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Gao, Ming
Wang, Jun
Song, Xiukai
et al.

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PAPER IN FOREFRONT

An effervescence-assisted switchable fatty acid-based microextraction with solidification of floating organic droplet for determination of fluoroquinolones and tetracyclines in seawater, sediment, and seafood

Ming Gao[1] · Jun Wang[1] · Xiukai Song[2] · Xin He[2] · Randy A. Dahlgren[3,4] · Zhenzhong Zhang[1] · Shaoguo Ru[1] · Xuedong Wang[4]

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Abstract

This study developed a new effervescence-assisted switchable fatty acid-based microextraction combined with solidification of a floating organic-droplet (EA-SFAM-SFO) for simple and rapid determination of fluoroquinolones and tetracyclines in seawater, sediment, and seafood. Five medium-chain fatty acids (pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, and nonanoic acid) were tested as an extraction solvent, given their ability to change between hydrophobic and hydrophilic forms by pH adjustment. As nonanoic acid had the highest extraction recovery (>92%) for the six antibiotics and the ability to transform from liquid to a solidified floating state at low temperature, it was selected as the optimum extraction solvent. The prominent advantages of the newly developed method are: (1) reaction between the procedures salt and fatty acid changed extraction solvent from the hydrophobic to hydrophilic state; (2) bubbling with CO_2 greatly increased the contact area between fatty acid and analytes resulting in improved extraction recovery; and (3) solidification of the fatty acid at a low temperature provided good separation and avoided the use of specialized equipment. Single-factor screening and optimization of the main factors were conducted using Plackett-Burman design and central composite design, respectively. The main parameters were optimized as follows: 258 μL fatty acid, 406 μL H_2SO_4 (98%), 3.9 min vortex time, and 354 μL Na_2CO_3 (2 mol $L^{-}1$). Under optimized conditions, limits of detection were 0.007–0.113 μg L^{-1} or μg kg^{-1} and extraction recoveries were 82.2%–116.7% for six fluoroquinolone and tetracycline antibiotics in seawater, sediments, and seafood. The newly developed method combines the advantages of effervescence-assisted dispersion, hydrophobic/hydrophilic switchable solvent, and liquid/solid transition induced by low temperature. Overall, the new method is simple, quick, and environment-friendly with low detection limits and high recoveries. Thus, the newly developed method has excellent prospects for sample pretreatment and analysis of antibiotics in marine environmental and food samples.

Keywords Fluoroquinolones · Tetracyclines · Medium-chain saturated fatty acids · Effervescence-assisted switchable fatty acid-based microextraction combined with solidification of floating organic droplet (EA-SFAM-SFO) · Marine environmental samples

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✉ Shaoguo Ru
rusg@ouc.edu.cn

✉ Xuedong Wang
zjuwxd@163.com

[1] College of Marine Life, Ocean University of China, Qingdao, Shandong 266000, China

[2] Shandong Marine Resource and Environment Research Institute, Yantai, Shandong 264006, China

[3] Department of Land, Air, and Water Resources, University of California, Davis, CA 95616, USA

[4] Key Laboratory of Watershed Sciences and Health of Zhejiang Province, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China

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Introduction

Fluoroquinolones (FQs) and tetracyclines (TCs) are widely used antibacterial agents in human and veterinary medicines [1, 2], which impact marine ecosystems through many pathways such as wastewaters from aquaculture, livestock, and urban environments [3–9]. Studies revealed FQ and TC concentrations of 108.8 and 13.02 ng L^{-1} in seawater, 70.2 and 3.28 μg kg^{-1} in sediments, and 64.2 and 9.45 μg kg^{-1} in aquatic organisms, respectively [8, 10, 11]. Antibiotics in the ocean not only affect the growth of microorganisms and contribute to the production of antibiotic resistance genes [12, 13] but also cause gastrointestinal discomfort, liver toxicity, nervous system disorders, and retinopathy through food chain enrichment [14–17]. Under ambient conditions, antibiotic concentrations are in the pg-ng-μg L^{-1} range in seawater and sediment [8, 18]. High concentrations of inorganic salts in seawater, organic matter in sediment, and proteins and fats in seafood products pose potentially strong interferences for the detection of antibiotics [19]. Therefore, an efficient sample pretreatment is required to eliminate the effects of complex impurities in marine matrices and to improve detection sensitivity and recovery [20, 21].

In addition to traditional extraction methods such as liquid–liquid extraction (LLE) [19], solid phase extraction (SPE) [22, 23], and solid phase microextraction (SPME) [11], dispersive liquid–liquid microextraction (DLLME) was proposed for the determination of antibiotics in seawater, sediment, and seafood [8]. DLLME was first reported by Rezaee et al. (2006) [24] and has many advantages such as simple operation and the need for only small amounts of organic solvents. However, it has a series of disadvantages such as extractants with densities greater than water being hazardous halogenated hydrocarbons [25, 26], whereas extractants less dense than water are hard to separate from water, thereby increasing experimental error. In these cases, isolation of the extractant from the top of the aqueous phase required the use of specialized home-designed devices [27, 28]. These noncommercial devices increased the complexity of the operation, the cost of extraction, and were very difficult to clean for reuse. Solidified floating organic droplet microextraction (SFODM) was first reported by Leong and Huang (2008) [29], and uses a low-density solvent with a room temperature melting point as the extraction solvent. After centrifugation and freezing, the low-density solvent can be easily collected without the need for specialized equipment, but is only feasible when the solidification temperature is lower than that encountered during the extraction procedure. However, suitable extraction solvents for SFODM are limited, and only undecanol and dodecanol are commonly used [30, 31]; both of these solvents have strong toxicities.

Medium-chain fatty acids belong to a class of environmentally friendly solvents, which can switch between hydrophilic and hydrophobic forms. They have been widely promoted as a new "green" solvent in pretreatment applications in recent years [32, 33]. Shih et al. (2015) [34] reported a novel fatty acid-based, in-tube dispersive liquid–liquid microextraction (FA-IT-DLLME) technique for the determination of alkylphenols in aqueous samples, which achieved high extraction recovery, short extraction times, and low extraction solvent requirements. To determine ofloxacin in human urine, Vakh et al. (2016) [35] used hexanoic acid as an extraction solvent and developed a fully automated effervescence-assisted switchable solvent-based liquid phase microextraction (EA-SS-LPME) with enhanced extraction recoveries achieved through bubbling of CO_2 to increase interaction with analytes.

In this study, five room temperature medium-chain fatty acids were screened as potential extraction solvents and only nonanoic acid achieved high extraction recoveries for all six target antibiotics. Thus, we used nonionic acid as the extraction solvent and adopted the advantages of EA-SS-LPME and SFODM to develop for the first time an effervescence-assisted switchable fatty acid-based microextraction combined with solidification of floating organic-droplet (EA-SFAM-SFO) method for the determination of four fluoroquinolones and two tetracyclines in seawater, sediments, and seafood. The newly developed method has excellent prospects for sample pretreatment and analysis of trace level fluoroquinolones and tetracyclines in complex environmental and food matrices.

Experimental

Reagents and materials

Analytical standards included six antibiotics, ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (ENR), oxytetracycline (OTC), and tetracycline (TC) with purity >99.0%, and five medium-chain fatty acids, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, and nonanoic acid, all purchased from J&K Chemical, Shanghai, China.

Stock standard solutions (1000 μg mL^{-1}) for the six antibiotics were prepared every month by dissolving each compound in methanol and stored at 4 °C for further use. Working solutions were diluted with Milli-Q ultrapure water (Millipore, Bedford, MA, USA) and methanol (v/v = 50:50) to prepare a secondary mixed stock solution of 10 μg mL^{-1}. All of the working solutions were prepared every week and stored at 4 °C for further use.

Sample collection and processing

According to the state standard of the People's Republic of China (GB 17378.3-2007), seawater and sediment samples were collected in Laizhou Bay (Weifang, China) in the

summer of 2017. The seawater sample was filtered through a 0.45-μm membrane filter and stored at –4 °C until analysis. The sediment sample was air-dried at room temperature before grinding and sieving. Fish, shrimp, shellfish, and squid were purchased from local fishing boats (Weifang, China) and stored at –20 °C after grinding (JX-FSTPRP-24 grinder, Shanghai, China). All samples were analyzed within 1 wk of sample collection.

