

# Determination of Sulfonamides in Fish Using a Modified QuEChERS Extraction Coupled with Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

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**Abstract** In the present work, an improved QuEChERS (quick, easy, cheap, effective, rugged and safe) protocol coupled with ultra-performance liquid chromatography-tandem mass spectrometry analytical method was developed and validated for determination of trace sulfonamides residues in fish tissues. The QuEChERS protocol was modified in terms of extraction solvent, as well as a graphene-based dispersive solid-phase cleanup procedure. Some extraction conditions such as solvent, acidity, and the amount of graphene sorbent were evaluated. The matrix effect related to the QuEChERS extraction from fish tissues was studied and compared with conventional QuEChERS protocol. The target analytes were quantified by a triple-quadrupole linear ion-trap mass spectrometer in multiple-reaction monitoring mode. The matrix-matched calibration curves were performed at seven concentration levels and good linear relationship ( $R^2 > 0.999$ ) was observed within the range of 0.5–200  $\mu\text{g kg}^{-1}$ . Recoveries of the spiked samples ranged between 70.7 and 100.9 % with relative standard deviation lower than 9.2 %. The limits of quantification were between 2.62 and 9.03  $\mu\text{g kg}^{-1}$ . The method was successfully applied to the analysis of real samples.

**Keywords** QuEChERS · Ultra-performance liquid chromatography-tandem mass spectrometry · Graphene · Sulfonamides · Fish

## Introduction

The QuEChERS (quick, easy, cheap, effective, rugged and safe) method was a simple and efficient sample preparation approach pioneered by Anastassiades et al. (2003). It exhibited great superiority for extraction of pesticides in fruits, vegetables, as well as some high-fat matrices, including milk, eggs, salmon, shrimp, etc. (Lehotay 2005; Lehotay et al. 2005; Jeong et al. 2012; Forsberg et al. 2011; Smoker et al. 2010; Wang et al. 2012a).

Generally, the QuEChERS method consisted of an initial extraction with acetonitrile, followed by partitioning with magnesium sulfate, either alone or in combination with other salts, such as sodium chloride. After extraction, a cleanup step was performed using dispersive solid-phase extraction (d-SPE) with primary secondary amine (PSA) to remove interfering matrix components. Recently, the QuEChERS methodology had usually been modified and successfully applied for the extraction of pesticides (Chamkasem et al. 2013), as well as various trace antibiotics, such as quinolones, tetracyclines, macrolides, and sulfonamides (Bourdat-Deschamps et al. 2014; Kung et al. 2015; Abdallah et al. 2015). To ensure food safety and human health, many countries, including the USA, the European Union, and China have set a maximum residue limit (MRL) of 100  $\mu\text{g kg}^{-1}$  for the sum of sulfonamides (SAs) in edible tissues (US Food and Drug Administration 2009; European Commission 2010; Ministry of Agriculture of the People's Republic of China 2002). Moreover, many analytical methods have been developed for the determination of SAs in various matrices, including liquid chromatography

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coupled with mass spectrometry (LC-MS), capillary electrophoresis (CE), and immunochemical methods (Wang et al. 2012b; Jiménez et al. 2010; Parab and Amritkar 2012; Lopes et al. 2012).

Recently, a novel and fascinating carbon nanomaterial, graphene, has attracted increasing attention for both fundamental science and applied research (Zhang et al. 2005; Bunch et al. 2007). The unique nanosheet morphology of graphene is conducive to achieve fast adsorption equilibrium and analyte elution (Su et al. 2009). These properties render graphene as an extraordinarily superior media in sample pretreatment procedures, including SPE (Liu et al. 2011a, b; Huang et al. 2012), stir rod sorptive extraction (SRSE) (Luo et al. 2011a), magnetic solid-phase extraction (MSPE) (Luo et al. 2011b, 2015), matrix solid-phase dispersion (MSPD) (Liu et al. 2011c), as well as solid-phase microextraction (SPME) (Luo et al. 2012; Chen et al. 2010).

Following the general research trend devoted to the development of sample pretreatment procedures in food analysis, we reported here a modified QuEChERS approach based on graphene-based cleanup process. Given the complex matrix of fish tissues, in the present work, the QuEChERS method was optimized in terms of extraction solution, solvent acidity, as well as graphene-based dispersive SPE cleanup process. The matrix effect related to the QuEChERS extraction from fish tissues was evaluated. In addition, the results were compared with conventional QuEChERS protocol. Quantification of target SAs was performed on ultra-performance liquid chromatography coupled with a triple-quadrupole linear ion-trap mass spectrometer (UPLC-MS/MS) in positive multiple-reaction monitoring (MRM) mode. In addition, the proposed method was carefully validated and applied to real sample analysis.

## Experimental

### Reagents and Chemicals

The standards of 12 SAs, i.e., Sulfapyridine (SPD), Sulfathiazole (ST), Sulfisoxazole (SIZ), Sulfadimidine (SM2), Sulfisomidine (SM2'), Sulfamonomethoxine (SMM), Sulfametoxydiazine (SMD), Sulfamethoxypyridazine (SMP), Sulfachloropyridazine (SPDZ), Sulfaquinolaxine (SQX), Sulfadimoxine (SDM), Sulfadimethoxine (SDM'), as well as two deuterium isotope-labeled internal standards (IS) SDM-D<sub>3</sub> and SDM'-D<sub>6</sub>, were supplied by Sigma-Aldrich Chemie (Steinheim, Germany, purity >98 %).

