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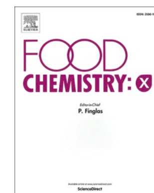

Rapid determination of caine-based anesthetics and their metabolite residues in fish using a modified QuEChERS method coupled with UPLC-MS/MS

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ABSTRACT

Caine-based anesthetics are frequently used in the production and transportation of aquatic products, but residues in fish threaten human health. A rapid, sensitive, and effective method was developed for detecting caine-based anesthetics and their metabolite residues in fish using ultra-high performance liquid chromatography-tandem mass spectrometry coupled with a modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) method. The sample was extracted with 0.05 % (*v/v*) formic acid-80 % acetonitrile, 4 g NaCl was added for liquid partitioning, and 50 mg C_{18} was used for purification. Matrix-matched calibrations showed good correlation coefficients with $R^2 \geq 0.9942$. The limits of detection were 0.5–4.4 ng/g. The recoveries ranged from 71.4 %–115.8 %, and the relative standard deviation for intra-day and inter-day precision was less than 8.5 % and 9.2 %, respectively. This method effectively analyzed the residues of caine-based anesthetics and their metabolites in fish and could be applied to aquatic product anesthetic regulation.

1. Introduction

Anesthetics are commonly used to lessen the stress of aquatic goods to achieve a high survival rate of aquatic products during farming and live shipment (Hoseini et al., 2018; Priborsky & Velisek, 2018). Caine-based anesthetics, such as tricaine methanesulfonate (MS-222), benzocaine, procainamide, lidocaine, and bupivacaine, are frequently used because of their short anesthetic times, long durations, and quick resuscitation times (Priborsky & Velisek, 2018; Zhang et al., 2022). However, multiple caine-based anesthetics were reported to cause cytotoxicity, behavioral alterations, methemoglobinemia, and neurotoxicity (Kubrova et al., 2021), posing a threat to human health. MS-222 and benzocaine have been approved in some countries, but a period of withdrawal time before marketing is needed (Kiessling et al., 2009; Purbosari et al., 2019). Regrettably, China has not yet clearly stated the type and residue limits of anesthetics allowed in aquatic products. Therefore, attention should be paid to the residues of anesthetics in aquatic products.

The matrix of fish is complex and consists of a variety of intricate matrices, which increases the difficulty of detecting trace residues. The main analytical methods currently used for analyzing anesthetics residues in fish include immunochromatographic (ICS) (Lei et al., 2023), high-performance liquid chromatography (HPLC) (Xia et al., 2023), gas chromatography–mass spectrometry (GC/MS) (Rafson et al., 2024) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Wang et al., 2024). However, the sensitivity and specificity are hardly satisfactory for some methods, and LC-MS/MS is increasingly becoming the technique of choice due to its high sensitivity and accuracy. What's more, QuEChERS (quick, easy, cheap, effective, rugged, and safe) is a simple, rapid, and inexpensive pretreatment method favored in sample preparation of drug residue detection in fish (Aissaoui et al., 2024; Desmarchelier et al., 2018). However, few studies focused on the high-throughput determination of multi-anesthetic residues in fish based on LC-MS/MS coupled with QuEChERS.

Currently, research on anesthetics in aquatic products mainly focuses on evaluating anesthetic effects, and there is a lack of attention to the residues of anesthetics in aquatic products. Several studies have proposed analytical methods that concentrate only on a few kinds of fish anesthetic residues, primarily detecting eugenols, MS-222, and benzocaine (Q. Huang et al., 2022; Y. Huang et al., 2021; Luo et al., 2024; Xia et al., 2023). Current high throughput screening studies of residues in fish have focused on sedative residues, with fewer types of anesthetic

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Table 1
Detailed information about the retention times and MRM parameters of 12 target compounds.

