacin	93106-60-6	$C_{19}H_{22}FN_3O_3$	359.39	8.79	360.1717	245.1090,316.1823	

Table 1 (continued)

Antibiotic	CAS	Molecular formula	Molecular weight	RT (min)	Precursor ion (m/z)	Product ions (m/z)	Structural formula
Norfloxacin	70458-96-7	$C_{16}H_{18}FN_3O_3$	319.33	8.54	320.1406	233.1084,276.1505	
Pefloxacin	70458-92-3	$C_{17}H_{20}FN_3O_3$	333.35	8.37	334.156	233.1091,290.1666	
Ciprofloxacin	85721-33-1	$C_{17}H_{18}FN_3O_3$	331.34	8.70	332.1404	314.1305, 231.0571, 288.1509	
Ofloxacin	82419-36-1	$C_{18}H_{20}FN_3O_4$	361.37	8.36	362.1516	261.1043,318.1618	
Sarafloxacin	98105-99-8	$C_{20}H_{17}F_2N_3O_3$	385.36	9.31	386.1315	299.0995, 342.1414, 368.1210	
Enoxacin	74011-58-8	$C_{15}H_{17}FN_4O_3$	320.32	8.39	321.1377	232.0522,303.1255	
Lomefloxacin	98079-51-7	$C_{17}H_{19}F_2N_3O_3$	351.35	8.99	352.1487	265.1143,308.1574	

(Continued)

Table 1 (continued)

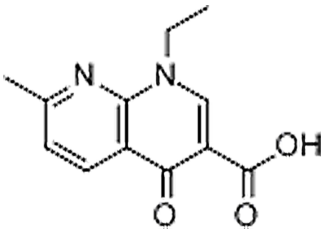
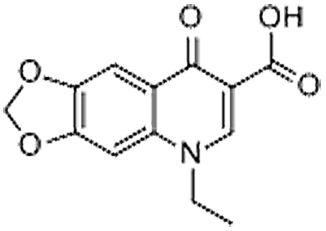
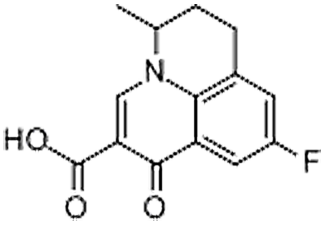
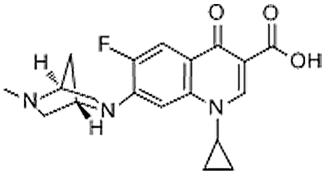
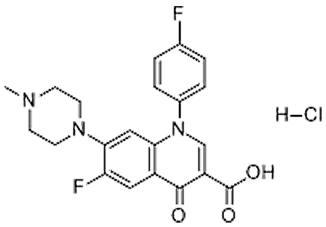
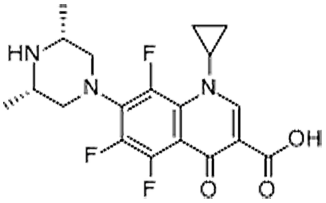
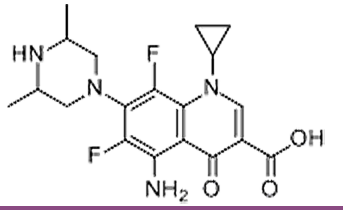
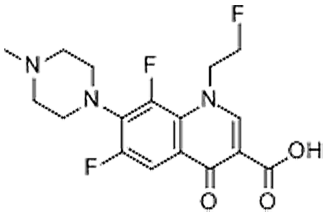
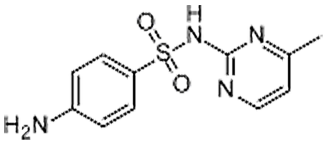
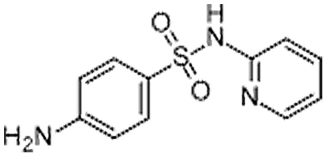
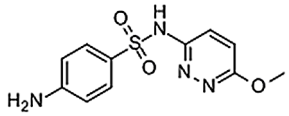
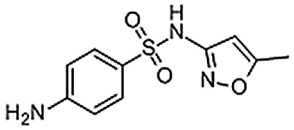
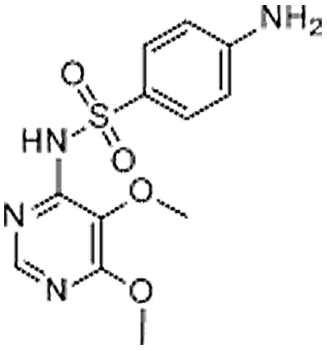
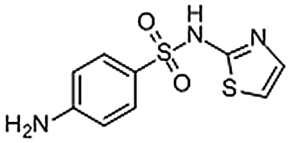
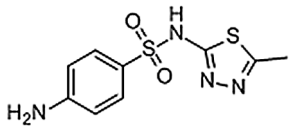
Antibiotic	CAS	Molecular formula	Molecular weight	RT (min)	Precursor ion (m/z)	Product ions (m/z)	Structural formula
Nalidixic acid	389-08-2	$C_{12}H_{12}N_2O_3$	232.24	12.02	233.0928	187.0508,215.0816	
Oxolinic acid	14698-29-4	$C_{13}H_{11}NO_5$	283.21	10.79	262.0717	244.0619	
Flumequine	42835-25-6	$C_{14}H_{12}FNO_3$	261.25	12.32	262.0882	202.0298,244.0764	
Danofloxacin	112398-08-0	$C_{19}H_{20}FN_3O_3$	357.38	8.82	358.1561	245.1083,340.1449	
Difluoxacin hydrochloride	91296-86-5	$C_{21}H_{20}ClF_2N_3O_3$	435.85	9.11	400.1471	299.0991, 358.1569, 382.1362	
Orbifloxacin	113617-63-3	$C_{19}H_{20}F_3N_3O_3$	395.38	9.06	396.1537	295.1054,352.1635	
Sparfloxacin	110871-86-8	$C_{19}H_{22}F_2N_4O_3$	392.40	9.83	393.1739	292.1250,349.1827	

Table 1 (continued)