HPLC grade of acetonitrile (ACN), methanol (MeOH), and formic acid (purity >98 %), were obtained from Merck (Darmstadt, Germany). Anhydrous magnesium sulfate, sodium chloride, and acetic acid of analytical grade were obtained from Hangzhou Huipu Chemical & Apparatus Co., Ltd (Hangzhou, China). Graphene was obtained from Nanjing

XFNano Materials Tech Co., Ltd. (Nanjing, China, diameter, 0.5–2 μm; thickness, 0.8–1.2 nm; single layer ratio, ~80 %; purity, ~99.8 %). Primary secondary amine (PSA) sorbents obtained from Agela Technologies Inc. (Shanghai, China, size: 40–60 μm). Ultra-pure water with a resistivity of 18.2 MΩ cm<sup>-1</sup> was purified using a Milli-Q system (Millipore, Bedford, USA).

### Preparation of Standard Solutions

Individual stock solutions were prepared by dissolving 10 mg of each standard in 100 mL of acetonitrile. The standard mixture containing 12 SAs was obtained by diluting 1 mL of each standard in 100 mL of acetonitrile, reaching 1 μg mL<sup>-1</sup>. They were later diluted with acetonitrile to achieve series working concentrations. The internal standards (SDM-D<sub>6</sub> and SDM'-D<sub>3</sub>) were prepared at 200 ng/mL in acetonitrile. All of the standard solutions were prepared before analysis and stored at 4 °C away from light.

### Fish Samples

The grass carp fish samples were purchased from local supermarkets. The approximate size of the same species of fish (*n* = 20) collected was similar and ranged from 22.0 to 25.5 cm (total length) and 245.5 to 285.2 g. Samples were transported to our laboratory within 20 min, packed in separate insulated polystyrene boxes with ice. Gutting and filleting was carried out in the laboratory manually. All tissues were stored at -20 °C prior to analysis.

### Graphene-Based QuEChERS Procedures

The fish tissues were homogenized using an electric blender. The fully homogenized sample (1.0 g) was weighed into a 50-mL centrifuge tube. With the addition of 10 mL ACN-MeOH (80:20, *v/v*, 1 % acetic acid) containing 25 ng mL<sup>-1</sup> of the internal standards solution, the tube was shaken vigorously for 1 min. Sodium chloride (1.0 g) and anhydrous magnesium sulfate (4.0 g) were added into the mixture and the shaking step was repeated for 1.0 min, then centrifuged for 5.0 min at 5000 rpm. A total of 1.0 mL upper organic layer was transferred to a 5.0 mL micro-centrifuge tube containing 10 mg graphene and 150 mg anhydrous magnesium sulfate. The mixture was then shaken vigorously for 1.0 min and centrifuged for 5.0 min at 12,000 rpm. Finally, the organic layer was filtered through a 0.22 μm filter membrane and subjected to the chromatographic analysis.

### UPLC-MS/MS Analysis

The analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA), which was equipped with a binary

pump, a heated column compartment, a PDA detector, and an Empower workstation. The analyte separation was achieved on a Waters Acquity BEH C8 column (2.1 mm × 50 mm, 1.7 μm, Waters, Milford, MA). A gradient UPLC program was performed for separation with the mobile phase A (0.1 % formic acid aqueous solution) and mobile phase B (ACN). The following gradient program was used for the analysis: 5 % B (initial), 5–7 % B (0–3 min), 7–15 % B (3–8 min), 15–25 % B (8–12 min), 25–5 % B (12–13 min), 5–5 % B (13–16 min). The flow rate was kept at 0.4 mL min<sup>-1</sup>. The injection volume was 2 μL, and the column temperature was maintained at 25 °C.

A triple-quadrupole linear ion-trap mass spectrometer (4000 Q-trap, Applied Biosystems, Foster City, CA) equipped with a TurboIon Spray™ interface was used. Ionization was achieved using electrospray ionization (ESI) source operating in the positive mode and the data were collected in the multiple-reaction monitoring (MRM) mode. Typical ESI parameters were used as follows: ion spray voltage (IS), 5.0 kV; ion source gas 1 (GS1), 35 psi; ion source gas 2 (GS2), 55 psi; curtain gas (CUR), 35 psi; ion source temperature (TEM), 500 °C. The MRM transitions, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were summarized in Table 1. All instrument control, data acquisition, and the processing were performed using the associate Analyst 1.5.1 software.