No.	Compound	Formula	Retention Time (min)	Precursor Ion	Product Ion	Cone Voltage (V)	Collision Energy (eV)
1	3-Aminobenzoic acid	$C_7H_7NO_2$	2.24	138.0	77.1*/65.1	20	17/20
2	4-Aminobenzoic acid	$C_7H_7NO_2$	2.67	138.0	77.1*/65.1	20	17/20
3	Benzocaine	$C_9H_{11}NO_2$	5.91	166.1	138.1*/94.1	22	14/17
4	MS-222	$C_{10}H_{15}NO_5S$	5.65	166.1	138.1*/94.1	22	14/17
5	4-Acetamidobenzoic acid	$C_9H_9NO_3$	3.22	180.1	94.1*/77.1	20	17/29
6	Prilocaine	$C_{13}H_{20}N_2O$	4.03	221.2	86.2*/136.2	18	15/19
7	Lidocaine	$C_{14}H_{22}N_2O$	3.97	235.3	86.2*/58.2	22	18/34
8	Procainamide	$C_{13}H_{21}N_3O$	2.04	236.2	163.2*/120.1	20	16/30
9	Chloroprocaine hydrochloride	$C_{13}H_{20}C_{12}N_2O_2$	3.70	271.2	100.2*/154.1	20	13/29
10	Ropivacaine	$C_{17}H_{26}N_2O$	4.56	275.3	126.2*/84.2	17	22/40
11	Bupivacaine	$C_{18}H_{28}N_2O$	5.06	289.3	140.2*/98.2	23	20/36
12	Cinchocaine	$C_{20}H_{29}N_3O_2$	6.18	344.4	271.3*/215.2	20	21/29

[*] represents quantitative ions.

residues (Hong et al., 2022; Wang et al., 2024). A paucity of studies exists in the literature on the multi-residues of caine-based anesthetics in fish and even fewer on the detection of their metabolites. Consequently, there is a pressing need to develop a sensitive, selective, and straightforward method for the high-throughput determination of caine-based anesthetics and their metabolite residues in aquatic products.

This study aims to develop a rapid, sensitive, and effective method for detecting 9 caine-based anesthetics and 3 of their metabolite residues in fish using UPLC-MS/MS coupled with a modified QuEChERS method. The chromatographic conditions, MS parameters, extraction reagent, QuEChERS salting-out and dehydrating agents, and QuEChERS sorbents were optimized to achieve a more suitable high-throughput determination. The established method was validated and applied to detect the residues of 9 caine-based anesthetics and 3 of their metabolites in market fish samples. This work provides a reference for the high-throughput detection of anesthetic residues in aquatic products, thus contributing to protecting food safety.

2. Materials and methods

2.1. Reagents and materials

Standards of 3-aminobenzoic acid, 4-aminobenzoic acid, benzocaine, MS-222, 4-acetamidobenzoic acid, prilocaine, lidocaine, procainamide, chloroprocaine hydrochloride, ropivacaine, bupivacaine, and cinchocaine were purchased from Anpel Laboratory Technologies Inc. (Shanghai, China), with all of the standards were of high purity grade (> 95 %). Methanol, acetonitrile (ACN), and formic acid (FA) (HPLC grade) were purchased from Thermo Fisher Scientific Ltd. (Fair Lawn, NJ, USA). QuEChERS extraction salt packets (4 g anhydrous Na_2SO_4 and 1 g NaCl) were purchased from Jin Yang Filter Material Company (Hebei, China). N-propyl-ethylenediamine (PSA) adsorbent was purchased from Dikma Technologies Inc. (Beijing, China). C_{18} adsorbent was obtained from Supelco (Bellefonte, PA, USA). Ultra-pure water (18 MΩ cm) was obtained by a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other reagents and chemicals were of analytical grade. Fifteen different species of fish samples were collected in March 2024 from three local supermarkets in Fuzhou, Fujian.

2.2. Instrumentation

ACQUITY UPLC I–Class/Xevo TQ–XS Triple quadrupole UPLC/MS (Waters, USA), analytical balance (Sartorius, Germany), automatic vortex mixer (Heidolph, Germany), Allegra X-30 centrifuge (Beckman Coulter, USA), intelligent silent ultrasonic cleaner (Xiaomei Ultrasonic Instruments Ltd.), and precision pipette (Socorex, Switzerland) were used.

2.3. Standard preparation

Stock solutions of 3-aminobenzoic acid, 4-aminobenzoic acid, benzocaine, MS-222, 4-acetamidobenzoic acid, prilocaine, lidocaine, procainamide, chloroprocaine hydrochloride, ropivacaine, bupivacaine, and cinchocaine (1.00 mg/mL) were prepared in methanol and stored at −20 °C in the dark. The mixed intermediate standard solutions for 9 caine-based anesthetics (1 μg/mL) and 3 metabolites (3-aminobenzoic acid, 4-aminobenzoic acid, and 4-acetamidobenzoic acid, 5 μg/mL) were prepared from the stock solutions in methanol. The standard working solutions were stored at −4 °C. The chemical structures of 12 target compounds are shown in Fig. S1.