Samples preparation

As illustrated in Fig. 1, 10 mL of seawater sample was added to a 15 mL high-density polyethylene (HDPE) tube, then 100~300 μL fatty acid and 100~700 μL Na_2CO_3 (2 mol L^{-1}) were added. When the solution became alkaline (~pH = 9.2), the fatty acid was converted to a hydrophilic form.

An aliquot of 2.0 g sediment sample and 10 mL ultrapure water were added to a 15 mL HDPE tube, and after vortexing, 100~300 μL fatty acid and 100~700 μL 2 mol L^{-1} Na_2CO_3 were injected. The sample was vortexed and centrifugation was carried out for 5 min with the supernatant transferred to 10 mL ultrapure water. A 1.0 g homogenized seafood sample was pipetted into a 15 mL HDPE tube, and then 1.0 mL n-hexane was injected to dissolve fat, which was then removed by vortex and centrifugation. Following the addition of 10 mL ultrapure water to the seafood sample and vortexing, 100~300 μL fatty acid and 100~700 μL 2 mol L^{-1} Na_2CO_3 were injected, and then 2.0 g Na_2SO_4 was added to remove protein impurities. After vortex and centrifugation, the supernatant was transferred to 10 mL of ultrapure water.

After the above procedures, 100~500 μL H_2SO_4 (98%) was added to the 10 mL solution, which was then vortexed for 0~5 min at 3200 rpm (50 Hz, 115 w) (SI-0246; Scientific Industries, New York, USA), centrifuged for 5 min at 3200 g (TDL-50C, Anting Low Speed Centrifuge, Shanghai, China), and solidified in an ice bath for 10 min. About 1 mm thickness concave surface solidified organic droplet which formed at the aqueous phase's surface of the polyethylene plastic tube with a diameter of 1.5 cm was collected using a medical spoon, and then melted (~250 μL) at room temperature for antibiotic detection by HPLC-UV (Agilent-1260 HPLC; Agilent, Santa Clara, USA).

Instrumentation

A Zorbax Eclipse-C_{18} column (250 mm × 4.6 mm, 5 μm particle size) was used for separating the test analytes with the following operating conditions: flow rate, 0.5 mL min^{-1}; column temperature, 40 ± 1 °C; mobile phase, methanol-acetonitrile-water (15:10:75, v/v/v; water consisting of 3.4 mL orthophosphoric acid and 6.0 mL triethylamine per liter); detection wavelength, 278 nm; and injection volume, 50.0 μL.

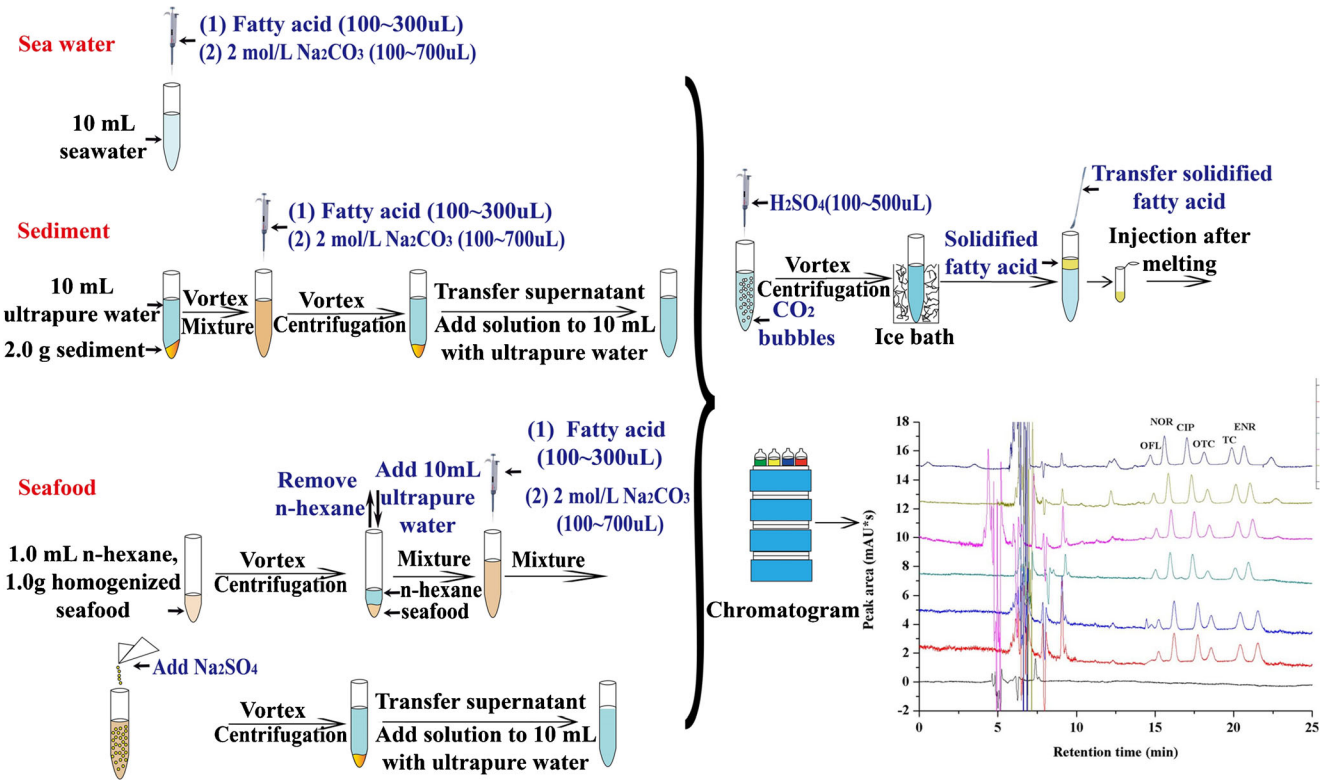

Fig. 1 Schematic representation of the EA-SFAM-SFO method. **Note:** Each step in the EA-SFAM-SFO procedure is described in the text

Plackett-Burman (PB) design

A two-level PB factorial design consisting of 12 runs was established to rapidly screen the significant factors from several operational factors by eliminating the interactions. According to previous studies [36, 37], the investigated variables included fatty acid volume **(A)**, H_2SO_4 volume (98%) **(B)**, Na_2CO_3 volume (2 mol L^{-1}) **(D)**, centrifugation time (3200 g) **(F)**, and vortex time (3200 rpm) **(H)**. In addition, according to previous studies [37, 38], the standard error (SE) of the PBD was estimated by the effects of dummy factors, and Equation 1 is as follow:

$$(SE)e = \sqrt{\frac{\sum E^2_{\text{dummy}}}{n\text{dummy}}} \quad (1)$$

where $\sum E^2_{dummy}$ is the sum of squares of the dummy factors, and n_{dummy} is the number of dummy factors. In this case, at least three dummy factors should be selected [39, 40]. Therefore, three dummy factors C, E, and G were introduced to investigate the effects. Each variable was investigated at two levels, low (−1) and high (+1). Table S1 in the Electronic Supplementary Material (ESM) summarizes the variable levels in the PB design.

Central composite design (CCD)

After single-factor screening using a Plackett-Burman design, four key factors, fatty acid volume, H_2SO_4 volume, Na_2CO_3 volume, and vortex time, were identified for further optimization by CCD. Single factor optimization determined the best extraction conditions

To be 200 μL fatty acid, 300 μL H_2SO_4, 2.5 min of vortex time, and 400 μL Na_2CO_3 (ESM Fig. S1). Using Design-Expert 8.0.5 software (Minneapolis, MN, USA), CCD was performed to investigate multi-factor interactions, which included 22 treatments at five levels ($-\alpha, -1, 0, +1, +\alpha$) for four factors using two blocks to optimize values for each factor based on extraction recovery (ER) (ESM Table S2 and Table 1). The 22 treatments included eight half-fraction factorial design points ($N_f = 2^{f-1}$), eight "star points" ($N_\alpha = 2f$), and six center points. A quadratic polynomial model Equation 2 was used to predict the response of dependent variables for the ERs of FQs and TCs:

$$Y = b0 + \sum_{i=1}^{4} bixi + \sum_{ij=1(i\neq j)}^{6} bijxixj + \sum_{i=1}^{4} biix_i^2 \quad (2)$$

where Y is the dependent variable; x_i is the independent variable; b_0 is the intercept; b_i is the coefficient for linear effects; b_{ij} is the coefficient for interaction effects; and b_{ii} is the coefficient for