### Method Validation

For method validation studies, linear dynamic range, precision, recovery, limits of detection and quantification were evaluated. Linearity was studied using matrix-matched calibration by analyzing blank fish extracts spiking all SAs at a concentration range between 0.5 and 200 μg kg<sup>-1</sup> (the blank tissue samples were used for preparation of matrix-matched standards and recovery studies, which were screened to have no studied SAs residues). Limits of detection (LODs) and quantification (LOQs) were determined as the lowest injected SA concentrations that yielded signal-to-noise (*S/N*) ratios of 3 and 10 (when the quantification ion was monitored), respectively. The trueness and precision of the method was tested via recovery and reproducibility experiments. The recovery was determined for three replicates in the spiking concentrations of 50, 100, and 150 μg kg<sup>-1</sup> of SAs standards (adding 50, 100, and 150 μL of the 1 μg mL<sup>-1</sup> SAs standard mixture to 1.0 g of blank tissue matrices to achieve the fortification levels, respectively). Precision of the method was evaluated as intra- and inter-day reproducibility by measuring corresponding relative standard deviations (RSDs). The intra-day precision was evaluated by repeated analyses of 12 SAs at three fortified concentrations on three sequential runs in six replicates. The inter-day precision and reproducibility were obtained in the same laboratory, but conducted by different operators over six separate days.

## Results and Discussions

### Optimization of the Graphene-Based QuEChERS Procedures

Generally, the conventional QuEChERS method contains two steps, a salting-out extraction and a dispersive SPE cleanup (Anastassiades et al. 2003). In the present work, the proposed QuEChERS protocol was modified in terms of extraction solvent, as well as a graphene-based dispersive solid-phase cleanup procedure. Some extraction conditions such as solvent, acidity, and the amount of graphene sorbent were evaluated. The matrix effect related to the QuEChERS extraction from fish tissues was studied and compared with conventional QuEChERS protocol.

**Influence of Extraction Solvent** The traditional QuEChERS involved an initial extraction with acetonitrile, however, it was reported that addition of methanol could improve the extraction efficiency, especially for veterinary drugs from foods of animal origin (Lopes et al. 2012). In addition, the solvent acidity was demonstrated to be of great importance for enhancement of the recovery. Therefore, several types of extraction solvents were evaluated in this work, including (A) ACN-MeOH (80:20, *v/v*) containing 1 % acetic acid; (B) ACN-MeOH (80:20, *v/v*); (C) ACN-MeOH (50:50, *v/v*) with 1 % acetic acid; (D) ACN-MeOH (50:50, *v/v*); and (E) ACN containing 1 % acetic acid. Figure 1 shows the results of experiments designed to determine the effect of extraction solvent, and/or acidification on the partitioning of the SAs on the upper layer. The recoveries of the analytes were calculated based on their concentrations in the supernatant. Among all the extraction solvents tested, the mixture ACN-MeOH (80:20, *v/v*) containing 1 % acetic acid provided the highest recoveries for most studied SAs. Comparing the results of solvents A and E, the effect of adding proper proportion of methanol was of great importance, which was well consistent with the ones presented in the literature (Lopes et al. 2012). However, high levels of methanol had the opposite effect. When 50 % MeOH was used, the recoveries of most SAs (except SDM') were decreased by 20 % or even more, which might be attributed to the high content of methanol to bring more of the matrix interferences.

Additionally, the use of acid in QuEChERS is common (Lehotay 2005), and it was tested in this study for fish samples. The 1 % acetic acid was added and compared. Results shown in Fig. 1 (solvent A and B) indicated that recoveries of all SAs were greatly improved. Moreover, combinations of salts (MgSO<sub>4</sub> and NaCl) were added to induce phase separation. It was reported that salting-out effect resulting from the addition of NaCl depends on the nature of the solvents involved in the partitioning step (Anastassiades et al. 2003). Therefore, addition of the proper amounts and combination

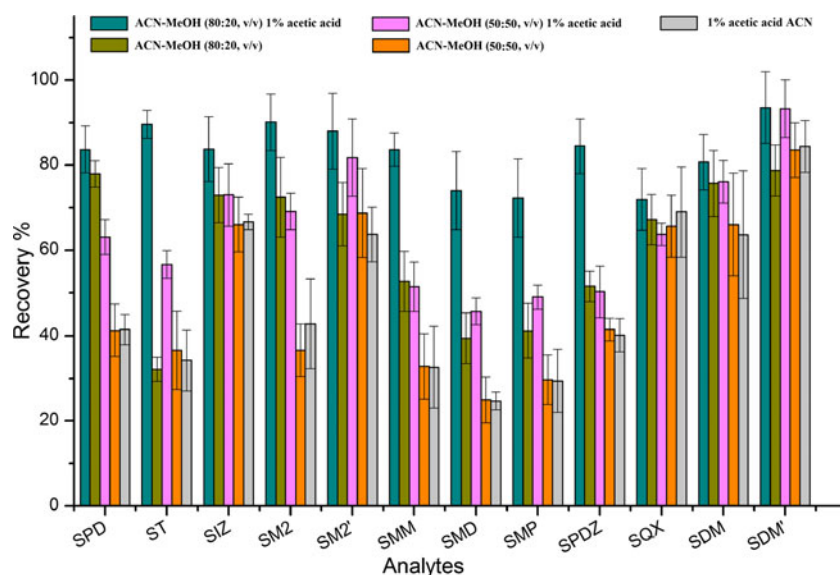
**Table 1** Optimized MS/MS parameters of studied SAs