Antibiotic	CAS	Molecular formula	Molecular weight	RT (min)	Precursor ion (m/z)	Product ions (m/z)	Structural formula
Fleroxacin	79660-72-3	$C_{17}H_{18}F_3N_3O_3$	369.34	8.10	370.1374	269.0893,326.1469	
Sulfamerazine	127-79-7	$C_{11}H_{12}N_4O_2S$	264.30	7.30	265.0754	92.0496,156.0111	
Sulfapyridine	144-83-2	$C_{11}H_{11}N_3O_2S$	249.29	6.90	250.0652	92.0495,156.0111	
Sulfamethoxypyridazine	80-35-3	$C_{11}H_{12}N_4O_3S$	280.30	8.54	281.0703	92.0496, 126.0662, 156.0114	
Sulfamethoxazole	723-46-6	$C_{10}H_{11}N_3O_3S$	253.28	9.05	254.0603	92.0497,156.0113	
Sulfadoxine	2447-57-6	$C_{12}H_{14}N_4O_4S$	310.33	9.39	311.0817	92.0496,156.0115	
Sulfathiazole	72-14-0	$C_9H_9N_3O_2S_2$	255.32	6.48	256.0212	92.0495,156.0111	
sulfamethizole	144-82-1	$C_9H_{10}N_4O_2S_2$	270.33	8.20	271.0321	92.0495,156.0113	

(Continued)

Table 1 (continued)

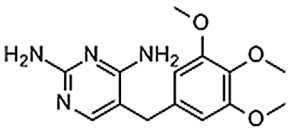
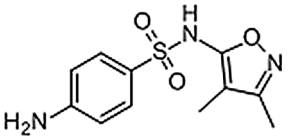
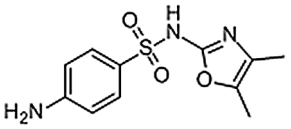
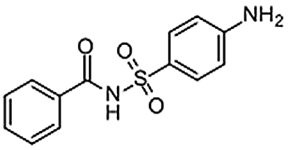
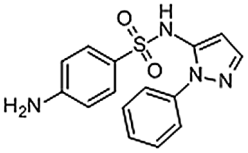
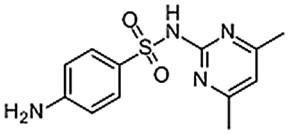
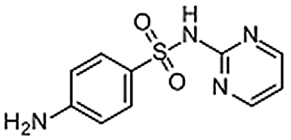
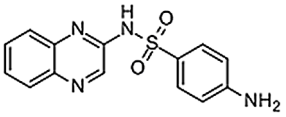
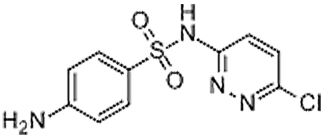
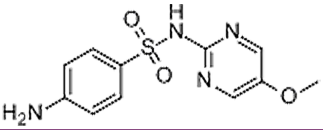
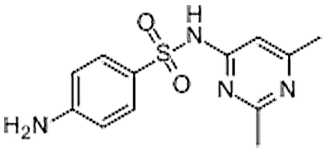
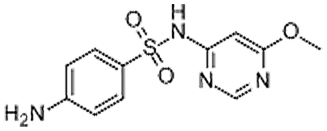
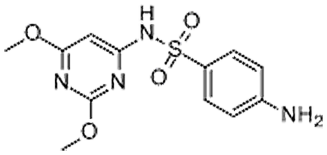
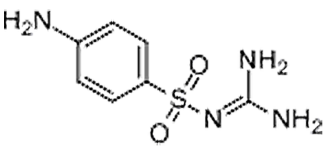
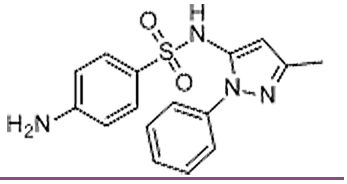
Antibiotic	CAS	Molecular formula	Molecular weight	RT (min)	Precursor ion (m/z)	Product ions (m/z)	Structural formula
Trimethoprim	738-70-5	$C_{14}H_{18}N_4O_3$	290.32	8.16	291.1467	123.0655, 261.0979, 275.1135	
Sulfisoxazole	127-69-5	$C_{11}H_{13}N_3O_3S$	267.30	8.09	268.0757	92.0495,156.0112	
Sulfamoxole	729-99-7	$C_{11}H_{13}N_3O_3S$	267.30	9.41	268.0756	92.0500, 113.0710, 156.0113	
Sulfabenzamide	127-71-9	$C_{13}H_{12}N_2O_3S$	276.31	9.80	277.0643	92.0496,156.0113	
Sulfaphenazole	526-08-9	$C_{15}H_{14}N_4O_2S$	314.36	10.13	315.0914	156.0111,158.0710	
Sulfamethazine	57-68-1	$C_{12}H_{14}N_4O_2S$	278.33	8.30	279.0917	124.0828, 156.0119, 186.0330	
Sulfadiazine	68-35-9	$C_{10}H_{10}N_4O_2S$	250.28	5.23	251.0596	92.0496,156.0112	
Sulfaquinoxaline	59-40-5	$C_{14}H_{12}N_4O_2S$	300.34	10.81	301.076	146.0713,156.0114	
Sulfachlorpyridazine	80-32-0	$C_{10}H_9ClN_4O_2S$	284.72	8.87	285.0206	92.0497,156.0115	
Sulfameter	651-06-9	$C_{11}H_{12}N_4O_3S$	280.30	9.16	281.0701	92.0493, 126.0657, 156.0107	

Table 1 (continued)

Antibiotic	CAS	Molecular formula	Molecular weight	RT (min)	Precursor ion (m/z)	Product ions (m/z)	Structural formula
Sulfisomidine	515-64-0	$C_{12}H_{14}N_4O_2S$	278.33	5.82	279.0917	124.0867,186.0328	
Sulfamonomethoxine	1220-83-3	$C_{11}H_{12}N_4O_3S$	280.30	8.05	281.0706	126.0660,156.0111	
Sulfadimethoxine	122-11-2	$C_{12}H_{14}N_4O_4S$	310.33	10.54	311.0817	92.0494,156.0764	
Sulfaguanidine	57-67-0	$C_7H_{10}N_4O_2S$	214.24	1.89	215.0601	92.0494,156.0112	
Sulfapyrazole	852-19-7	$C_{16}H_{16}N_4O_2S$	328.39	10.73	329.107	156.0121,172.0870	

antibiotic standards and six internal isotope standards (roxiyhromycin-D7, enrofloxacin-D5 hydrochloride, sulfadoxine-D3, ciprofloxacin-D8, norfloxacin-D5, and sulfadimethoxine-D6, purity:93.6%) were obtained from Dr. Ehrenstorfer GmbH (Germany). Methanol, acetonitrile, ethyl acetate were purchased from Merck (UPLC-grade; Darmstadt, Germany). Anhydrous sodium sulfate of analytical reagent grade and HPLC-grade formic acid, acetic acid, sodium chloride, octadecylsilane (C18), alumina-N (ALU-N), primary-secondary amine (PSA) and leucine enkephalin was provided by ANPEL (China).