Table 1 Design matrix and responses for the CCD

Run	Block	A: Volume of fatty acid (μL)	B: Volume of H_2SO_4 (μL)	C: Vortex time (min)	D: Volume of Na_2CO_3 (μL)	Recovery (%)
1	1	200	300	2.5	400	99.15
2	1	260	180	1	580	63.12
3	1	200	300	2.5	400	94.23
4	1	260	180	4	580	79.05
5	1	140	420	1	580	56.57
6	1	260	420	1	220	55.28
7	1	140	420	4	580	70.79
8	1	140	180	1	220	53.53
9	1	140	180	4	220	66.99
10	1	200	300	2.5	400	100.79
11	1	260	420	4	220	69.18
12	1	200	300	2.5	400	105.15
13	2	200	300	2.5	700	91.74
14	2	300	300	2.5	400	79.75
15	2	200	500	2.5	400	86.74
16	2	200	300	0	400	49.63
17	2	200	300	2.5	400	95.37
18	2	200	300	2.5	100	54.92
19	2	200	300	5	400	93.27
20	2	200	100	2.5	400	57.65
21	2	200	300	2.5	400	104.18
22	2	100	300	2.5	400	41.38

squared effects. The model determined by Design-Expert 8.0.5 (Minneapolis, MN, USA) was evaluated by analysis of variance (ANOVA) to obtain response surfaces for factor optimization. The optimal extraction data is calculated by the software Design-Expert 8.0.5 with setting the average target ER of six target compounds at 100% and the acceptable SD deviation at ±20%.

Experimental evaluation

Central composite design evaluation: ANOVA was used to evaluate the level of the model impact factor and the corresponding results.

Methods evaluation: The method was evaluated based on coefficients of determination (R^2), linear range (LR), limits of detection (LOD), limits of quantification (LOQ), intra- and inter-day precision, and extraction recovery (ER) based on Equation 3):

$$ER = \frac{C_{found} - C_{real}}{C_{added}} \times 100\% \quad (3)$$

where C_{found} was the concentrations of analyte in the final solution, C_{real} was the concentration of the analyte in the real sample (blank sample) without adding any standards, and C_{added} was the concentration of a known amount of standard spiked into the sample. C_{found} and C_{real} were calculated using calibration line.

Method stability evaluation: The precision study was carried out in six parallel experiments by determining the intra- and inter-day relative standard deviations (RSDs) at four fortification levels (10, 50, 100, and 500 μg L^{-1} or μg kg^{-1}) of FQs and TCs. In intra-day stability study, each sample of four fortification levels was detected once per hour, and in inter-day stability study, each sample of four fortification levels was detected at 10 o'clock every day, the RSDs of intra- and inter-day were calculated after six parallel experiments.

Analysis of samples: the EA-SFAM-SFO method was applied for determining four FQs and two TCs in real-world seawater, sediment, and seafood samples in six parallel experiments. The ER was used to assess the analytical performance of the optimized method.

Results and discussion

Selection of fatty acids

Five medium-chain fatty acids (pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, and nonanoic acid) were evaluated as extraction solvents with the resulting ERs for four FQs and two TCs (spiked level of 10 μg L^{-1} /μg kg^{-1}) shown in Fig. 2a. The ERs for the six antibiotics extracted by pentanoic acid and octanoic acid were too low (mean ER <20%) for further consideration. Although the ER for hexanoic acid reached 98.5% for OFL, it was unacceptably low for the other five antibiotics, especially for OTC and TC. In contrast, the ERs for heptanoic acid and nonanoic acid were relatively high for five antibiotics, but this was not the case for heptanoic acid extraction of CIP (~70%). The best ERs were obtained for nonanoic acid with ERs >90% for all analytes, which was comparable or superior to traditional extraction solvents [41]. Additionally, it has a lower density than water and coagulates upon chilling in an ice bath. As a consequence, nonanoic acid is a suitable extraction solvent in SFOM procedures, which decreases matrix effects (i.e., impurity peak influence) and increases ERs compared with common DLLME methods. Notably, the ability of nonanoic acid to change between hydrophilic and hydrophobic properties with pH adjustment makes it an ideal candidate as a switchable solvent microextraction. As a result, nonanoic acid was selected as extraction solvent for subsequent experiments.

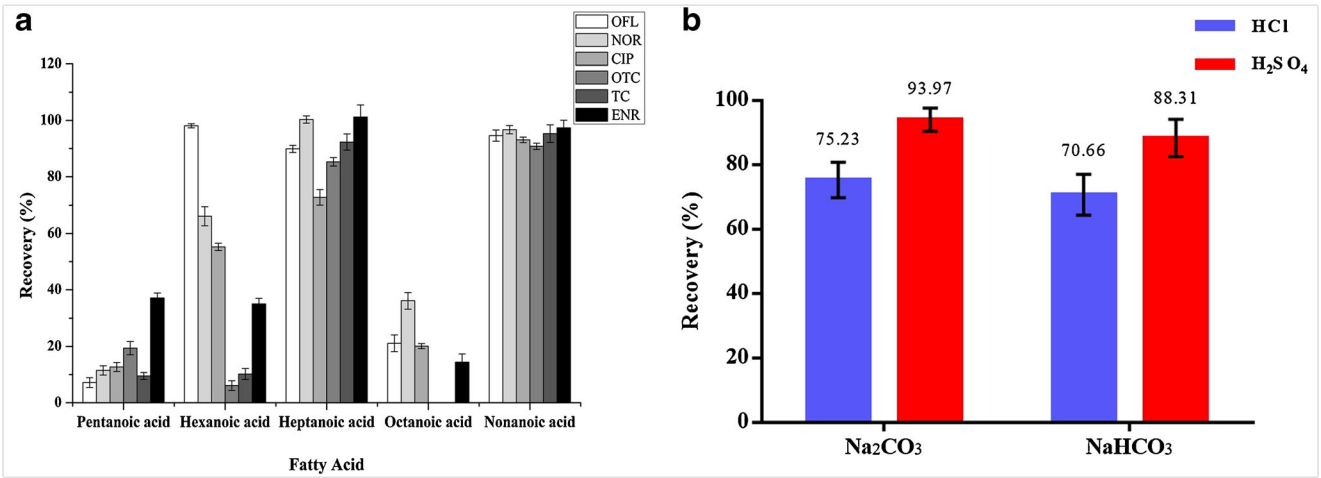

Fig. 2 (1) **a**, extraction recovery of five medium-chain fatty acids for four FQs and two TCs; **b**, selection of pH regulator. (2) Samples were fortified with FQs and TCs at 10 μg L^{-1} or μg kg^{-1}; (3) 200 μL fatty acid, 300 μL acidic regulator, 2.5 min of vortex time, and 400 μL alkaline regulator

Selection of pH regulator

Shih et al. (2015) [34] reported that approximately 99.9% of the fatty acid was dissociated into its anion form and acted as an anionic surfactant when the pH was higher than its pK_a by at least three pH units. In contrast, when the pH was at least three pH units below its pK_a, more than 99.9% of the fatty acid was in its neutral hydrophobic form. The pK_a of nonanoic acid was 4.96, so strong alkali and strong acid were required for changing the hydrophobic/hydrophilic properties of nonanoic acid. Additionally, the bubbling of CO_2 facilitated mixing to increase the contact area for increasing ERs. Therefore, Na_2CO_3 and $NaHCO_3$ were chosen as a potential alkaline regulator; HCl and H_2SO_4 were chosen as candidates for acidic regulator. The ER of FQs and TCs in 10 μg L^{-1} /μg kg^{-1} spiked level was used to evaluate the ability of pH regulator. As illustrated in Fig. 2b, the ERs for the combinations of Na_2CO_3-H_2SO_4 and Na_2CO_3-HCl were 93.97% and 88.31%, respectively, which were higher than those of $NaHCO_3$-H_2SO_4 or $NaHCO_3$-HCl (75.23% and 70.66%, respectively). These results