Analytes	Abbreviation	Precursor/ production pairs ( <i>m/z</i> )	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
Sulfapyridine	SPD	250.1/155.9	70.0	3.4	24.3	11.3
		250.1/108.0	70.0	3.4	24.3	11.3
Sulfathiazole	ST	256.1/156.0	72.6	4.3	21.5	9.8
		256.1/108.0	72.6	4.3	35.0	9.8
Sulfisoxazole	SIZ	268.1/156.0	61.7	5.0	20.6	9.1
		268.1/113.0	61.7	5.0	35.0	9.1
Sulfadimidine	SM2	279.2/186.0	70.3	3.9	26.3	11.9
		279.2/155.9	70.3	3.9	26.3	11.9
Sulfisomidine	SM2'	278.9/186.0	77.3	4.0	25.2	11.5
		278.9/108.0	77.3	4.0	40.0	11.5
Sulfamonomethoxine	SMM	281.1/156.1	80.2	9.8	26.4	9.8
		281.1/108.1	80.2	9.8	40.0	9.8
Sulfametoxydiazine	SMD	281.1/156.0	66.4	3.9	25.3	9.0
		281.1/108.0	66.4	3.9	35.0	9.0
Sulfamethoxy pyridazine	SMP	281.1/156.2	78.5	4.1	25.2	10.2
		281.1/108.1	78.5	4.1	35.0	10.2
Sulfachloropyridazine	SPDZ	285.1/156.0	63.3	5.9	22.4	11.2
		285.1/108.0	63.3	5.9	35.0	11.2
Sulfaquinoxaline	SQX	301.1/156.0	82.0	4.0	25.0	10.4
		301.1/108.0	80.0	3.0	40.0	6.0
Sulfadimoxine	SDM	311.0/156.1	70.5	3.7	26.0	9.3
		311.0/107.9	73.8	4.0	40.0	7.0
Sulfadimethoxine	SDM'	311.1/156.0	85.8	4.0	26.0	11.2
		311.1/108.0	85.8	4.0	35.0	11.2
Sulfadimoxine-D <sub>3</sub>	SDM-D <sub>3</sub>	313.8/155.7	80.0	4.0	26.0	10.0
Sulfadimethoxine-D <sub>6</sub>	SDM'-D <sub>6</sub>	316.9/161.6	72.0	4.0	26.0	10.0

Ionization mode (ESI+); ion spray voltage (IS), 5.0 kV; curtain gas (CUR), 35 psi; ion source gas1 (GS1), 35 psi; ion source gas2 (GS2), 55 psi; temperature (TEM), 500 °C; interface heater, on; collision activated dissociation (CAD), medium, RT retention time

*DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential

**Fig. 1** Recovery (%) of the comparison among different solvents and acidification in the extraction of 12 SAs (spiked at 50 µg kg<sup>-1</sup>) from fish



of salts can be used to control the percentage of water in the organic phase (and vice versa for organic solvent in the water phase), thus enabling a certain degree of adjustment in the polarity of the phases.

**Graphene-Based Dispersive SPE Cleanup** Traditionally, dispersive SPE cleanup has been carried out as follows: an aliquot of the sample extract is added to a vial containing a small amount of SPE sorbent (normally PSA), and the mixture is briefly shaken or mixed in a vortex mixer to distribute the SPE material evenly and, thus, make the cleanup process easier. The sorbent is then separated by centrifugation, and an aliquot of the final extract is taken for analysis. In this work, the proposed cleanup method was based on dispersive SPE using graphene as sorbents. Therefore, after the first salting-out extraction, the organic phase was further cleaned up and dried by mixing with the graphene and  $\text{MgSO}_4$  (the role of  $\text{MgSO}_4$  is to absorb the trace amount of water in the organic extract).

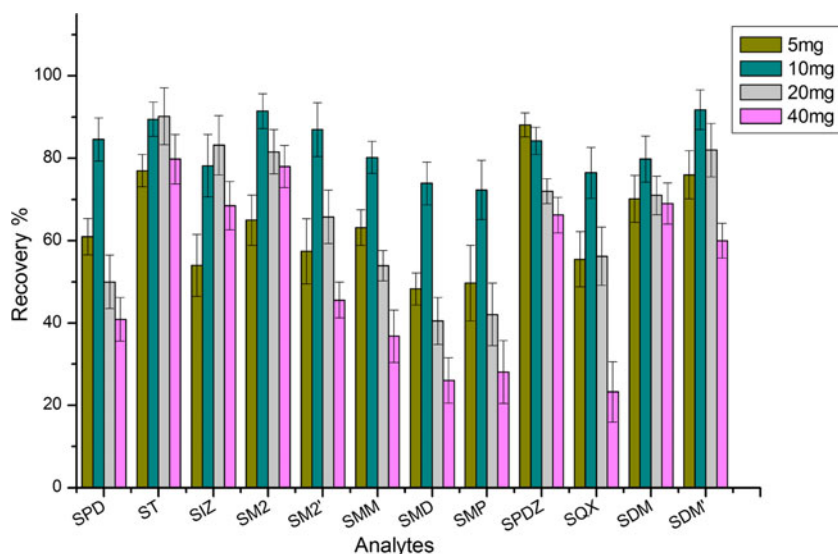
The amount of solid-phase sorbent as the most important factor has shown to affect the recoveries in the QuEChERS method. To evaluate the effect of this parameter, different amounts of graphene were investigated in the same dispersive SPE cleanup procedure. The experiments were performed using 1 mL of the organic extract at the spiked level of  $50 \mu\text{g kg}^{-1}$  that was placed into 5 mL centrifuge tubes containing 150 mg  $\text{MgSO}_4$  and different amounts of graphene (i.e., 5, 10, 20, 40 mg). As shown in Fig. 2, when the amount of graphene increased from 5 to 10 mg, most recoveries of the analytes were improved, except for SPDZ which was 4 % lower. However, by increasing the amount of graphene from 10 to 20 mg, the recoveries for most studied SAs were reduced to the range of 40–90 %. Moreover, the recoveries decreased further to 22–80 % when the amount of graphene was increased to 40 mg. It was obvious that when the graphene