Individual stock solutions (100 μg/mL) were prepared by dissolving each antibiotic standard in methanol and then stored at −18 °C. Mixed standard solution (1 μg/mL) were diluted from the stock solutions with methanol. Calibration curves were obtained by diluting mixed standard solution with acetonitrile—water solvent (25:75 v/v) at the final concentration of 1, 5, 10, 25, 50, 100 ng/mL. The concentrations of 6 isotope internal standards in each calibration standard solution were 20 ng/mL.

Sample treatment

Three main species of aquatic products including grass Carp, Penaeus vannamei and *Scylla serrata*, which acted as common food in Fujian province were involved in this research.

After collection from supermarkets, 32 fresh samples of aquatic products were treated according to Practice of sampling plans for aquatic products (GB/T 30891-2014) including amount, size, transport and storage of sampling. To prevent antibiotic degradation, they were immediately stored in the refrigerator at −20 °C prior to analysis. Each kind of aquatic samples (2 ± 0.01 g) was thawed at room temperature and weighed into a 50 mL centrifuge tube. Afterwards, each tube was added with 50 μL mixed antibiotic standard solution (1 μg/mL) and then was mixed and placed for 15 min.

Antibiotic extraction and clean-up optimization

The targeted residues were extracted using a modified QuEChERS method, which were optimized in terms of extractants, salting-out agents and sorbents. Antibiotics were extracted by 10 mL ACN with 3% acetic acid. Then, salting-out agent (3 g of anhydrous Na_2SO_4 and 1 g of NaCl) were successively placed into the tube and swirled for 1 min. Subsequently, the tube was centrifuged for 5 min at 10,000 rpm 4 °C. A 6.5 mL supernatant was transferred to a 15 mL centrifuge tube containing the sorbents of 200 mg C18 and 50 mg PSA. The tube was swirled for 2 min and then centrifuged for 10 min at 5,000 rpm 4 °C. Five milliliters aliquot of supernatant was pipetted to a 25-mL evaporation flask and dried using a rotary evaporator under a nitrogen flow at 50 °C. The residue was fully resuspended in 1 mL of acetonitrile-water solvent (25:75 v/v) by ultrasonication and oscillation. The solution was subsequently filtered through 0.22 μm nylon membrane before final placement into an auto-sampler vial for the UPLC-QToFMS analysis.

Instrumental conditions

Instrumental

ACQUITY H-CLASS UPLC and Xevo G2-S Q-ToF mass spectrometer (Waters, Milford, MA, USA) with electrospray ionization source were used. A 3–30K high speed refrigerated centrifuge (SiGMA, Ronkonkoma, NY, USA), MS3 digital vortex mixer (IKA, Königswinter, Germany), laborata 4000 efficient rotary evaporator (Heidolph, Schwabach, Germany), multi Reax oscillator (Heidolph, Schwabach, Germany), N-EVAP™ 112 (Organomation Associates, Berlin, MA, USA) and Milli-Q water purification system (Millipore, Burlington, MA, USA) were used for sample preparation.

LC conditions

The separation of mixed antibiotic standard solutions were achieved on a Waters Acquity UPLC BEH C18 silica column (100 mm × 3.0 mm, 1.7 μm). A gradient LC elution method was employed by 0.1% formic acid aqueous solution as mobile phase A and methanol as mobile phase B.

The gradient elution was as follows: 10% B at 0–3 min, 10–100% B at 3–15 min, 100% B at 15–18 min, 100–10% B at 18–18.1 min and 10% B at 18.1–21 min. The injection volume, flow rate, sample manager and column temperature were set at 10 μL, 0.3 mL/min, 10 °C and 40 °C, respectively. All target antibiotics were eluted, and the column was cleaned and equilibrated.

MS conditions

MS experiments were operated using electrospray ionization (ESI) in the positive mode. The optimum MS parameters were as follows: mass collection range 50–1,000 Da; capillary voltage 3.0 kV; ion source temperature 120 °C; desolvation temperature 450 °C; cone gas flow 50 L/h; desolvation gas flow rate 800 L/h and core voltage 40 V.

QToFMS screening for 49 antibiotic residues was performed using MS^E mode. The simultaneous acquisition of accurate-mass full-spectrum at low and high collision energy are allowed in MS^E mode, where the low collision energy (LE) spectrum provides useful information on the parent molecules and the main fragment ions were obtained commonly in the high collision energy (HE) function. In this study, LE was set as 6 V and HE was set from 10 eV to 40 eV. Leucine enkephalin, a commonly used peptide, was employed here as a reference material to tune MS instruments in every 10 s.

RESULTS AND DISCUSSION

Optimization of LC condition

The effect of the two types of mobile phases in the separation process were compared between 0.1% formic acid-acetonitrile and 0.1% formic acid water-methanol. As shown in Fig. 1, using 0.1% formic acid water-acetonitrile as the mobile phases, it is difficult to separate sulfamonomethoxine and sulfamethoxypyridazine completely. It was found that when methanol was used, better resolution and higher overall signal response were obtained. Therefore, 0.1% formic acid water-methanol was selected as the mobile phase in this experiment.

Optimization of the QuEChERS process

Sample extraction

For the purpose of optimizing extraction of the antibiotic residues for different substrates of aquatic products including grass Carp, Penaeus vannamei and *Scylla serrata*, ethyl acetate and acetonitrile mixed with different amounts of acetic acid were compared. As shown in Fig.2, 3% acetic acid acetonitrile was used as the extractant, and the average recoveries of 49 antibiotics in three matrices were 75.3%, 76.7%, 81.8%, respectively, which were higher than using 1% acetic acid-acetonitrile (v:v), 5% acetic acid-acetonitrile (v:v), and ethyl acetate for the extraction. Intriguingly the acidity of the extractant has a great effect on the quinolones. The sequence of recoveries of quinolones from low to high was ethyl acetate, acetonitrile, 1% acetic acid acetonitrile, 3% acetic acid acetonitrile, 5% acetic acid acetonitrile when each of them was performed as the extractant. The possible reason is that quinolones, which are amphoteric, are easily soluble in acidic or alkaline such as acetic acid solutions. From these results, 3% acetic acid acetonitrile was chosen as the optimum composition of solvents for the extraction buffer.