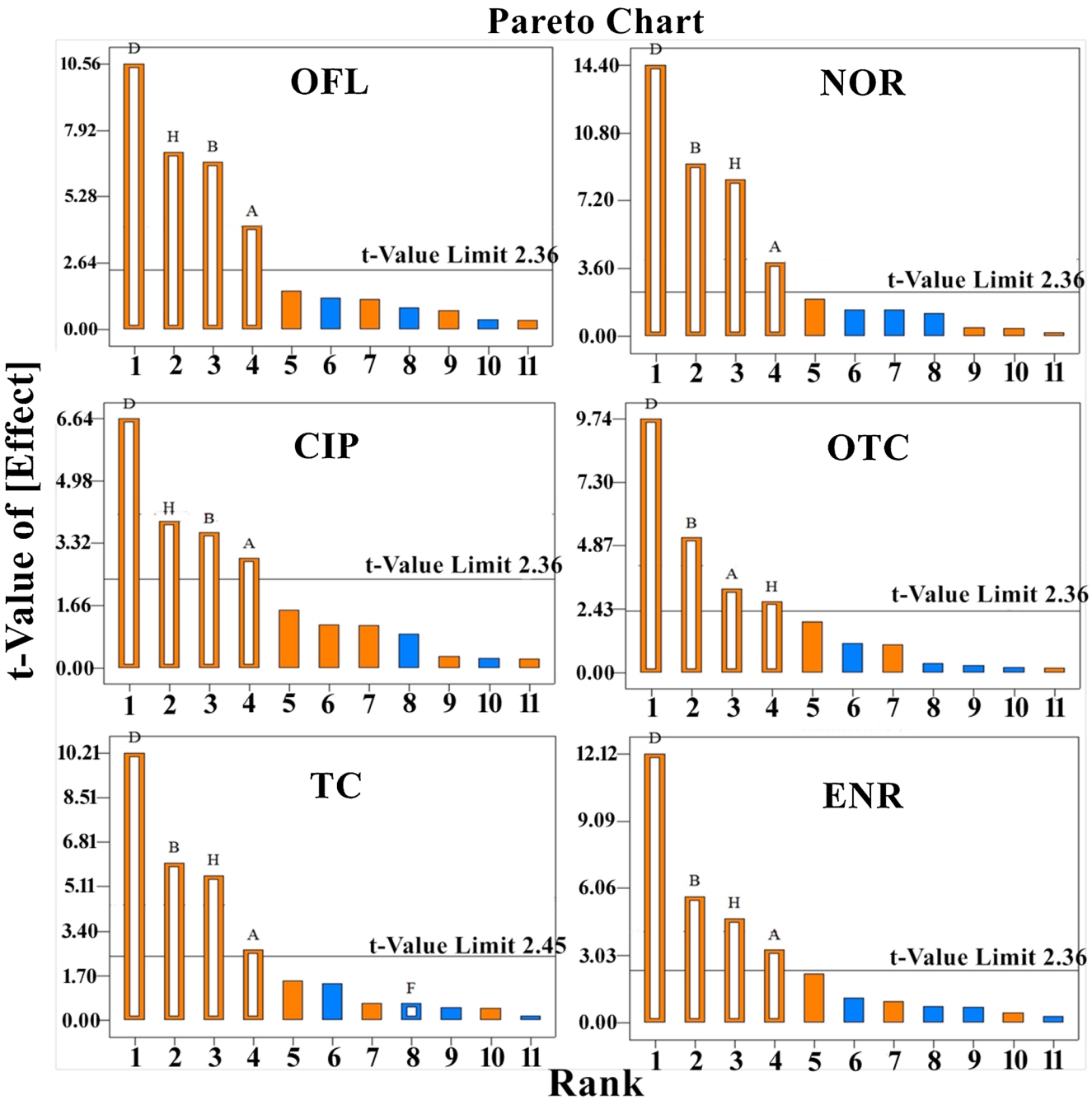

Fig. 3 Standardized Pareto charts ($p < 0.05$) of main factor effects. **Note:** (1) **A**, volume of fatty acid; **B**, volume of H_2SO_4; **D**, volume of Na_2CO_3; **H**, vortex time. (2) Samples were fortified with FQs and TCs at 10 μg L^{-1} or μg kg^{-1}

demonstrated that the ERs for antibiotics were mainly dependent on the alkaline regulator, presumably because the solubility of Na_2CO_3 in water at room temperature is about three times that of $NaHCO_3$ [42, 43], which leads to a higher extraction efficiency. Therefore, Na_2CO_3 and H_2SO_4 were selected for the EA-SFAM-SFO method.

Screening of variables by PB design

Standardized Pareto charts ($p < 0.05$) of main factor effects are illustrated in Fig. 3. Critical values representing statistically significant effects for factors at the 95% confidence level were 2.36, 2.36, 2.36, 2.36, 2.45, and 2.36 for the response of OFL, NOR, CIP, OTC, TC and ENR, respectively. Fatty acid volume **(A)**, H_2SO_4 volume **(B)**, Na_2CO_3 volume **(D)**, and vortex time **(H)** were significant factors with positive effects (white hollow bar), whereas the other variables displayed either negative effects (solid bar) or no effects at the 95% confidence level. In addition to acidity/alkalinity considerations of the system, solvent volume and extraction time can affect the final extraction recovery. As the extraction solvent, the volume of fatty acid strongly affected ERs. Similarly, the volumes of H_2SO_4 and Na_2CO_3 affected the pH of the extraction solution and CO_2 creation, which altered ERs. Vortex time also influenced ERs through its effects on mixing of the fatty acid extraction solution with the analytes. Therefore, PB design identified the fatty acid volume, H_2SO_4 volume, Na_2CO_3 volume, and vortex time as significant operational variables for extraction of FQs and TCs from seawater, sediment, and seafood.

Optimization result for significant operational variables by CCD

Some interactions might occur between the four major factors, which can also affect the final ER. Therefore, the CCD that optimized all of the major factors at the same time was more suitable than signal factor optimization. The significant results for the CCD model equation and related terms were evaluated by ANOVA (ESM Table S3). The model was highly significant (p = 0.002) and the lack of fit was not significant (p = 0.1502), indicating that other factors in this experiment had little effect on the overall model [44]. The p-values for A, B, C, D, AD, BD, A^2, B^2, C^2, and D^2 were all lower than 0.05, indicating that fatty acid volume **(A)**, H_2SO_4 volume **(B)**, vortex time **(H)**, and Na_2CO_3 volume **(D)** all affected ERs, as was also indicated by the results of the PB design. Equation 4 illustrates the effects of all factors on ER. Herein, Y is ER, b_0 is the intercept, and b_1 to b_{14} are parameter coefficients.

$$Y = bo + b_1A + b_2B + b_3C + b_4D + b_5AB + b_6AC + b_7AD + b_8BC + b_9BD + b_{10}CD + b_{11}A^2 + b_{12}B^2 + b_{13}C^2 + b_{14}D^2 \quad (4)$$

with b_0 = 98.64, b_1 = 11.51, b_2 = 8.73, b_3 = 9.65, b_4 = 11.05, b_5 = 7.98, b_6 = 0.27, b_7 = 10.09, b_8 = −0.16, b_9 = 9.17, b_{10} = 0.35, b_{11} = −12.53, b_{12} = −8.34, b_{13} = −8.54 and b_{14} = −7.93.

The relationship between the related effect and the response is indicated by "+" or "−" for each coefficient. A "+" indicates the coefficient and extraction recovery has a positive relationship, whereas a "−" means a negative relationship. The strength of the relationship between the coefficient and ER **(Y)** was indicated by the absolute value of the coefficient. As shown in Fig. S2a (see ESM), most data points were located near the regression line, suggesting a good correlation between predicted and actual responses, and that the model well simulates the experimental factor effects and their interactions on ER. Moreover, the residual plots were randomly

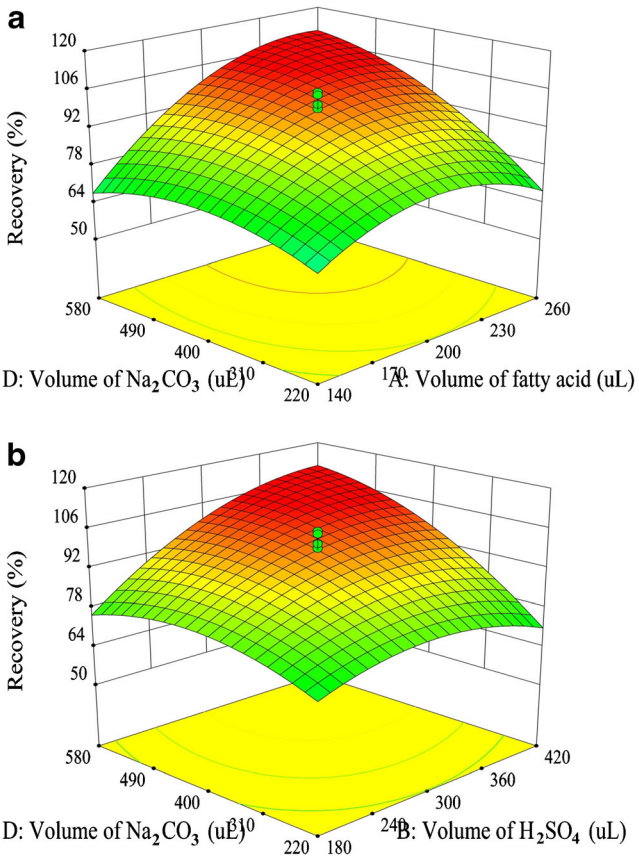

Fig. 4 (1) **a**, 3D response surface for fatty acid volume and Na_2CO_3 volume at a constant concentration of 300 μL H_2SO_4 and 2.5 min vortex time on the average extraction recovery; **b**, 3D response surface for H_2SO_4 volume and Na_2CO_3 volume at a constant concentration of 200 μL fatty acid and 2.5 min vortex time on the average extraction recovery. (2) Samples were fortified with FQs and TCs at 10 μg L^{-1} or μg kg^{-1}

scattered (ESM Fig. S2b), indicating that the variance of the experimental measurements was constant for all values of Y.