2.4. Sample preparation

Freshwater fish samples were cleaned with deionized water. After removing the fish bones, the fish muscle and skin were placed in a tissue homogenizer for mincing and mixing and then stored in a refrigerator at −18 °C. 2.0 g of the homogenized sample was weighed into 50 mL centrifuge tubes, and 10.0 mL of 0.05 % (*v/v*) FA-80 % acetonitrile was added. The sample was extracted by shaking for 5 min and sonication for 10 min (Yang et al., 2024). Then 4 g NaCl was added, vortexed for 1 min, and centrifuged at 9000 rpm for 5 min. Next, 50 mg C_{18} was added to 1.0 mL of the supernatant for purification. After shaking for 2 min, the mixture was centrifuged at 9000 rpm for 5 min. The supernatant was filtered through a 0.22 μm filter before UPLC-MS/MS analysis.

2.5. UPLC-MS/MS analysis conditions

Target compounds were analyzed using a Waters Acquity I-Class UPLC system coupled to a Xevo TQ-XS tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). The chromatographic separation was performed on an Acquity UPLC® BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm) with gradient elution using 0.05 % FA (A) and acetonitrile (B) as mobile phase at 30 °C. The gradient elution was performed as follows: 0–3.5 min, 5 %B-30 %B; 3.5–7.0 min, 30 %B-60 %B; 7.0–8.5 min, 60 %B-90 %B; 8.5–9.0 min, 90 %B-5 %B; 9.0–10.0 min, 5 %B-5 %B. The flow rate was set at 0.25 mL/min with an injection volume of 2 μL. Multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI+) was used for mass spectrometric analysis. The optimized parameters for the ionization source were set as follows: capillary voltage 3.0 kV; desolvation temperature 500 °C; source temperature 150 °C; desolvation gas flow rate 1000 L/h; cone gas flow rate 150 L/h; nebulizer gas flow rate 7.0 bar; collision gas flow rate 0.15 mL/min.

2.6. Method validation

Method validation experiments were conducted, including matrix effect (ME), linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. All of the 12 target compounds were