Purification procedure

Five most commonly used sorbents were investigated in this experiment, including PSA, C18, ALU-N, PSA-C18 mixture, PSA-ALU-N mixture. The purification effects on grass Carp, Penaeus vannamei and *Scylla serrata* were shown in Fig. 3. It is obvious that

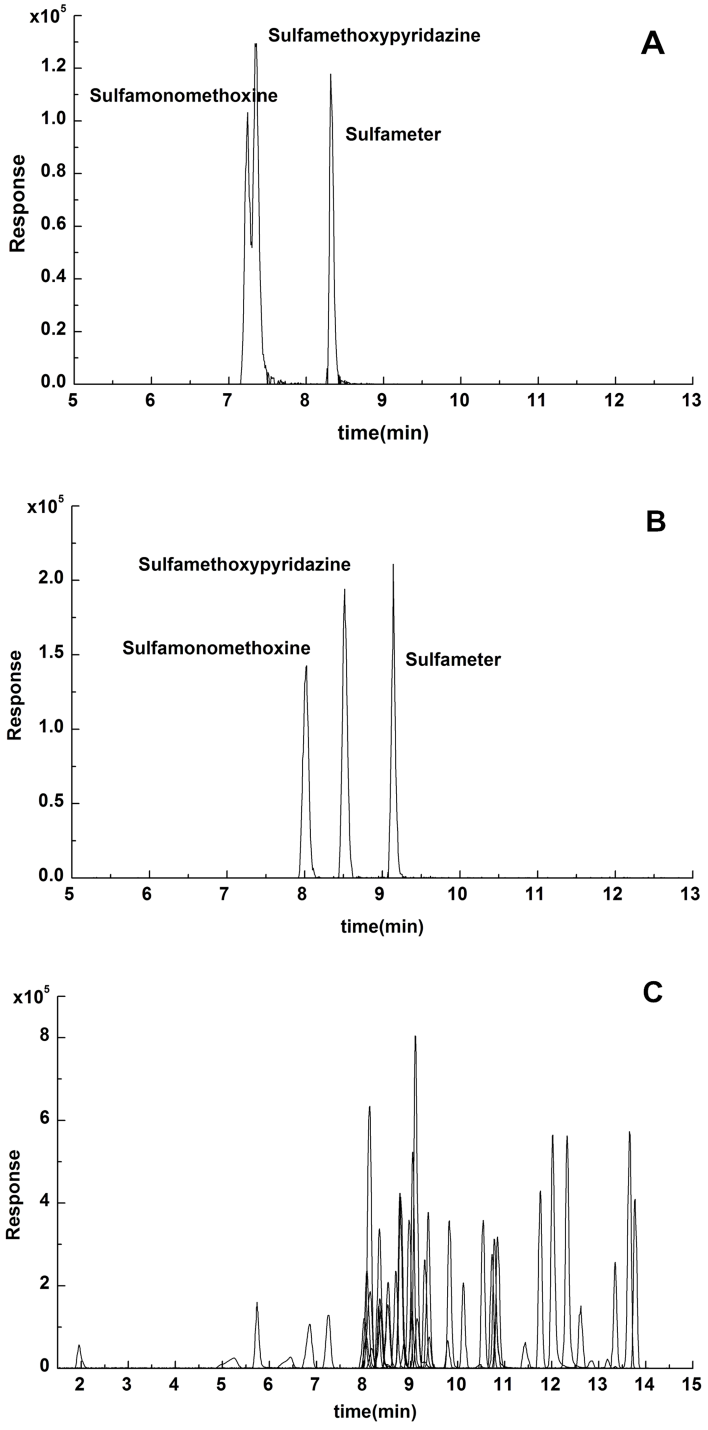


Figure 1 Chromatogram of the three isomers of sulfamonomethoxine, sulfamethoxypyridazine and sulfameter with (A) 0.1% formic acid water-acetonitrile and (B) 0.1% formic acid water-methanol as the mobile phase, respectively. (C) Overlapping extracted ion chromatograms of 49 antibiotics with 0.1% formic acid water-methanol as the mobile phase. Using 0.1% formic acid water-acetonitrile as the mobile phases, it is difficult to separate sulfamonomethoxine and sulfamethoxypyridazine completely.

Full-size DOI: 10.7717/peerj-achem.8/fig-1

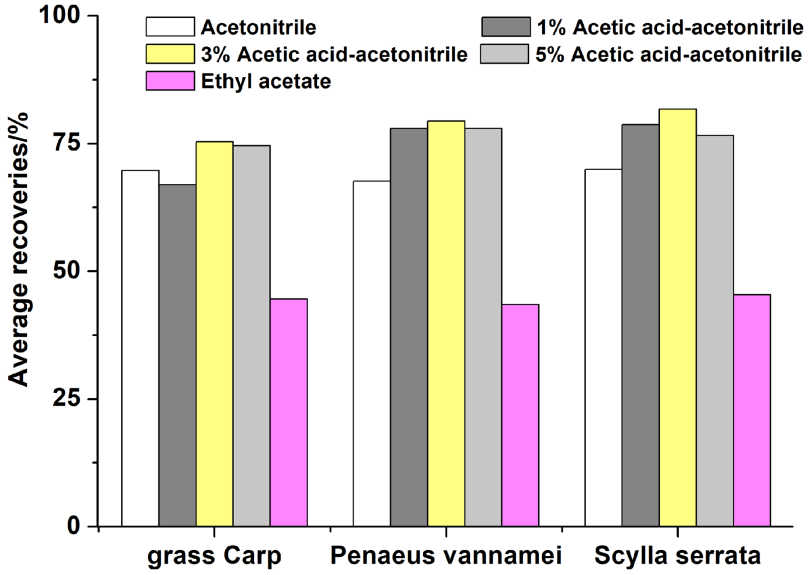


Figure 2 Effects of different extracting solvents on the recoveries of the 49 antibiotics. Using 3% acetic acid acetonitrile as the extractant, the average recoveries of 49 antibiotics in three matrices were 75.3%, 76.7%, 81.8%, respectively, which were higher than using 1% acetic acid-acetonitrile (v:v), 5% acetic acid-acetonitrile (v:v), and ethyl acetate for the extraction.