The optimal extraction data is calculated by the software Design-Expert 8.0.5 with setting the average target ER of six target compounds at 100% and the acceptable SD deviation at ±20%. In order to extract more details concerning experimental factors on ER, 3D response surfaces were plotted to represent the relationship between ER and the four experimental factors (Fig. 4). For example, Fig. 4a describes the 3D response surface for the effect of fatty acid volume and Na_2CO_3 volume on ER under fixed conditions of 300 μL H_2SO_4 and 2.5 min vortex time. The ERs for FQs and TCs increased with increasing fatty acid volume from 100 to 258 μL and Na_2CO_3 volume from 100 to 354 μL. However, with a further increase in fatty acid volume from 258 to 300 μL and Na_2CO_3 volume from 354 to 700 μL, the ERs for FQs and TCs declined. The 3D response surface for the effects of H_2SO_4 volume and Na_2CO_3 volume on ER when fatty acid volume and vortex time were set at 200 μL and 2.5 min, respectively, are shown in Fig. 4b. The maximum ER was

Table 2 The analytical performance of the EA-SFAM-SFO method

Sample	Analytes	Regression equations	Correlation coefficients (R^2)	Linear range (μg L^{-1}/μg kg^{-1})	LOD (μg L^{-1}/ μg kg^{-1})	LOQ (μg L^{-1}/ μg kg^{-1})
Sea water	OFL	y=0.2002x-0.8019	0.9998	0.072–500	0.022	0.072
	NOR	y=0.3151x-0.7647	0.9999	0.024–500	0.007	0.024
	CIP	y=0.6237x-1.1124	0.9999	0.026–500	0.008	0.026
	OTC	y=0.3271x+4.4884	0.9989	0.059–500	0.018	0.059
	TC	y=0.5020x+2.4902	0.9992	0.042–500	0.013	0.042
	ENR	y=0.5213x+2.5254	0.9995	0.038–500	0.011	0.038
Sediment	OFL	y=0.2036x-1.8278	0.9995	0.350–500	0.105	0.350
	NOR	y=0.3395x+2.2502	0.9972	0.119–500	0.036	0.119
	CIP	y=0.6210x–0.6805	0.9995	0.127–500	0.038	0.127
	OTC	y=0.3271x+4.4884	0.9989	0.297–500	0.089	0.297
	TC	y=0.5020x+2.4902	0.9992	0.199–500	0.060	0.199
	ENR	y=0.5213x+2.5254	0.9995	0.181-500	0.054	0.181
Fish	OFL	y=0.1803x+0.1176	0.9964	0.377-500	0.113	0.377
	NOR	y=0.2935x+0.0231	0.9986	0.146–500	0.044	0.146
	CIP	y= 0.5992x-0.1063	0.9990	0.158–500	0.047	0.158
	OTC	y=0.3326x+0.9310	0.9994	0.326–500	0.098	0.326
	TC	y=0.4832x+0.4775	0.9973	0.167–500	0.050	0.167
	ENR	y=0.5176x+0.4646	0.9985	0.137–500	0.041	0.137
Shellfish	OFL	y= 0.2029x-1.3583	0.9995	0.291–500	0.087	0.291
	NOR	y=0.3178x-1.2988	0.9996	0.097–500	0.029	0.097
	CIP	y=0.6208x-0.5072	0.9995	0.105–500	0.031	0.105
	OTC	y=0.3400x+1.8775	0.9974	0.237–500	0.071	0.237
	TC	y=0.5087x+1.1281	0.9967	0.169–500	0.051	0.169
	ENR	y=0.5235x+2.0801	0.9979	0.151–500	0.045	0.151
Shrimp	OFL	y= 0.2027x-1.3289	0.9976	0.329–500	0.099	0.329
	NOR	y=0.3228x-1.8719	0.9985	0.111–500	0.033	0.111
	CIP	y=0.6414x-2.8311	0.9992	0.119–500	0.036	0.119
	OTC	y=0.3697x-1.4665	0.9983	0.278–500	0.083	0.278
	TC	y=0.5565x-4.2588	0.9969	0.186–500	0.056	0.186
	ENR	y=0.5651x-2.6003	0.9987	0.170–500	0.051	0.170
Squid	OFL	y=0.2456x-18.5320	0.9936	0.294–500	0.088	0.294
	NOR	y=0.3239x-4.1763	0.9998	0.099–500	0.030	0.099
	CIP	y=0.6456x-9.6500	0.9998	0.107–500	0.032	0.107
	OTC	y=0.2818x+22.183	0.9949	0.247–500	0.074	0.247
	TC	y=0.5399x-12.294	0.9990	0.167–500	0.050	0.167
	ENR	y=0.5777x-19.46	0.9981	0.152–500	0.046	0.152

observed at 406 μL H_2SO_4 and 354 μL Na_2CO_3. With further increases in H_2SO_4 (406 to 500 μL) and Na_2CO_3 (354 to 700 μL) volumes, ERs decreased sharply. After rigorous analyses of the four interacting factors (Fig. 4), the optimum operational conditions were determined to be 258 μL fatty acid, 406 μL H_2SO_4, 3.9 min vortex time, and 354 μL Na_2CO_3, which were calculated by Equation 4 and the software Design-Expert 8.0.5, setting the target ER = 100% and the acceptable SD deviation is±20%. The 3D response surface of AB, AC, BC, and CD, the *p* values of which were all higher than 0.05, are illustrated in Fig. S3 (see ESM).

Method evaluation

Under optimized conditions, the performance of the EA-SFAM-SFO method was evaluated for regression equations, determination coefficient (R^2), linear range (LR), limits of detection (LOD at S/N = 3), and limit of quantification (LOQ at S/N = 10) (Table 2). Coefficients of determination (R^2) for linearity of standard curves for the six antibiotics were in the range 0.9936–0.9999. The LRs were (μg L^{-1} or μg kg^{-1}): 0.072–500 for OFL, 0.024–500 for NOR, 0.026–500 for CIP, 0.059–500 for OTC, 0.042–500 for TC, and 0.038–500 for ENR. The LODs for seawater, sediment, and seafood were in the range (μg L^{-1} or μg kg^{-1}) 0.022–0.113 for OFL, 0.007–0.044 for NOR, 0.008–0.047 for CIP, 0.018–0.098 for OTC, 0.013–0.060 for TC, and 0.011–0.054 for ENR. The precision study was carried out in six parallel experiments by determining the intra- and inter-day RSDs (relative standard deviations) at four fortification levels (10, 50, 100, and 500 μg•L^{-1} / μg•kg^{-1}) of FQs and TCs. The RSDs ranged from 1.06% to 5.96% for intra-day analysis and from 1.57% to 6.88% for inter-day analysis (Table 3).