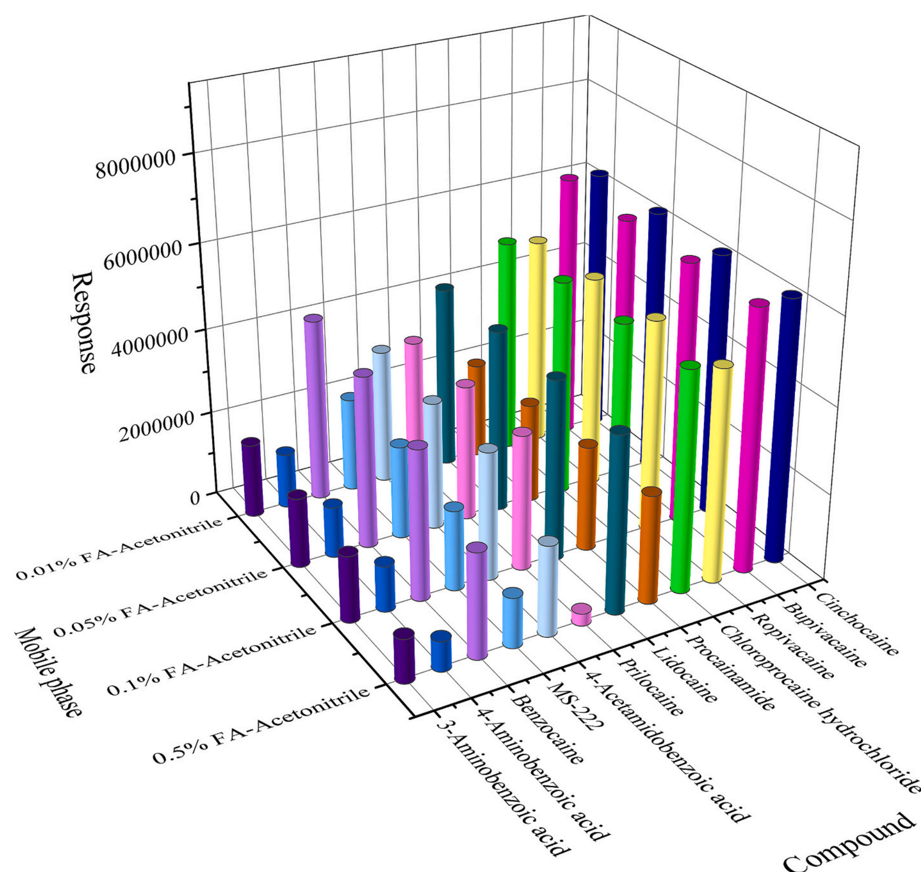

Fig. 1. Effect of different concentrations of FA in the mobile phase on the corresponding response of target compounds.

quantified by an external standard method. The ME value was calculated as follows: ME = slope matrix/slope solven (Q. Huang et al., 2022). The slope matrix and slope solvent mean the slope of the matrix-matched standard calibration curve and the slope of the solvent standard calibration curve, respectively. The LOD and LOQ were determined by spiking blank samples with low concentration standards and calculating the signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively (Xing et al., 2023). The precision was evaluated as the relative standard deviation (RSD) of repeat measurements. The accuracy of the method was assessed by adding the target analytes at three different concentrations (9 caine-based anesthetics at 2 ng/g, 10 ng/g, and 50 ng/g, and 3 metabolites at 5 ng/g, 25 ng/g and 250 ng/g) in six replicates to matrix blanks and then processed according to the sample preparation procedure.

3. Results and discussion

3.1. Optimization of chromatographic conditions and mass spectrum

Mass spectrometry parameters were optimized to obtain the maximal abundance of precursor and product ions. All of the 12 compounds are more suitable for the positive ion mode to obtain a proton to form the parent ion $[M + H]^+$. This may be attributed to the fact that most caine-based anesthetics belong to amide compounds, and the molecular structure contains electronegative atoms such as O and N, which have strong proton affinity (Szostak & Szostak, 2018). Detailed information on the retention times and MRM parameters, such as precursor and product ions, collision energies, and cone voltages for the 12 target compounds, are listed in Table 1.

Methanol and acetonitrile are commonly used as organic solvents in the LC-MS/MS method mobile phases. A comparative analysis was conducted on the effects of methanol-water and acetonitrile-water as mobile phases on individual analytes, revealing superior peak shapes when employing acetonitrile-water as the mobile phase. However, adopting acetonitrile-water as a mobile phase proved inadequate to achieve baseline separation between the isomer MS-222 and benzocaine. This deficiency was substantially rectified by incorporating FA into the mobile phase. Consequently, investigations were carried out to evaluate the impact of FA concentrations (0.01 %, 0.05 %, 0.1 %, and 0.5 %) on the response signals of each component, as shown in Fig. 1.

Fig. 1 illustrates that the response signal of procainamide and chloroprocaine hydrochloride decreased with increasing FA concentration while the signals of other components increased. Concentrations of 0.01 %, 0.05 %, and 0.1 % FA displayed negligible effects on signal fluctuations, whereas prilocaine exhibited a marked decrease in response signal at 0.5 % FA concentration. All compounds exhibited favorable response signals at the 0.05 % FA concentration. Consequently, 0.05 % FA-acetonitrile was selected as the definitive mobile phase. This differs from the research which indicated that 0.1 % FA was commonly added in the mobile phase in the analysis of veterinary drug residues (Saleh et al., 2024; Wen et al., 2023). The discrepancy may be attributed to the fact that the compounds under investigation were disparate. A Waters Acquity UPLC® BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm) with gradient elution was used in the separation of 12 target compounds as it gave an excellent separation of two pairs of isomers (3-aminobenzoic acid and 4-aminobenzoic acid, benzocaine and MS-222). The total ion chromatogram (TIC) of the 12 compounds investigated is given in Fig. S2.