Full-size DOI: 10.7717/peerj-achem.8/fig-2

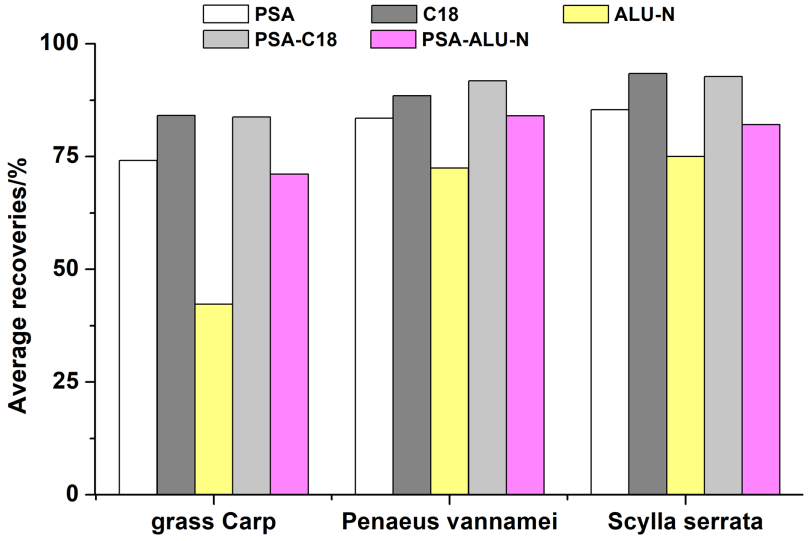


Figure 3 Effects of five different sorbents on the average recoveries of the 49 antibiotics in grass Carp, Penaeus vannamei and *Scylla serrata*. The highest average recoveries of all 49 antibiotics in three matrices were achieved using PSA-C18, overall.

Full-size DOI: 10.7717/peerj-achem.8/fig-3

ALU-N gets an inferior purification effect probably because ALU-N has a certain adsorption effect on antibiotics especially quinolones. The highest average recoveries of all 49 antibiotics in three matrices were achieved using PSA-C18, overall.

Afterwards, the amounts of salting-out agents (anhydrous Na_2SO_4 and NaCl) and sorbents (PSA and C18) were optimized using $L_9(3^4)$ orthogonal experimental design

Table 2 Orthogonal design for sorbents and salting agents.

Levels	Factors		
	PSA (mg)	C18 (mg)	Na_2SO_4: NaCl (g:g)
1	50	100	4:1
2	100	200	3:1
3	150	300	2:1

at three levels (Table 2). The results indicated that satisfactory recoveries of 49 antibiotics were observed when 3g Na_2SO_4/1 g NaCl and 50 mg PSA/200 mg C18 were conducted.

After optimization, the average recoveries of 49 antibiotics in grass Carp, Penaeus vannamei and *Scylla serrata* reached 83.4%, 88.4%, and 88.8% respectively, while this procedure provided the best results for the majority of target antibiotics. In summary, this improved QuEChERS process for antibiotic extraction in aquatic products is fast, effective, economical and eco-friendly.

Method validation

Identification

As listed in Table 1, each of the 49 target antibiotics was measured in MS^E mode by one precursor ion and at least two product ions. Meanwhile, retention time was also required to provide vital information to identify specific antibiotics.

Linear range, regression equation, limits of detection and limits of quantitation

The series of solvent-based standard solutions were prepared according to "Chemicals and Solutions" and were then determined by UPLC-QToFMS. The calibration curves were obtained from the relationship between the analyte concentration (X, μg/L) and the analyte peak areas/internal standard peak area, providing the linear equation and the correlation coefficient for each analyte. The linear ranges were 1–100 μg/L for each examined analyte with correlation coefficients of greater than 0.9888. The limits of detection (LODs) were evaluated with signal-to-noise ratio (S/N) of 3 and the limits of quantification (LOQs) were evaluated with S/N of 10. LODs and LOQs of solvent-based calibration curves were in the range of 0.01–1.33 μg/L and 0.04–4.42 μg/L, respectively.

Matrix effects

Aquatic products are rich in proteins and unsaturated fatty acids, as well as they contain a variety of vitamins, minerals, trace elements and so on. Complex components cause ubiquitous matrix effects (signal suppression and enhancement) during the LC–MS/MS analysis which may strongly affect the quantitative accuracy and reproducibility in this study (*Guo et al., 2016*). Here, the matrix effects of three subtracts were evaluated by

Table 3 Matrix effects, LODs and LOQs for all matrices tested.

Antibiotic	grass Carp		Penaeus vannamei		*Scylla serrata*	
	Matrix effect (%)	LOD/LOQ (μg/kg)	Matrix effect (%)	LOD/LOQ (μg/kg)	Matrix effect (%)	LOD/LOQ (μg/kg)
Lincomycin hydrochloride	31.79	0.21/0.71	41.39	0.83/2.77	43.54	0.77/2.55
Clindamycin hydrochloride	−25.94	0.31/1.04	−1.62	0.28/0.94	−24.43	0.31/1.03
Azithromycin	−11.18	0.12/0.41	−7.69	0.26/0.86	0.31	0.17/0.58
Clarithromycin	17.16	0.05/0.16	32.76	0.08/0.25	29.59	0.17/0.56
Roxithromycin	−9.19	0.07/0.23	−18.25	0.09/0.30	−32.34	0.14/0.45
Tylosin	51.20	0.18/0.61	50.99	0.26/0.86	47.37	0.42/1.39
Erythromycin	−8.76	2.40/8.00	−1.99	1.12/3.73	7.00	1.78/5.93
Tilmicosin	27.82	0.36/1.18	15.82	0.48/1.59	36.00	0.66/2.20
Spiramycin	−0.50	1.32/4.40	−7.63	1.65/5.50	−2.03	1.38/4.60
Virginiamycin M1	28.51	0.48/1.60	31.29	0.39/1.29	42.49	0.24/0.81
Enrofloxacin	6.17	0.33/1.09	4.81	0.41/1.35	5.34	0.40/1.34
Norfloxacin	4.56	0.56/1.86	12.74	0.74/2.47	15.72	1.35/4.51
Pefloxacin	26.43	0.60/1.99	27.39	0.55/1.85	7.24	1.14/3.81
Ciprofloxacin	−14.44	0.20/0.65	−8.25	0.33/1.11	−12.74	0.49/1.63
Ofloxacin	−29.83	0.65/2.18	−25.24	0.25/0.84	−43.51	0.51/1.69
Sarafloxacin	−5.64	0.38/1.27	5.55	0.15/0.49	7.29	0.42/1.40
Enoxacin	12.29	1.44/4.80	5.83	1.54/5.15	12.33	2.09/6.98
Lomefloxacin	3.40	0.29/0.98	5.78	0.26/0.85	15.48	0.61/2.04
Nalidixic acid	−3.27	0.26/0.88	8.22	0.22/0.75	0.68	0.19/0.62
Oxolinic acid	−10.55	0.18/0.60	2.98	0.38/1.26	−4.17	0.56/1.88
Flumequine	−15.30	0.22/0.74	5.03	0.15/0.51	−24.12	0.33/1.09
Danofloxacin	−5.79	0.20/0.68	−7.85	0.66/2.20	−0.75	0.65/2.15
Difluoxacin hydrochloride	−17.38	0.16/0.53	−5.70	0.08/0.28	−3.54	0.13/0.45
Orbifloxacin	4.17	0.13/0.43	4.59	0.11/0.36	−0.25	0.16/0.53
Sparfloxacin	−5.40	0.23/0.77	−21.83	0.20/0.65	−35.49	0.34/1.13
Fleroxacin	3.25	0.31/1.03	−14.68	0.80/2.65	−29.59	0.69/2.31
Sulfamerazine	2.09	0.29/0.98	30.66	0.17/0.57	18.46	0.23/0.78
Sulfapyridine	1.84	0.23/0.77	12.30	0.30/0.99	−10.27	0.24/0.80
Sulfamethoxypyridazine	−12.69	0.55/1.83	0.76	0.58/1.95	28.26	0.10/0.34
Sulfamethoxazole	1.90	0.12/0.41	10.38	0.27/0.89	4.80	0.45/1.50
Sulfadoxine	−3.65	0.21/0.69	9.84	0.19/0.63	0.73	0.12/0.40
Sulfathiazole	6.85	0.24/0.79	13.20	0.52/1.73	13.02	0.15/0.49
Sulfamethizole	−5.47	0.60/2.01	7.40	0.72/2.41	3.91	0.40/1.32
Trimethoprim	0.25	0.10/0.34	−1.28	0.08/0.26	−1.78	0.10/0.32
Sulfisoxazole	−11.43	0.21/0.69	3.77	0.20/0.66	15.40	1.18/3.94
Sulfamoxole	−19.74	0.37/1.23	−24.75	0.50/1.66	−26.04	0.20/0.67
Sulfabenzamide	−11.43	0.42/1.41	−0.98	0.83/2.76	0.55	1.00/3.34
Sulfaphenazole	1.42	0.29/0.97	27.97	0.35/1.17	29.30	0.80/2.67
Sulfamethazine	−5.14	2.13/7.11	17.09	2.00/6.66	21.71	2.04/6.79