Table 3 Precision and accuracy data for the determination of FQs and TCs in sea water, sediment and seafoods (n=6)

Compound	Concentration(μg•L^{-1} / μg•kg^{-1})		RSD(%)	
	Spiked	Detected	Intra-day	Inter-day
OFL	10	9.85	4.91	6.57
	50	51.22	4.03	5.88
	100	97.91	3.98	5.24
	500	483.62	3.05	4.19
NOR	10	9.97	2.66	2.87
	50	49.54	2.97	2.93
	100	96.38	2.33	4.05
	500	493.57	2.08	3.17
CIP	10	10.12	2.14	4.17
	50	19.56	2.45	5.14
	100	97.33	1.62	3.69
	500	498.50	2.57	2.86
OTC	10	9.28	5.96	6.88
	50	19.02	4.93	5.52
	100	38.07	5.34	6.86
	500	475.63	3.60	3.87
TC	10	9.91	4.01	6.13
	50	19.83	2.57	3.68
	100	49.66	2.38	4.35
	500	507.35	1.06	1.57
ENR	10	9.85	2.46	4.52
	50	51.29	2.91	4.37
	100	105.15	1.69	1.63
	500	500.67	2.06	2.99

Analysis of real-world seawater, sediment, and seafood samples

Figure 5 illustrates typical chromatograms of four FQs and two TCs in real-world marine environmental samples by the newly developed EA-SFAM-SFO method. Figure 5a shows the typical chromatogram of the fish sample at a LOQ spiked level of OFL, and Fig. 5b illustrates chromatograms of seawater, sediment, and seafood samples at fortification levels of 10 μg L^{-1}/μg kg^{-1}. At the three fortification levels, ERs for the six antibiotics were in the range 90.6%–109.8% for OFL, 85.8%–111.5% for NOR, 90.6%–107.9% for CIP, 82.2%–114.6% for OTC, 90.7%–116.7% for TC, and 90.1%–109.7% for ENR (Table 4). Concentrations of NOR, CIP, and TC in nonfortified samples were all below their respective LODs in seawater, sediment, and seafood (fish, shellfish, shrimp, and squid). However, OFL and ENR were detected at 0.81 and 1.22 μg L^{-1}, respectively, in seawater, and OTC was detected as high as 3.15 μg kg^{-1} in fish. These results demonstrate that the newly developed EA-SFAM-SFO method has excellent prospects for analyzing trace levels of FQs and TCs in marine environmental and seafood samples with high precision and accuracy.

Comparison of the EA-SFAM-SFO method with others

To verify the efficacy of the newly developed EA-SFAM-SFO method, the optimized method was applied to the determination of four FQs and two TCs in seawater, sediment, and four kinds of seafood. Typical chromatograms for marine environmental samples at a LOQ fortification level of OFL and 10 μg L^{-1}/μg kg^{-1} displayed good peak symmetry and separation, indicating that the method was effective for accurate quantification of six common antibiotics (Fig. 5). The newly developed EA-SFAM-SFO method was also compared with other methods from the literature to compare its relative efficacy with respect to SPE [22, 23, 45], SPME [11, 46], PLE [47], DMI-MSPD [48], enzyme linked immunosorbent assay (ELISA) [49], etc. (Table 5). The LODs for EA-SFAM-SFO were in the range 0.007–0.113 μg L^{-1}/μg kg^{-1}, which was

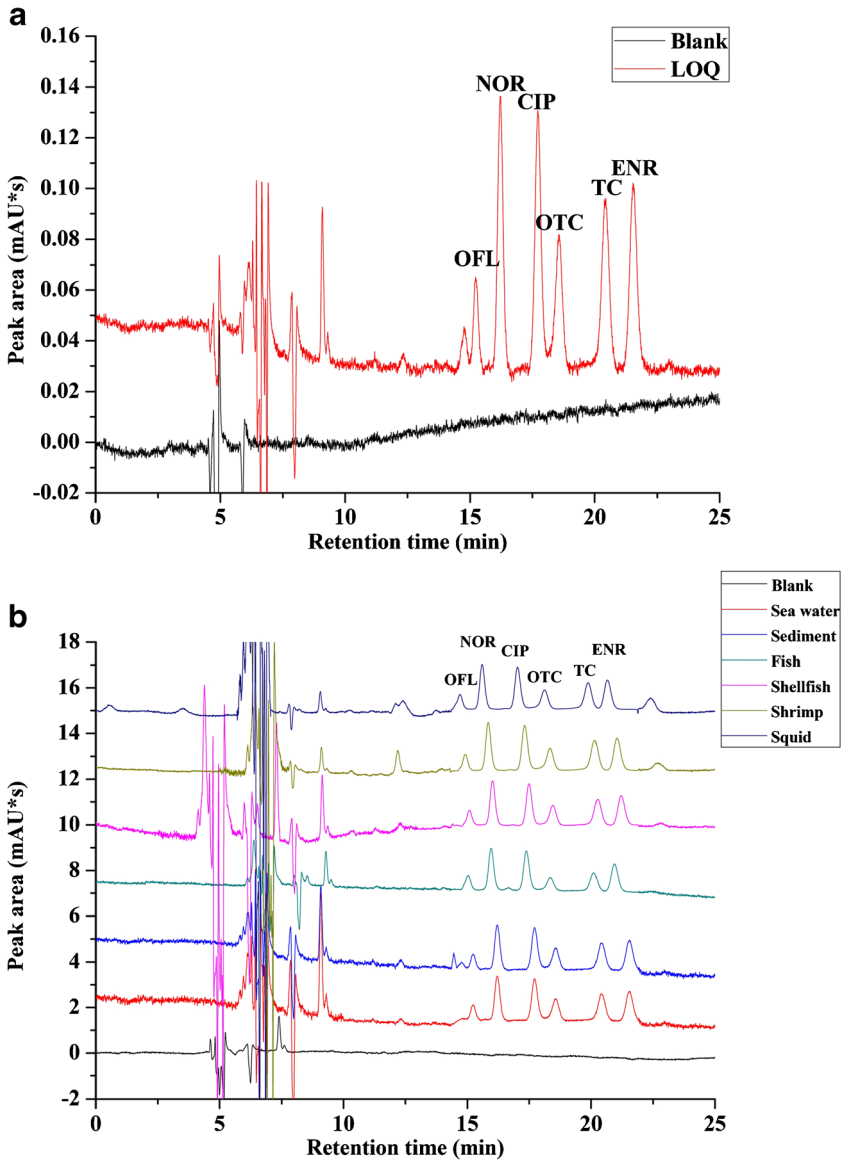

Fig. 5 Chromatogram of analytes obtained from the newly developed EA-SFAM-SFO method under optimized conditions. **Note:** Experimental conditions: (1) **a**, fish sample was fortified with FQs and TCs at a LOQ level of OFL; **b**, marine samples were fortified with FQs and TCs at 10 μg L^{-1} or μg kg^{-1}. (2) 258 μL fatty acid, 406 μL H_2SO_4, 3.9 min vortex time, and 354 μL Na_2CO_3

comparable with those of SPME (0.1–0.2 μg kg^{-1}) [46] and DMI-MSPD (0.06–0.22 μg kg^{-1}) [48], but substantially lower than most other pretreatment methods. The ERs were in the range 82.2%–116.7%, which was comparable with methods like SPE (90%–132%) [50], but better than many traditional pretreatments such as SPE (77%–88%) [22], DMI-MSPD (66.4%–102.7%) [48], ELISA (61.7%) [49], SPME (63.8%–87.4%) [11], and PLE (47%–89%) [47]. Large amounts of CO_2 were produced through the reaction of H_2SO_4 with Na_2CO_3, which increased the contact area between the nonanoic acid and extraction solution, resulting in improved extraction recovery. In addition, this method reduces errors associated with the collection of extraction media, with solidified fatty acids providing a highly efficient extraction agent to further improve extraction recovery [35]. Sample preparation time (23.9 min) was much shorter than SPE (150 h, 22 h, and 260 min) [11, 22, 23], SPME (55 min) [46], and TPATPS (14 h) [51]. SPE and SPME require intensive washing of the extraction column with organic solvent [11, 22] or a long vortex time (~22 h) [23] to improve extraction recovery, whereas ATPS requires 2 h to form the aqueous two-phase system and 12 h to separate the extraction solution and extraction solvent. These time-consuming steps greatly increase processing time, limiting rapid sample throughput. Although DMI-MSPD (5 min), ELISA (7 min), and PLE (3 min) have much shorter pretreatment time requirements, their LODs are much higher and their ERs very low [47–49].