amount increased, the analytes would be more easily adsorbed, leading to the significant recovery losses. Consequently, 10 mg (1 mL extract) was used as the optimum amount for the graphene-based dispersive SPE cleanup in the further studies since acceptable recoveries (71–93 %) and good cleanup performances were obtained at this amount.

**Comparison with Traditional PSA Cleanup** To evaluate the efficiency of the proposed graphene-based dispersive SPE procedure, its performance was compared with traditional PSA cleanup (mostly, PSA 50 mg/mL). For this purpose, parallel experiments were performed under the same conditions: 1 mL of the organic extract at the spiked level of  $50 \mu\text{g kg}^{-1}$  that was placed into 5 mL centrifuge tubes containing 150 mg  $\text{MgSO}_4$  and graphene (10 mg), or PSA (50 mg), respectively. Results indicated that the recoveries for all studied SAs were in the range of 67–86 % by PSA cleanup, which was slightly lower than those by graphene. However, the performance of PSA cleanup was not good enough to remove the interfering substances in the matrices: With graphene-based dispersive SPE cleanup, the maximum baseline intensity of MRM chromatogram was lower than 100 counts per second (cps). While when PSA cleanup procedure was applied, this value reached ~1000 cps, more than 10-folds higher.

To further illustrate the comparative results of graphene-based dispersive SPE method and conventional PSA cleanup procedure, the matrix effect (%ME) of the two protocols were compared. The occurrence of matrix effects is regarded as signal suppression or enhancement of the analyte due to the co-extraction of matrix components. Matrix effect can lead to a significant increase or decrease in the response of an analyte in a sample compared to a pure standard solution. In this work, matrix effect were determined by calculating the signal suppression/enhancement as  $\% \text{ME} = 100 \times (R_s - R_b) / R_0$  (the

**Fig. 2** Effect of dispersive sorbents on the recoveries of the 12 SAs spiked at  $50 \mu\text{g kg}^{-1}$



response of the spiked extracts ( $50 \mu\text{g kg}^{-1}$ ,  $R_s$ ) after non-spiked sample signal ( $R_b$ ) subtraction was compared to the response factor of a standard prepared in ACN ( $R_0$  with the same concentration) (Matuszewski et al. 2003). No matrix effect is observed when ME is equal to 100 %. Values above 100 % indicate ionization enhancement, and values below 100 % show ionization suppression.

As it is displayed in Fig. 3, %ME values of graphene-based cleanup are significantly higher than those by PSA (>80 % for most studied SAs, except for SDM). These results are consistent with the above comparison, where many interfering matrix constituents are removed by graphene-based cleanup. This may be due to the porous, wrinkled, and three-dimensional structure of the graphene provides larger surface area, which increases the adsorption sites available and then leads to the improvement of cleanup efficiency. Graphene can be considered as a reversed phase sorbent that is effective at trapping (binding) and removing fat and salt from samples. While PSA is a structure that has chelating effect due to the presence of the secondary amine, as well as the primary one. This kind of interaction is probably not strong enough to retain most matrix interferences present in the fish extracts.