(Continued)

Table 3 (continued)

Antibiotic	grass Carp		Penaeus vannamei		*Scylla serrata*	
	Matrix effect (%)	LOD/LOQ (μg/kg)	Matrix effect (%)	LOD/LOQ (μg/kg)	Matrix effect (%)	LOD/LOQ (μg/kg)
Sulfadiazine	−9.30	0.60/2.02	1.00	0.42/1.39	28.47	0.76/2.53
Sulfaquinoxaline	−22.11	0.37/1.25	−16.77	0.42/1.39	−2.79	0.89/2.96
Sulfachlorpyridazine	−3.70	0.25/0.82	18.58	0.60/1.99	19.14	0.36/1.20
Sulfameter	−17.09	0.64/2.15	−4.26	0.48/1.60	0.49	0.67/2.25
Sulfisomidine	−24.53	1.86/6.20	−21.60	1.89/6.31	−13.95	2.27/7.56
Sulfamonomethoxine	−9.24	0.28/0.92	−24.85	0.35/1.18	−38.07	0.66/2.20
Sulfadimethoxine	−5.97	0.34/1.13	−6.84	0.24/0.81	−7.14	0.20/0.66
Sulfaguanidine	−13.11	1.54/5.14	−12.19	1.82/6.07	−10.28	2.00/6.67
Sulfapyrazole	−15.58	0.15/0.5	−17.05	0.22/0.72	−19.00	0.16/0.52

comparing the calibration curves of the target antibiotics prepared in solvent and in the matrix (*Hernando et al., 2007*), which is calculated as:

$$\text{Matrix effect } (\%) = (\text{Slope}_{\text{matrix-matched standard curve}}/\text{Slope}_{\text{solvent-based standard curve}} - 1) \times 100$$

Three sets of blank matrix samples were introduced to the mixed standard solution of different concentrations (1, 5, 10, 25, 50, 100 μg/L). As listed in Table 3, among the three matrices of grass Carp, Penaeus vannamei and *Scylla serrata*, matrix effects could still encountered in determining several antibiotics such as lincomycin hydrochloride, clindamycin hydrochloride and tylosin. Therefore, matrix-matched standard curves were applied to mitigate matrix effects for quantification of 49 antibiotics. The results of the regression analysis showed that the correlation coefficients (R^2) of the matrix-matched standard curves of 49 antibiotics in grass Carp, Penaeus vannamei and *Scylla serrata* ranged from 0.9900 to 0.9999, 0.9851 to 0.9998, 0.9908 to 0.9997, respectively which indicated excellent linearity.

Based on data obtained from matrix-matched standard curves of 49 antibiotics in grass Carp, Penaeus vannamei and *Scylla serrata*, the range of the LODs were 0.05–2.40 μg/kg, 0.08–2.00 μg/kg and 0.10–2.27 μg/kg, respectively. And LOQs were in the range of 0.16–8.00 μg/kg, 0.25–6.66 μg/kg and 0.32–7.56 μg/kg, respectively. Hereby, the results of all the LODs and LOQs exhibited in Table 3 in this research were satisfactory as compared with the MRLs.

Recovery and precision

In order to investigate the accuracy and precision of this method, recovery experiments were conducted at different spiking levels of 10, 50, 100 μg/kg (Table 4). Among the 49 antibiotics, except for lincomycin hydrochloride whose recoveries were less than 60%, the recoveries of other antibiotics in three matrices were generally greater than 70%. These results indicated that this method had a satisfactory stability and could meet the actual detecting requirements of 49 antibiotics in aquatic products.

Table 4 Recoveries and repeatability (expressed as %RSD) results for all matrices tested.