The enrichment factor (EF) was calculated by Equation 5 [52]:

$$\mathrm{EF} = \frac{ER\%}{100} \times \frac{V_0}{V_{inj}} \tag{5}$$

Table 4 The fortified recoveries of FQs and TCs by the EA-SFAM-SFO method in sea water, sediments and seafoods

Analytes	Added ($\mu g \cdot L^{-1}$ /$\mu g \cdot kg^{-1}$)	Sea water (mean±SD, n=3)			sediment (mean±SD, n=3)			Fish (mean±SD, n=3)			Shellfish (mean±SD, n=3)			Shrimp (mean±SD, n=3)			Squid (mean±SD, n=3)		
		Blank	Detected ($\mu g \cdot L^{-1}$)	ER (%)	Blank	Detected ($\mu g \cdot kg^{-1}$)	ER (%)	Blank	Detected ($\mu g \cdot kg^{-1}$)	ER (%)	Blank	Detected ($\mu g \cdot kg^{-1}$)	ER (%)	Blank	Detected ($\mu g \cdot kg^{-1}$)	ER (%)	Blank	Detected ($\mu g \cdot kg^{-1}$)	ER (%)
OFL	10	0.81±0.37	10.47±0.32	96.6	ND	10.09±1.33	100.9	ND	9.06±0.68	90.6	ND	10.36±1.28	103.6	ND	9.85±1.42	98.5	ND	10.83±0.56	108.3
	50		51.05±0.66	100.5		49.66±0.96	99.3		48.29±1.04	96.6		54.91±1.49	109.8		53.44±1.54	106.9		54.62±1.44	109.2
	100		105.13±1.43	104.3		93.68±1.29	93.7		98.55±1.13	98.6		105.35±1.46	105.4		92.58±2.01	92.6		102.56±1.63	102.6
NOR	10	ND	9.25±0.61	92.5	ND	8.58±0.49	85.8	ND	10.83±1.96	108.3	ND	9.18±1.35	91.8	ND	10.29±0.38	102.9	ND	11.15±1.71	111.5
	50		49.38±0.99	98.8		48.66±0.92	97.3		50.04±1.66	100.1		51.21±1.33	102.4		48.09±0.96	96.2		50.15±1.34	100.3
	100		106.25±1.12	106.3		91.74±1.46	91.7		104.97±2.36	105.0		100.52±2.07	100.5		90.28±1.61	90.3		97.66±2.08	97.7
CIP	10	ND	10.79±0. 85	107.9	ND	10.16±0.93	101.6	ND	9.65±0.88	96.5	ND	9.06±1.21	90.6	ND	9.87±0.83	98.7	ND	9.32±1.63	93.2
	50		48.87±0.84	97.8		49.28±0.77	98.6		49.07±0.63	98.1		48.87±1.34	97.7		49.37±1.06	98.7		49.25±0.98	98.5
	100		103.47±1.26	103.5		107.84±2.44	107.8		98.02±1.22	98.0		98.26±1.65	98.3		105.22±1.85	105.2		104.26±1.33	104.3
OTC	10	ND	9.39±0.37	93.9	ND	9.51±0.89	95.1	3.15±1.04	11.37±0.93	82.2	ND	10.52±1.29	105.2	ND	11.35±1.33	113.5	ND	9.19±1.58	91.9
	50		49.26±1.09	98.5		49.48±1.26	99.0		49.05±1.33	91.8		51.62±1.66	103.2		48.27±0.96	96.5		57.29±3.14	114.6
	100		97. 63±1.06	97.6		99.37±1.43	99.4		107.72±2.38	104.6		108.27±2.31	108.3		107.29±2.05	107.3		100.27±0.49	100.3
TC	10	ND	9.08±0.87	90.8	ND	9.14±1.25	91.4	ND	9.35±0.88	93.5	ND	9.08±0.96	90.8	ND	9.07±0.92	90.7	ND	10.85±0.46	108.5
	50		48.12±0.96	96.3		58.34±0.77	116.7		49.10±1.26	98.2		49.22±1.18	98.4		49.83±1.08	99.7		48.17±1.65	96.3
	100		108.01±0.84	108.0		107.32±1.72	107.3		103.71±0.98	103.7		100.67±1.67	100.7		93.65±1.79	93.7		101.51±0.96	101.5
ENR	10	1.22±0.77	10.26±0.63	102.6	ND	10.97±1.08	109.7	ND	9.29±0.92	92.9	ND	10.21±0.92	102.1	ND	9.29±0.92	92.9	ND	9.01±0.67	90.1
	50		50.36±0.78	100.7		48.96±1.26	97.9		48.12±1.49	96.2		50.28±1.35	100.6		48.12±1.49	96.2		48.16±1.33	96.3
	100		94.81±0.82	94.8		91.63±2.35	91.6		98.70±0.94	98.7		104.66±1.69	104.7		93.66±0.96	93.7		93.74±2.37	93.7

Note: (1) ER indicates extraction recovery; (2) Each treatment includes three replicates; (3) Each detected value is mean ± SD (standard deviation)

Table 5 Comparison of the EA-SFAM-SFO method with others for determination of FQs and TCs in sea water, sediment and seafood

Methods	Matrices	Antibiotics	LOD (μg L^{-1} /μg kg^{-1})	RR (%)	R^2	Linear range (μg L^{-1}/μg kg^{-1})	Enrichment factor	Extraction time (min)	Organic solvents	References
SPE-HPLC-MS	Seawater	OFL NOR	0.45-3	77-88			770-880	>2.5 h, SPE (102.8 min)-N_2 drying (1 h)	12 mL methanol	[22]
SPME-HPLC-MS/MS	Seawater	OTC TC	0.63-1.2	95.03-99.91			19.0-20.0	>30 min, Oscillate (2 min)-SPE (8.3 min)-vacuum drying (20 min)	10 mL isopropanol	[8]
	Sediment		1.09-2.31	60.79-80.78			12.2-16.2		6 mL methanol,	
	Shellfish		0.94-1.23	60.79-80.78			12.2-16.2		0.5 mL *n*-hexane	
SPME-LC-MS/MS	Shellfish	OFL NOR CIP ENR	0.1-0.2		0.9974-0.9990	0.5-500		>55 min, static extraction (10 min)-SPE (10min)-Vacuum drying (30 min)-N_2 drying-centrifuge (5 min)	11 mL methanol	[46]
SPE-LC-MS/MS	Fish	CIP ENR	2	90-132	0.9986			~1 h, incubate (10 min)-vortex (1 min)-ultrasonic (35 min)-centrifuge (10 min)-N_2 drying	2 mL methanol	[50]
DMI-MSPD-HPLC-FLD	Fish	OFL NOR CIP ENR	0.06-0.22	64.4-102.7				mixture time 5 min	6 mL acetonitrile	[48]
ELISA	Fish	NOR	4-1800	61.7				7 min, shake (2 min)-centrifuge (5 min)	10 mL ethyl acetate	[49]
SPE-LC-MS/MS	Sediment	ENR	1.22-1.38	97.7-101.8				~22 h, sonificate (80 min)-vortex (20 h)-centrifuge (40 min)-vacuum drying (20 min)	16 mL methanol	[23]
SPME-LC-UV	Sediment	NOR CIP ENR	3.4-4.6	63.8-87.4			6.4-8.7	260 min, ultrasonic (15 min)-centrifuge (10 min)-SPE (210 min)-dry (15 min)	5 mL acetonitrile, 10 mL methanol	[11]
SPE-LC-MS/MS	Shrimp	OTC TC		75.4-97.1		25-400	6.0-7.8	>45 min, centrifuge (15 min)-shake (10 s)-column drying (5 min)-N_2 drying (15 min)-evaporate (10 min)	6 mL methanol, 1 mL acetonitrile	[45]
PLE-HPLC-UV	Fish	OTC TC	5.0-10.0	47-82				3 min, heat time (3 min)	Special instrument, (80 °C), 85 ba	[47]
	Shrimp		5.0-10.0	53-89		15-300				
TPATPS-HPLC-UV	Shrimp	CIP	6.8	94.6-95.4	0.9995	100-1000		>14 h, stir (5 min)-centrifuge (10 min)-ATPS forming (2 h)-water bath (12 h)	3 mL ethylene glycol/ propylene glycol(V/V=50/50)	[51]
EA-SFAM-SFO-HPLC-UV	Seawater	OFL NOR CIP OTC TC ENR	0.007-0.022	90.8-108.0	0.9989-0.9999	0.024-500	35.2-41.9	23.9 min, centrifuge (10 min)-vortex (3.9 min)-ice bath (10 min)	1.0 mL *n*-hexane, fatty acid 258 μL	this work
	Sediment		0.036-0.105	85.8-116.7	0.9972-0.9995	0.119-500	33.3-45.2			
	Fish		0.041-0.113	82.2-108.3	0.9964-0.9994	0.137-500	31.9-42.0			
	Shellfish		0.029-0.087	90.6-109.8	0.9967-0.9996	0.097-500	35.1-42.6			
	Shrimp		0.033-0.099	90.3-113.5	0.9969-0.9992	0.111-500	35.0-44.0			