### Optimization of UPLC-MS/MS conditions

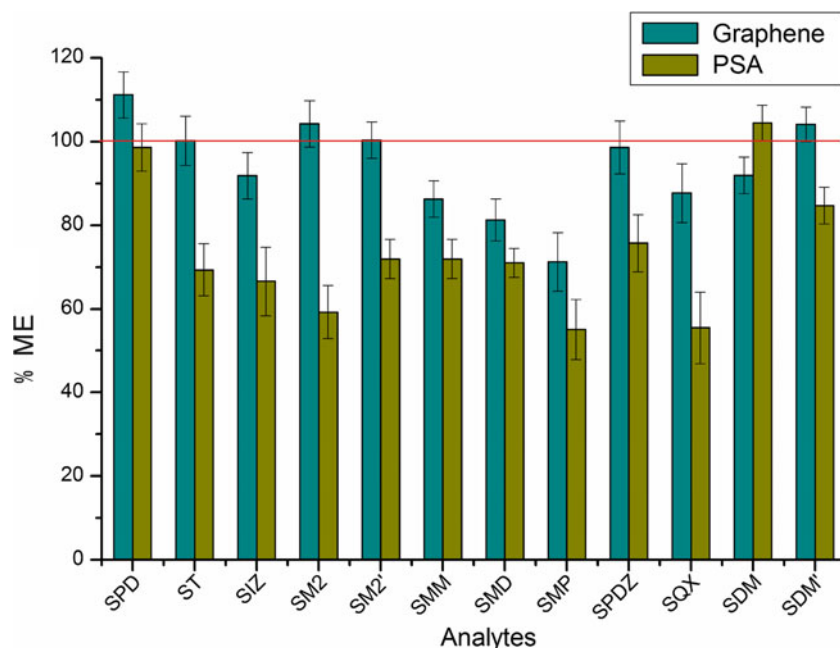
The selection of mobile phase is a paramount condition that would influence separation efficiency and MS ionization. It can directly determine the chromatographic peak shape and method sensitivity. In this work, the analytical sensitivity in condition of samples eluted with MeOH/water, ACN/water,

ACN/water (0.1 % formic acid), were compared. The results indicated that ACN gave rise to better elution strength and shorter retention time. The addition of formic acid (0.1 %) in water solution played an important role in improving chromatographic separation and promoting the ionization efficiency of mass spectrometry. Results of multiple injections indicated that under such situation good peak shape and high sensitivity of SAs could be achieved. Therefore, ACN and ultra-pure water (0.1 % formic acid) were finally chosen as mobile phases for the final chromatographic separation.

Electrospray ionization (ESI) was tested in both positive- and negative-ion modes. SAs showed much higher response signals using positive-mode ESI than in the negative mode. Therefore, the ESI source in positive mode was chosen for SAs detection. In order to optimize the gas setting for SAs, flow injection analysis (FIA) method was adopted by introducing  $5 \mu\text{L}$  standard solution of SAs ( $1 \mu\text{g mL}^{-1}$ ) into the mass spectrometer using the initial chromatographic conditions. The optimum conditions including: curtain gas (CUR), ion source gas 1 (GS1), ion source gas 2 (GS2), temperature (TEM) and collision activated dissociation (CAD) were summarized in the “[Experimental](#)” section.

Acquisition parameters of the mass spectrometer were optimized by direct continuous pump infusion of standard working solutions of the SAs ( $1 \mu\text{g mL}^{-1}$ ) at a flow rate of  $10 \mu\text{L min}^{-1}$  in the mass spectrometer. Full-scan spectra were acquired over the  $m/z$  range of 100–500 amu with a dwell time of 1.0 s and a step size of 0.1 amu for identification of the precursor ions. The selected protonated molecular ion  $[M + H]^+$  and product ions were summarized in Table 1. Breakdown curves were recorded

**Fig. 3** Matrix effects (% ME) by different dispersive SPE cleanup (spiked level:  $50 \mu\text{g kg}^{-1}$ )



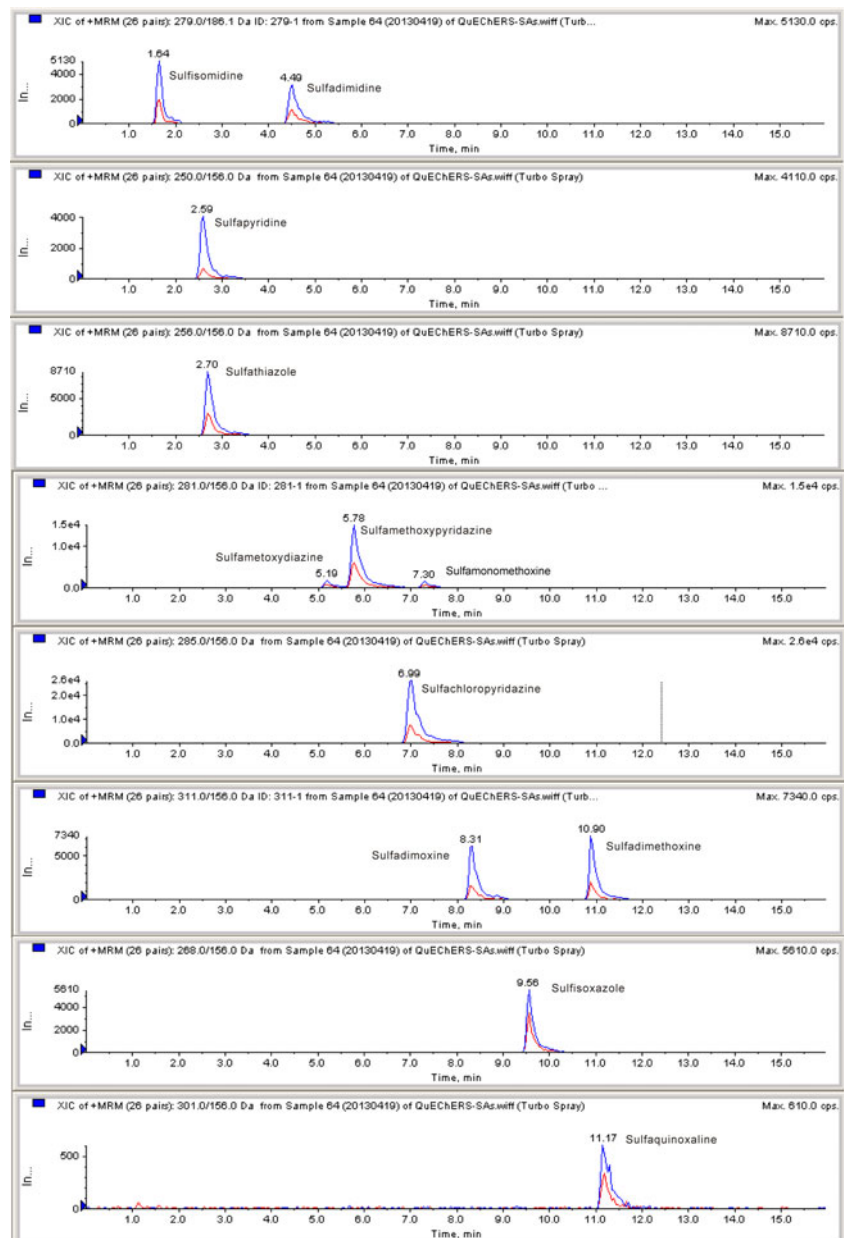
under different collision energy conditions to select the main characteristic fragments for quantification and analyte confirmation purposes. The DP was optimized for achieving the highest signal response of  $[M + H]^+$ . Further identification of the most abundant fragment ions and selection of the optimum CEs for SAs was carried out in the product ion scan mode. SAs show a very typical fragmentation pattern, which includes ions with  $m/z$  156 and 108. Based on this point, the MRM transition ( $[M + H]^+ > 156.0$ ) was used as a quantifier for 10 SAs, while the mass transition ( $[M + H]^+ > 186.0$ ) was used for SM2 and SM2' determination. The chromatograms for each SA under optimum condition were shown in Fig. 4.