Antibiotic	Spiked levels (μg/kg)	grass Carp		Penaeus vannamei		*Scylla serrata*	
		Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
Lincomycin hydrochloride	10	54.1	5.4	37.9	6.7	44.0	10.4
	50	55.5	2.7	37.4	4.8	32.2	11.6
	100	50.7	3.1	39.6	5.4	39.3	15.7
Clindamycin hydrochloride	10	76.4	10.9	76.6	5.5	81.4	4.2
	50	73.8	5.7	76.0	5.3	73.0	11.5
	100	74.9	4.1	100.5	3.5	82.8	6.4
Azithromycin	10	100.0	10.4	104.8	6.0	111.2	3.7
	50	81.8	5.4	101.6	4.6	95.6	5.5
	100	100.2	7.7	116.0	2.0	104.3	3.1
Leucomycin	10	81.2	7.0	86.8	4.9	63.8	3.7
	50	82.8	7.4	88.7	5.7	69.4	3.5
	100	73.4	8.0	93.4	7.8	77.9	5.6
Clarithromycin	10	89.8	7.3	95.8	5.7	98.6	5.3
	50	96.6	4.4	102.0	2.4	95.9	6.4
	100	88.7	3.8	100.4	4.7	105.1	2.2
Roxithromycin	10	91.0	2.2	94.8	2.2	89.1	5.0
	50	79.8	3.6	87.4	5.0	73.5	6.3
	100	84.6	3.8	90.3	2.6	83.1	6.0
Tylosin	10	77.3	7.7	87.6	6.1	104.7	5.4
	50	76.8	4.6	91.4	5.1	99.2	3.7
	100	74.1	3.6	103.3	3.2	101.3	6.4
Erythromycin	10	88.3	9.1	97.8	14.0	93.1	5.5
	50	76.1	4.7	78.0	8.3	75.7	5.1
	100	78.0	3.1	66.6	5.5	64.8	5.0
Tilmicosin	10	93.9	7.3	97.9	6.9	89.1	6.5
	50	80.8	3.4	95.3	4.8	100.7	3.1
	100	97.2	7.2	101.4	3.5	106.4	2.8
Spiramycin	10	74.7	11.3	91.7	8.6	100.7	4.8
	50	60.2	10.8	74.7	5.1	73.1	3.4
	100	64.6	4.5	85.9	11.1	71.9	5.5
Virginiamycin M1	10	73.0	12.7	102.4	4.1	103.4	4.9
	50	75.7	6.6	98.2	3.4	88.7	8.9
	100	68.1	6.0	107.4	4.8	91.0	4.6
Enrofloxacin	10	99.2	4.9	109.1	2.6	101.4	4.4
	50	90.4	2.4	107.1	3.0	100.4	2.8
	100	95.6	4.7	104.5	3.1	101.8	1.7
Norfloxacin	10	104.0	4.3	84.4	6.0	87.6	4.5
	50	101.2	6.9	84.4	4.0	90.1	7.1
	100	103.6	5.3	87.1	4.4	93.3	5.6

(Continued)

Table 4 (continued)

Antibiotic	Spiked levels (μg/kg)	grass Carp		Penaeus vannamei		*Scylla serrata*	
		Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
Pefloxacin	10	86.1	5.3	105.1	8.1	108.4	4.2
	50	96.4	9.3	106.2	4.0	108.2	4.1
	100	101.0	5.8	104.1	4.1	105.1	2.7
Ciprofloxacin	10	78.9	2.8	86.0	2.4	94.3	5.5
	50	86.0	3.8	83.8	3.9	98.7	4.9
	100	91.7	4.5	90.5	5.7	103.1	5.4
Ofloxacin	10	83.8	4.0	101.8	6.6	95.3	6.8
	50	97.5	5.2	106.3	2.4	109.0	4.2
	100	92.4	5.5	97.4	4.8	105.1	3.7
Sarafloxacin	10	85.7	6.3	81.3	3.8	86.6	5.6
	50	91.7	3.7	85.5	5.1	94.4	5.4
	100	97.0	4.7	100.2	7.3	98.0	8.1
Enoxacin	10	92.3	6.8	96.6	9.7	89.0	5.5
	50	95.9	7.1	103.1	2.9	105.6	4.0
	100	98.0	5.6	100.4	2.6	104.0	5.3
Lomefloxacin	10	92.2	7.1	88.0	4.5	102.3	6.1
	50	89.4	8.6	84.7	4.5	102.8	4.3
	100	104.3	4.9	98.3	5.4	104.1	2.7
Nalidixic acid	10	77.8	6.6	74.3	7.5	66.7	4.0
	50	103.3	4.0	87.5	4.0	75.8	3.9
	100	106.5	2.7	97.4	2.6	82.4	3.6
Oxolinic acid	10	78.2	8.6	70.3	5.5	65.8	3.8
	50	99.2	11.0	86.4	5.0	76.8	4.2
	100	102.3	3.2	94.2	3.9	81.3	2.3
Flumequine	10	75.0	8.9	74.2	8.9	66.8	3.2
	50	106.2	5.1	88.7	3.6	80.9	4.4
	100	103.3	2.0	95.8	4.3	85.8	2.1
Danofloxacin	10	112.9	2.0	117.9	2.8	105.2	4.2
	50	95.6	2.7	101.5	4.2	105.2	2.4
	100	100.7	4.2	102.1	3.5	106.5	3.5
Difluoxacin hydrochloride	10	92.0	2.8	79.8	6.1	95.0	7.2
	50	94.8	3.5	79.8	6.1	104.6	3.3
	100	102.4	2.0	98.3	4.9	104.5	2.5
Orbifloxacin	10	72.8	11.5	74.0	3.8	79.3	4.3
	50	92.9	8.6	85.6	5.5	97.8	4.1
	100	99.9	3.9	96.7	3.0	102.7	3.2
Sparfloxacin	10	75.1	5.0	75.7	6.8	63.6	3.2
	50	79.8	4.5	93.5	3.1	88.9	5.6
	100	78.0	1.7	106.0	4.1	100.6	3.8

Table 4 (continued)