3.2. Sample preparation optimization

3.2.1. Optimization of extraction reagent

Caine-based anesthetics are often extracted using methanol and acetonitrile, while the addition of formic acid can facilitate the targets' extraction. Fig. 2 displays the findings of an investigation of the effects

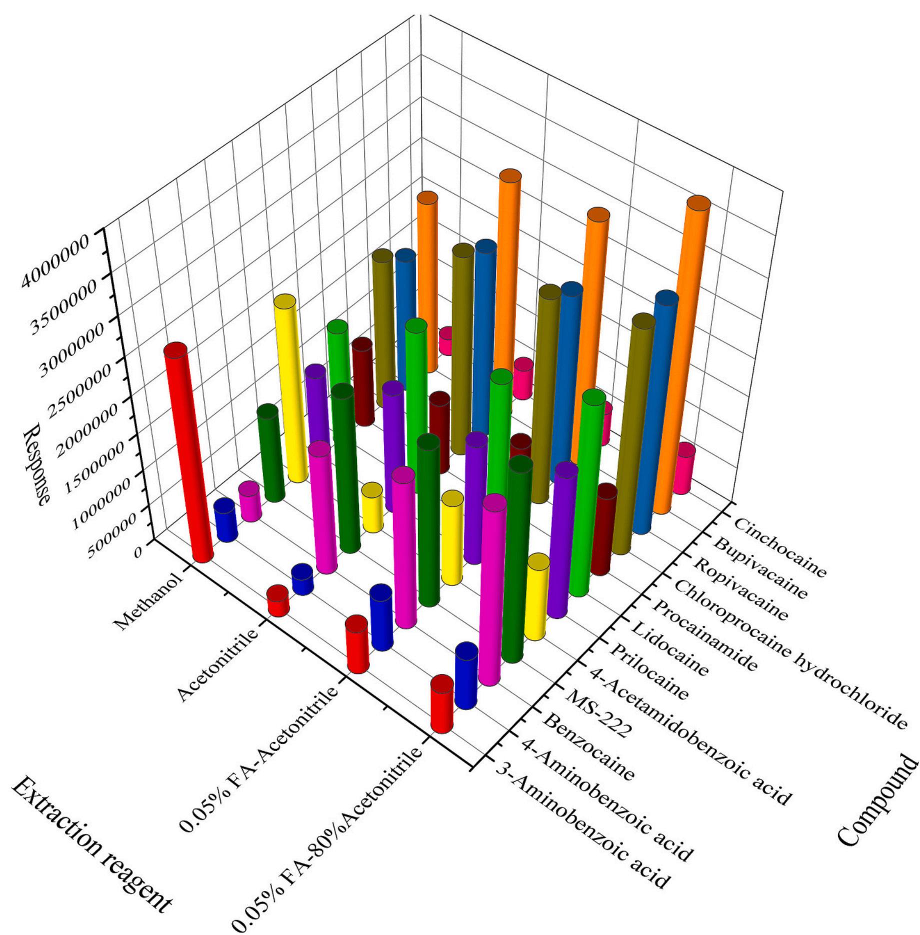

Fig. 2. Effect of different extraction reagents on the response signals of target components.