## Validation of the Method

The analytical performance of the graphene-based QuEChERS coupled with UPLC-MS/MS protocol were carefully evaluated using spiked samples at levels of 0, 0.5, 1.0, 1.5 MRL ( $100 \mu\text{g kg}^{-1}$ ). The values of the linearity, recovery, precision, LOD, and LOQ were determined.

Considering the matrix effect of fish tissues, in the present work, the linearity was tested in the matrix-matched calibration curve, which was the most widely used method. The linearity of the chromatography response was tested with matrix-matched curves using

**Fig. 4** MRM chromatogram for the SAs standards under optimized UPLC-MS/MS conditions



**Table 2** Parameters of matrix-matched calibration curves and linearity, limit of detection (LOD) and limit of quantification (LOQ)

Analytes	Standard calibration equation ( $y = ax + b$ )			LOD $\mu\text{g kg}^{-1}$	LOQ $\mu\text{g kg}^{-1}$
	$a$	$b$	$R^2$		
SPD	4440	-5660	0.9996	1.34	4.48
ST	6090	-5840	0.9996	1.01	3.38
SIZ	2440	-22.2	0.9994	2.71	9.03
SM2	3340	-2270	0.9998	0.95	3.16
SM2'	6320	-15,600	0.9996	1.35	4.50
SMM	23300	-273	0.9998	0.88	2.90
SMD	24400	-31,900	0.9996	1.11	3.68
SMP	20500	9490	0.9996	0.89	2.98
SPDZ	25300	18,400	0.9998	1.00	3.33
SQX	578	-981	0.9996	2.63	8.76
SDM	0.0148	0.0334	0.9996	0.79	2.62
SDM'	0.0159	0.0517	0.9996	1.15	3.85

seven calibration points in the concentration range of 0.5–200  $\mu\text{g kg}^{-1}$ . The calibration curves were performed using the peak area of analyte versus the area of labeled analyte and the concentration of analyte with a weighting factor of  $1/x$ . Each point of the curves had been injected at least in triplicate. Table 2 shows the results of the calibration curves. Good linearity is found for all the analytes with  $R^2$  values better than 0.999, indicating satisfactory linearity in the concentration range studied.

LOD and LOQ were also calculated as the minimum detectable amount of analytes with a signal-to-noise ratio of 3 and 10, respectively (using the matrix-matched calibration curves). As shown in Table 2, the LODs and LOQs for 12 SAs ranged from 0.79 to 2.71  $\mu\text{g kg}^{-1}$  and 2.62 to 9.03  $\mu\text{g kg}^{-1}$ , respectively, indicating the satisfactory sensitivity for the proposed analytical protocol.