Antibiotic	Spiked levels (μg/kg)	grass Carp		Penaeus vannamei		*Scylla serrata*	
		Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
Fleroxacin	10	116.0	6.0	110.7	7.6	108.1	3.1
	50	111.9	4.2	105.5	5.4	104.4	3.4
	100	102.2	5.0	106.3	2.2	104.2	4.6
Sulfamerazine	10	67.1	6.4	86.3	5.8	90.6	2.6
	50	80.4	6.4	81.6	2.1	75.2	7.0
	100	72.7	3.0	81.4	6.6	71.6	4.0
Sulfapyridine	10	71.6	3.6	88.4	8.4	88.1	4.9
	50	78.4	8.5	76.7	2.7	82.2	6.1
	100	79.5	3.8	76.1	6.7	86.4	3.6
Sulfamethoxypyridazine	10	74.7	5.1	88.0	5.0	87.1	2.4
	50	73.9	5.1	75.2	2.6	76.4	2.8
	100	77.9	7.7	84.1	2.1	80.8	3.0
Sulfamethoxazole	10	73.7	4.2	85.9	9.5	82.7	3.5
	50	74.7	7.4	82.9	1.9	71.0	7.4
	100	76.4	3.6	78.8	6.0	69.9	3.9
Sulfadoxine	10	69.1	4.1	88.4	4.5	84.8	3.3
	50	75.2	4.0	82.0	5.3	71.0	4.3
	100	77.1	3.0	83.4	2.8	72.7	3.3
Sulfathiazole	10	72.3	3.4	88.6	10.1	73.1	8.8
	50	73.0	8.5	90.6	4.9	85.4	6.0
	100	71.8	2.4	86.4	4.6	90.7	4.2
Sulfamethizole	10	65.6	4.7	95.6	3.9	86.7	6.2
	50	72.6	5.3	75.2	3.8	85.6	6.3
	100	72.2	2.8	85.4	4.2	92.8	7.3
Trimethoprim	10	85.3	8.6	92.1	4.0	85.3	5.1
	50	105.6	5.1	95.2	3.0	90.8	4.3
	100	100.4	5.4	94.7	3.6	103.2	1.6
Sulfisoxazole	10	71.6	3.3	85.3	9.6	81.9	5.3
	50	78.7	7.4	74.3	5.1	85.9	5.3
	100	74.4	2.9	91.9	4.9	103.4	3.3
Sulfamoxole	10	76.9	3.5	94.3	3.8	89.0	4.6
	50	78.3	5.0	85.2	4.5	81.6	4.8
	100	78.4	3.4	91.1	3.5	87.0	3.7
Sulfabenzamide	10	81.6	3.2	94.8	5.6	86.9	4.6
	50	83.3	5.8	85.6	5.0	84.9	6.8
	100	76.9	6.0	94.4	2.1	90.6	6.1
Sulfaphenazole	10	83.8	2.4	90.6	3.1	84.7	3.9
	50	97.0	8.5	81.4	4.6	75.1	5.3
	100	84.1	2.6	86.7	5.7	73.3	2.9

(Continued)

Table 4 (continued)

Antibiotic	Spiked levels (μg/kg)	grass Carp		Penaeus vannamei		*Scylla serrata*	
		Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
Sulfamethazine	10	79.1	3.2	85.6	11.4	87.0	3.9
	50	79.6	5.9	76.8	2.0	74.6	4.8
	100	75.0	3.4	78.9	3.2	72.0	3.6
Sulfadiazine	10	80.3	4.3	92.6	7.1	90.4	4.9
	50	83.2	7.7	89.4	5.6	83.0	3.4
	100	79.1	2.7	86.7	5.2	82.5	3.1
Sulfaquinoxaline	10	80.3	4.3	85.0	3.9	88.9	3.9
	50	83.2	7.6	78.7	2.6	74.9	4.7
	100	79.1	2.7	86.2	1.6	75.7	5.4
Sulfachlorpyridazine	10	78.3	3.3	87.8	6.6	86.1	5.6
	50	72.9	3.3	83.6	2.7	74.8	4.7
	100	69.8	3.7	82.6	4.4	73.3	3.8
Sulfameter	10	82.0	4.4	90.9	3.7	83.1	2.7
	50	80.1	8.3	81.8	2.4	90.4	6.9
	100	75.5	5.3	89.6	2.9	90.3	7.6
Sulfisomidine	10	75.3	2.7	89.2	4.8	86.3	4.0
	50	74.8	4.4	81.9	4.6	86.7	6.5
	100	74.6	3.1	84.4	3.0	90.4	6.9
Sulfamonomethoxine	10	78.1	3.0	90.1	3.9	87.8	7.9
	50	77.2	7.2	94.0	4.9	96.9	4.5
	100	76.6	3.6	96.1	4.2	105.1	2.6
Sulfadimethoxine	10	79.8	6.7	91.7	3.9	91.9	2.3
	50	71.6	8.2	83.4	3.7	83.1	6.9
	100	74.7	5.1	88.7	4.3	92.4	6.5
Sulfaguanidine	10	75.8	6.0	68.2	9.9	91.8	4.3
	50	85.1	7.4	58.8	34.1	77.8	5.5
	100	77.3	4.8	64.8	5.0	67.4	8.9
Sulfapyrazole	10	81.3	2.1	93.3	3.2	82.1	4.1
	50	95.6	5.6	80.1	4.5	77.5	6.2
	100	85.7	2.7	80.7	2.7	84.9	5.8

Application to real samples

In this study, 32 samples of aquatic products (including 12 grass Carp, 11 Penaeus vannamei, and 9 *Scylla serrata*) bought from supermarkets were tested to display the applicability of this method. These samples were dealt with the improved QuEChERS procedure and screened by UPLC-QToFMS. All antibiotic residues were quantified using the matrix-matched calibration method, increasing the data accuracy. Results showed that difluoxacin hydrochloride was detected in the samples of Penaeus vannamei whose amounts ranged from 1.5 to 7.0 μg/kg. MRLs of difluoxacin hydrochloride was 300 μg/kg according to GB 31650-2019 announced by MOA, China. Overall, all the concentrations of

antibiotic residues in real samples were lower than their MRLs, while other target antibiotics were below their LOQs.

CONCLUSIONS

Summing up, in this study, a fast, convenient, effective, economical and eco-friendly strategy based on QuEChERS process was established to extract the antibiotics in aquatic products including grass Carp, Penaeus vannamei and *Scylla serrata*. Using UPLC-QToFMS platform and matrix-matched calibration method to screen and quantity the 49 antibiotic residues, the study achieved satisfactory recoveries, significant linearity and decent stability. Our method also possesses great potential in the analysis of various kinds of antibiotic residues in aquatic products.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Yao Gao conceived and designed the experiments, performed the experiments, analyzed the data, performed the computation work, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Tianwen Zhang conceived and designed the experiments, performed the experiments, analyzed the data, performed the computation work, prepared figures and/or tables, and approved the final draft.
- Shirong Huang performed the experiments, analyzed the data, performed the computation work, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Xinxin Lin analyzed the data, prepared figures and/or tables, and approved the final draft.
- Sisi Gong performed the experiments, performed the computation work, authored or reviewed drafts of the paper, and approved the final draft.
- Qiuhua Chen performed the experiments, analyzed the data, performed the computation work, prepared figures and/or tables, and approved the final draft.

- Dongren Huang conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Min Chen conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available as a Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj-achem.8#supplemental-information.

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