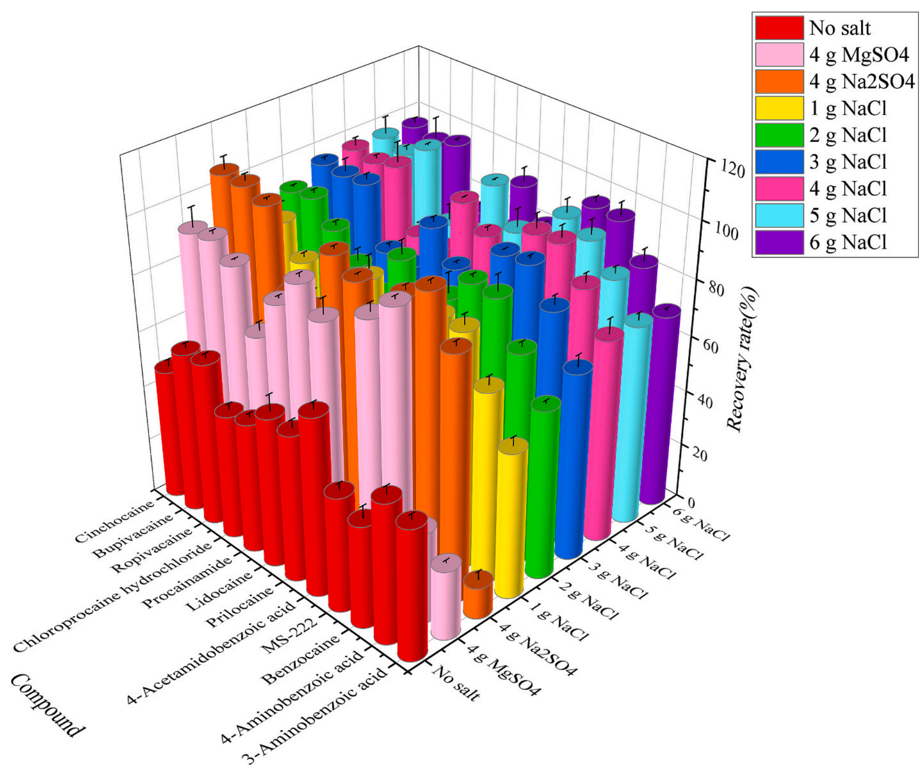

Fig. 3. Effect of different QuEChERS salting-out and dehydrating agents on the recoveries of target components.

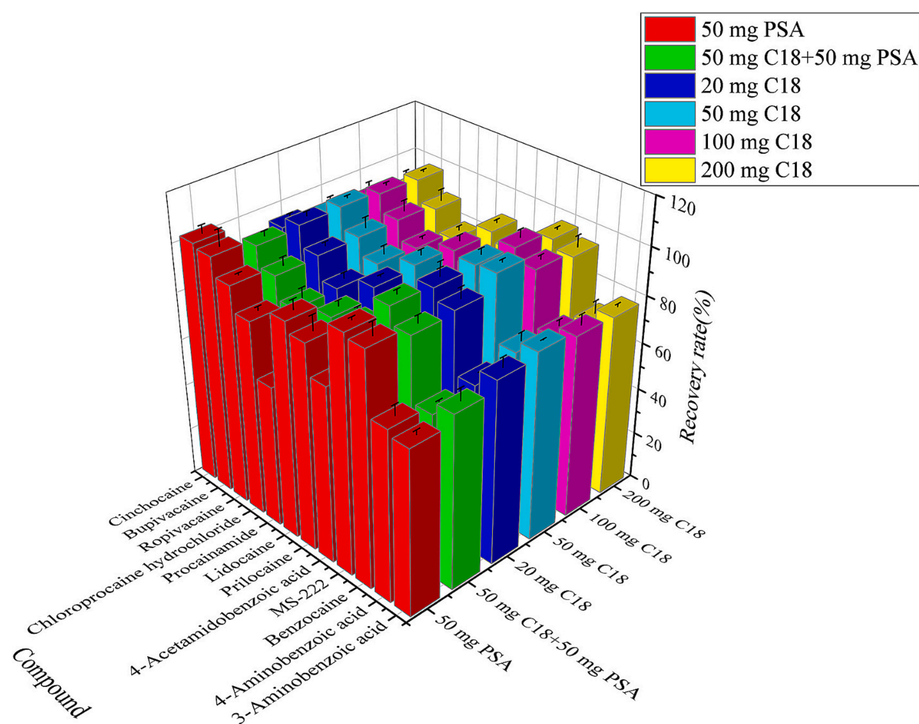

Fig. 4. Effect of different QuEChERS sorbent on the recoveries of target components.

of methanol, acetonitrile, 0.05 % (*v*/*v*) FA-acetonitrile, and 0.05 % (*v*/*v*) FA-80 % acetonitrile as extraction solvents on the corresponding signals of each component. Methanol was more suitable for extracting 3-aminobenzoic acid and 4-acetamidobenzoic acid, whereas most of the components had stronger response signals when extracted with acetonitrile. Additionally, when acetonitrile was used for extraction, the proteins were better precipitated, and the extracts were more clarified, which would be helpful for further purification. Thus, acetonitrile is a superior option than methanol. The response signals of all the components were enhanced to varying degrees by adding 0.05 % FA, especially for 3-aminobenzoic acid, 4-aminobenzoic acid, benzocaine, and 4-acetamidobenzoic acid. The probable reason for this is that most caine-based compounds belong to the primary amine group, and adding FA helps to protonate the amine group, which increases the extraction efficiency. Further optimization indicated that most components exhibited the maximum corresponding signals when 0.05 % (*v*/*v*) FA-80 % acetonitrile was used as the extraction reagent. Therefore, the extraction reagent for this experiment was ultimately determined to be 0.05 % (*v*/*v*) FA-80 % acetonitrile. The result differs from the previous approach, which typically employed acetonitrile as the extraction solution for the detection of eugenols and MS-222 (Luo et al., 2024; Wang et al., 2024; Xia et al., 2023). This may be attributed to the fish samples clumping together from the pure organic solutions during extraction, lowering the extraction effectiveness of most compounds.