To evaluate the accuracy of the proposed method, the recovery (%) was performed by spiking 12 SAs standards into fish tissue samples. Three different concentration levels of SAs standards (0.5, 1.0, and 1.5 MRL) were added to the homogenized tissue sample prior to QuEChERS procedure. The resulting samples were operated as described above. Triplicate experiments were performed at each level. As shown in Table 3, the recoveries of the method for the analytes are between 70.7 and 100.9 %, and the relative standard deviations (RSDs) ranged from 3.0 to 9.2 %, indicating that the proposed graphene-based QuEChERS protocol was reliable and applicable for real sample analysis. In addition, the precision of the method was also evaluated, as shown in Table 4 (see supplementary information), the RSDs of intra-day precision obtained varied from 2.3 to 6.9 %, and inter-day precision from 4.4 to 9.7 %. These

**Table 3** Recoveries of the proposed method at three different spiking levels

Analytes	50 $\mu\text{g/kg}$		100 $\mu\text{g/kg}$		150 $\mu\text{g/kg}$	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
SPD	85.0	4.5	86.2	6.8	87.7	5.5
ST	90.9	7.4	96.9	4.8	99.2	4.7
SIZ	81.4	5.5	89.1	3.0	89.5	3.6
SM2	92.6	3.8	88.8	4.1	96.4	4.3
SM2'	89.6	9.2	94.5	4.3	94.9	3.9
SMM	81.0	8.5	99.0	3.6	97.0	3.6
SMD	74.5	7.1	75.0	6.5	86.5	6.4
SMP	70.7	4.7	76.1	6.6	79.9	3.3
SPDZ	86.0	4.2	88.0	3.3	90.0	4.8
SQX	77.0	5.9	76.8	5.6	81.3	6.0
SDM	80.1	3.8	92.7	6.4	93.1	5.2
SDM'	92.7	5.7	98.9	5.3	100.9	3.2



**Table 4** The method precisions at three different spiking levels

Analytes	Intra-day precision (RSD%)			Inter-day precision (RSD%)		
	50 µg/kg	100 µg/kg	150 µg/kg	50 µg/kg	100 µg/kg	150 µg/kg
SPD	6.2	4.9	5.8	8.9	7.7	8.1
ST	6.9	5.8	5.2	9.5	8.2	5.9
SIZ	3.4	2.3	3.1	6.3	4.4	4.7
SM2	4.1	3.8	4.2	5.8	5.3	4.9
SM2'	5.0	4.6	2.7	9.7	5.4	4.8
SMM	3.2	3.6	3.5	9.3	5.9	5.2
SMD	5.1	4.1	3.3	9.7	7.4	7.8
SMP	6.2	4.4	3.6	7.2	8.0	5.6
SPDZ	4.1	2.9	3.3	6.9	4.6	5.6
SQX	5.2	5.7	4.3	7.3	6.6	6.7
SDM	4.8	3.9	4.2	8.5	6.3	5.1
SDM'	5.3	4.7	3.0	9.1	6.6	6.3

results confirmed the acceptable accuracy and repeatability of the proposed method.

#### Application of the Method

The validated method was applied to grass carp fish samples collected from different markets located in Hangzhou. In order to ensure the quality of the results when the proposed method was applied, an internal quality control was carried out on every batch of samples. The two MRM transitions as well as their ion-ratios to the quantitative ion of individual analytes were also examined for both confirmation and quantification of the detected ions in real samples. The samples were all pretreated as described above under the optimum experimental conditions. The contents of detected SAs were quantified by the matrix-matched calibration curves and the results were shown in Table 5. Among these samples, SM2 was detected in three of nine detected samples (ranging 5.4–30.6 µg kg<sup>-1</sup>), which indicated current status of the use of sulfonamide antibiotics. Additionally, other two samples tested showed positive results for SMM and SDM, however, with a concentration below the LOQ of the method. In the rest of the samples, nearly all the SAs detected were below the LOD. The recurrent presence of

SAs in grass carp samples of this study make evident the health concern and the needs of regulation. Additionally, the proposed graphene-based QuEChERS approach also exhibited great advantage in detecting the trace SAs in fish samples.

#### Conclusions

The present study comprises the optimization and validation of an analytical methodology for determination of SAs antibiotics in fish samples. The QuEChERS protocol was modified in terms of a simple extraction with ACN-MeOH (80:20, v/v, with 1 % acetic acid) and a cleanup step by means of graphene-based dispersive SPE prior to analysis of the extract by UPLC-MS/MS. Graphene proved to be a potential type of dispersive SPE sorbent material and is expected to be widely applied for monitoring of veterinary residues at trace levels in the future for sample cleanup. The validation parameters of the method in terms of analytical range, precision, and recovery showed that the proposed method meets the requirements for SAs analysis with sufficient sensitivity, accuracy (70.7–100.9 % average recoveries), and precision (RSDs < 10 %). This proposed method has successfully revealed the great potentials of the

**Table 5** Concentration (µg kg<sup>-1</sup>) of target analytes detected in fish samples

Analytes	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
SM2	–	5.4	–	15.5	–	–	30.6	–	–
SMM	–	–	–	–	–	–	–	–	<LOQ <sup>a</sup>
SDM	–	–	–	–	–	–	–	<LOQ <sup>a</sup>	–

<sup>a</sup><LOQ lower than method limit of quantification

graphene-based QuEChERS as an effective sample pretreatment tool in residue analysis.

### Compliance with Ethical Standards

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**Conflict of Interest** All the authors declare that they have no conflict of interest.

**Ethical Approval** This article does not contain any studies with animals performed by any of the authors.

**Informed Consent** Not applicable.

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