Table 5 (continued)

Methods	Matrices	Antibiotics	LOD ($\mu g\ L^{-1}$ /$\mu g\ kg^{-1}$)	RR (%)	R^2	Linear range ($\mu g\ L^{-1}$/$\mu g\ kg^{-1}$)	Enrichment factor	Extraction time (min)	Organic solvents	References
	Squid		0.030-0.088	90.1-114.6	0.9936-0.9998	0.099-500	35.0-44.4			

Note: (1) SPE-HPLC-MS indicates solid-phase extraction combined with high-performance liquid chromatography-tandem mass spectrometry detector;

(2) SPME-HPLE-MS/MS indicates solid-phase microextraction combined with high-performance liquid chromatography-tandem mass spectrometry detector;

(3) HPLC-UV indicates high-performance liquid chromatography-ultraviolet detector;

(4) LC-MS/MS indicates liquid chromatography-tandem mass spectrometry detector;

(5) SPME-LC-MS/MS indicates liquid chromatography-tandem mass spectrometry detector;

(6) HPLC-FLD indicates high-performance liquid chromatography-fluorescence detector;

(7) SPE-LC-MS/MS indicates solid-phase extraction combined with liquid chromatography-tandem mass spectrometry detector;

(8) DMI-MSPD-HPLC-FLD indicates dummy molecularly imprinted matrix solid-phase dispersion combined with high-performance liquid chromatography-fluorescence detector;

(9) ELISA indicates enzyme-linked immunosorbent assay;

(10) SPE-LC-UV indicates solid-phase extraction combined with liquid chromatography-ultraviolet detector;

(11) PLE-HPLC-UV indicates pressurized liquid extraction combined with high-performance liquid chromatography-ultraviolet detector;

(12) TPATPS-HPLC-UV indicates thermoseparating polymer aqueous two-phase system combined with high performance liquid chromatography-ultraviolet detector;l

(13) UPLC-MS/MS indicates ultra-performance liquid chromatography-tandem mass spectrometry detector;

(14) EA-SFAM-SFO -HPLC-UV indicates effervescence-assisted switchable fatty acid-based microextraction combined with solidification of floating organic droplet combined with high-performance liquid chromatography-ultraviolet detector

where ER is the extraction recovery, V_0 is the volume of sample solution for microextraction, and V_{inj} is the volume of injection phase after microextraction procedure.

The EFs were 35.2–41.9 for seawater, 33.3–45.2 for sediment, 31.9–42.0 for fish, 35.1–42.6 for shellfish, 35.0–44.0 for shrimp, and 35.0–44.4 for squid, demonstrating a high enrichment capacity for FQs and TCs by this newly developed EA-SFAM-SFO method (Table 5). As can be seen from Table 5, the EF of this method was higher than SPME (12.2~20.0, 6.4~8.7) [8, 11], and SPE (6.0~7.8) [45]. Although the SPE-HPLC-MS [22] had a very high enrichment factor in seawater (770~880), there was a need for 1 L seawater sample and 12 mL methanol in this method. The extraction time of this SPE method was over 2.5 h, which was not conducive to rapid determination of large quantities of samples.

Only 1.0 mL hexane and 258 μL nonanoic acid were used in the newly developed EA-SFAM-SFO method, which were much lower solvent requirements than SPE [22, 23] and SPME [8, 11, 46] that require about 10 mL methanol or isopropanol, and were also lower than DMI-DSPD [48], which needs 6 mL of acetonitrile, and ELISA [49], which needs 10 mL of ethyl acetate. In addition, the medium-chain fatty acids belong to a class of environmentally friendly solvents [32, 33], which made the newly developed EA-SFAM-SFO more "green" than traditional SPE, SPME, ELISA methods mentioned above, and conformed more to the requirements of green analytical chemistry. Although PLE [47] does not require organic solvents, the method requires specialized instrumentation to achieve the high temperature and high pressure extraction conditions. The need for specialized equipment increases the cost and operational complexity, making this method less desirable for rapid quantification of target antibiotics. This newly developed EA-SFAM-SFO method did not require specialized equipment, compared with the expensively solid phase extraction columns and ELISA kits, the nonanoic acid was inexpensive and easy to get, making it suitable for the rapid detection of large-scale samples. The solidification of nonanoic acid in an ice bath reduces experimental error caused by an inefficient collection of the extraction agent, but also avoids the need for specialized extraction devices, which simplifies operational steps and reduces experimental cost. The use of nonanoic acid, which solidifies upon chilling, further reduces complex matrix effects [34, 35], resulting in improved accuracy and recovery.

Conclusions

A new switchable fatty acid dispersive liquid–liquid microextraction based on solidification of a floating organic droplet was developed for the determination of four fluoroquinolone and two tetracycline antibiotics in seawater, sediment, and seafood. The newly developed methods combined the advantages of fatty acid hydrophilic/hydrophobic alteration, CO_2 suspension assisted, and floating organic drop solidification, along with parameter optimization by Plackett-Burman design and Central Composite Design. The method required only 23.9 min to extract antibiotics from a 10 mL sample solution and utilized minimal organic solvents (1.0 mL hexane and 258 μL nonanoic acid). The newly developed EA-SFAM-SFO method combines the advantages of switchable hydrophobic/hydrophilic forms and cold-induced liquid/solid state transition to realize high extraction efficiency for antibiotics. The effects of the main operational factors on ERs were evaluated by single-factor screening, and interactions between the key parameters were assessed by CCD. Under optimized conditions, the mean ERs for the six antibiotics was >90% in seawater, sediment, and seafoods, and LODs were as low as 0.007–0.113 $\mu g\ L^{-1}$ or $\mu g\ kg^{-1}$. Overall, the newly developed method has low detection limits, short extraction time, wide linear detection range, and environmentally friendly characteristics for the determination of trace-level antibiotic concentrations in complex marine, environmental, and seafood matrices.

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Compliance with ethical standards

Ming Gao declares that he has no conflict of interest. Jun Wang declares that he has no conflict of interest. Xiukai Song declares that he has no conflict of interest. Xin He declares that he has no conflict of interest. Randy A. Dahlgren declares that he has no conflict of interest. Zhenzhong Zhang declares that he has no conflict of interest. Shaoguo Ru has received research grants from the National Natural Science Foundation of China (41676100). Xuedong Wang has received research grants from the National Natural Science Foundation of China (21577107).

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Ming Gao is pursuing a Doctor's degree at College of Marine Life Sciences, Ocean University of China, Qingdao, China. His current research interests focus on separation and analysis of trace environmental and food pollutants.

Jun Wang is an Associate Professor of Environmental Ecology in College of Marine Life Sciences, Ocean University of China, Qingdao, China. His research interests include environmental toxicology and biological monitoring.

Xiukai Song is Head of Biological and Ecological Department of Marine Environment Monitoring Center of Shandong Province, Shandong Marine Resource and Environment Research Institute, Yantai, China. His current research interests include analysis of marine biology and marine biology diversity changes under long-term climate change, phytoplankton classification, and relationship with environmental factors.

Xin He is a researcher of Biological and Ecological Department of Marine Environment Monitoring Center of Shandong Province, Shandong Marine Resource and Environment Research Institute, Yantai, China. His current research interests include analysis of marine biology and marine biology diversity changes.

Randy Alan Dahlgren is a Professor of Soil Science and Biogeochemistry in the Department of Land, Air, and Water Resources, Davis, University of California, USA. His principal research interest is watershed-scale biogeochemistry, including interactions of hydrological, geochemical, and biological processes in regulating groundwater and surface water chemistry.

Shaoguo Ru is a professor of Environmental Ecology in College of Marine Life Sciences, Ocean University of China, Qingdao, China. His current research interests include biological screening methods and damage mechanism on aquatic organisms.

Zhenzhong Zhang is pursuing a Master's degree at College of Marine Life Sciences, Ocean University of China, Qingdao, China. His current research interests focus on ecotoxicology, antibody, and colloidal gold preparation.

Xuedong Wang is currently a researcher in College of Public Health and Management, Wenzhou Medical University, Wenzhou, China. He is also an Associate Dean of the Key Laboratory of Watershed Sciences and Health of Zhejiang Province. His current research interests focus on development of green analytical techniques and ecological restoration of pollutants.