3.2.2. Optimization of QuEChERS salting-out and dehydrating agents

The QuEChERS approach facilitates the enrichment of target compounds in the organic phase and effectively prevents the entry of strongly polar water-soluble contaminants into the extract by using salting and dehydrating agents to remove water and induce organic-water phase separation. QuEChERS salting-out and dehydrating agents that are frequently employed are Na_2SO_4, $MgSO_4$, and NaCl. The effects of QuEChERS extraction salt packs (4 g Na_2SO_4, 4 g $MgSO_4$, and 4 g NaCl) and no salt on recoveries of target compounds were compared through experiments (Fig. 3). The results showed that the recovery of each compound was less than 65 % with no salt. 4-Aminobenzoic acid could be adsorbed by $MgSO_4$ with a recovery of less than 35 %, while 4 g Na_2SO_4 and 4 g NaCl resulted in a higher recovery (≥85 %). 3-Aminobenzoic acid and 4-acetamidobenzoic acid could be absorbed by Na_2SO_4 and $MgSO_4$, greatly reducing their recoveries (<35 %). Better recoveries were obtained using 4 g NaCl as the QuEChERS salting-out and dehydrating agent, both >72 %. Moreover, 4 g NaCl was suitable for all other components, with a recovery of ≥76 %. Therefore, NaCl was chosen for the tests. The dose of NaCl (1 g, 2 g, 3 g, 4 g, 5 g, and 6 g) was further optimized for the recovery of each component (Fig. 3). The findings demonstrated that as the dosage of NaCl increased, the recoveries of each component generally exhibited a trend of rising and then falling or rising and then stabilizing. The highest recovery of 4-acetamidobenzoic acid was observed at 2 g NaCl, with subsequent decreases in recovery observed as the dosage of NaCl increased. The highest recoveries at 5 g NaCl were obtained for MS-222, procainamide, and ropivacaine. Nevertheless, all component recoveries were greater than 72 % at 4 g NaCl. As a result, 4 g NaCl was ultimately chosen as the QuEChERS salting-out and dehydrating agent for the 12 target compounds. In the existing literature, 1.0 g of NaCl and 3.0 g of $MgSO_4$ were employed as salting-out and dehydrating agents for the residual measurement of MS-222 in fish (Xie et al., 2019). The disparate optimized results may be attributed to the varying anesthetics analyzed and the disparate extraction reagents used.

3.2.3. Optimization of QuEChERS sorbents

The sample matrix needs to be cleaned up because fish samples have a complex matrix, and the co-extracts contain a range of components, such as lipids and proteins. The selection and dosage of the sorbent have an impact on the target compound recovery as well as the sensitivity of the analysis. N-propyl ethylenediamine (PSA), graphitized carbon black (GCB), and C_{18} are common QuEChERS sorbents. Of these, PSA can be used to remove polar organic acids, fatty acids, and other substances; GCB is primarily used for the adsorption of pigments from the plant sample; and C_{18} can be used to remove nonpolar compounds from the matrix. Since the substances used in this investigation were animal-derived, GCB was disregarded. The efficacy of six QuEChERS sorbents was evaluated in the presence of 50 mg PSA, varying dosages of C_{18} (20 mg, 50 mg, 100 mg, and 200 mg), and a combination of 50 mg PSA and

Table 2
The ME, linear range, calibration curves, LOD, and LOQ of 9 caine-based anesthetics and 3 metabolites.

No.	Category	Matrix effect	Linear range (ng/mL)	Calibration curves	R^2	LOD (ng/g)	LOQ (ng/g)
1	3-Aminobenzoic acid	0.92	5–500	$Y = 3.6274 \times 10^2x - 2.7150 \times 10^3$	0.9980	4.0	13.3
2	4-Aminobenzoic acid	0.98	5–500	$Y = 6.2614 \times 10^2x + 5.1846 \times 10^3$	0.9968	4.4	14.8
3	Benzocaine	0.71	2–200	$Y = 9.7654 \times 10^3x + 1.8026 \times 10^3$	0.9994	0.7	2.4
4	MS-222	1.16	2–100	$Y = 5.4088 \times 10^3x + 2.0339 \times 10^3$	0.9970	1.4	4.6
5	4-Acetamidobenzoic acid	1.08	5–500	$Y = 1.9330 \times 10^3x + 1.8359 \times 10^4$	0.9968	2.6	8.8
6	Prilocaine	1.30	2–100	$Y = 3.6265 \times 10^4x + 4.8602 \times 10^3$	0.9998	1.1	3.6
7	Lidocaine	1.32	2–100	$Y = 4.8316 \times 10^4x + 4.4125 \times 10^3$	0.9998	1.3	4.2
8	Procainamide	0.70	2–100	$Y = 3.0784 \times 10^3x - 1.5265 \times 10^3$	0.9942	1.5	5.1
9	Chloroprocaine hydrochloride	1.36	2–100	$Y = 6.4423 \times 10^4x + 6.8841 \times 10^3$	0.9996	1.0	3.3
10	Ropivacaine	1.21	2–100	$Y = 5.9577 \times 10^4x + 3.2296 \times 10^3$	0.9998	0.7	2.3
11	Bupivacaine	1.21	2–100	$Y = 7.4635 \times 10^4x + 8.9519 \times 10^3$	0.9998	0.5	1.8
12	Cinchocaine	1.23	2–100	$Y = 1.1216 \times 10^4x + 6.0621 \times 10^3$	0.9984	1.0	3.2

Table 3
Recovery and precision of 9 caine-based anesthetics and 3 metabolites ($n = 6$).

No.	Category	Low		Medium		High	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
1	3-Aminobenzoic acid	72.5	7.1	75.7	0.8	75.0	1.2
2	4-Aminobenzoic acid	81.6	3.0	84.2	1.7	85.2	1.7
3	Benzocaine	113.5	1.8	109.3	1.7	107.6	1.7
4	MS-222	115.8	2.1	106.2	1.2	105.3	0.9
5	4-Acetamidobenzoic acid	71.4	4.6	72.1	2.3	73.1	1.3
6	Prilocaine	109.2	6.0	106.2	0.9	104.4	0.5
7	Lidocaine	109.9	4.2	108.4	1.0	103.8	0.8
8	Procainamide	73.3	6.3	74.0	6.0	76.1	2.3
9	Chloroprocaine hydrochloride	110.0	8.5	105.1	1.7	101.5	1.2
10	Ropivacaine	104.0	6.2	101.0	1.2	107.0	1.1
11	Bupivacaine	106.1	7.8	106.4	1.4	104.1	1.5
12	Cinchocaine	108.7	4.9	108.6	2.8	107.7	1.9

Note: Low, medium, and high spike levels represent 2 ng/g, 10 ng/g, and 50 ng/g, respectively (3-aminobenzoic acid, 4-aminobenzoic acid, and 4-acetamidobenzoic acid at 5 ng/g, 25 ng/g and 250 ng/g, respectively).

50 mg C_{18} (Fig. 4). The findings indicated that PSA was not an appropriate purification choice for procainamide, 4-aminobenzoic acid, 4-acetamidobenzoic acid, and 3-aminobenzoic acid, as it gave recoveries less than 70 %. The recoveries of MS-222, prilocaine, ropivacaine, and bupivacaine were found to be unaffected by the dosage of C_{18}. The use of 50 mg C_{18} resulted in satisfactory recoveries for all compounds, with recoveries ranging from 76 % to 103 %. As the C_{18} dosage increased, the recoveries exhibited a corresponding decline for most compounds. Consequently, the QuEChERS sorbent for analyzing the 12 target compounds was determined to be 50 mg of C_{18}. Previous studies have frequently employed PSA and C_{18} as QuEChERS sorbents, particularly for the detection of sedatives and MS-222 (Hong et al., 2022; Xie et al., 2019). The disparate optimization outcomes in this study compared to previous ones can be primarily attributed to the distinct properties of the target compounds, with PSA being unsuitable for the three metabolites.

3.3. Result of method validation

3.3.1. Matrix effect, linearity, LOD and LOQ

The co-elution of matrix components during the chromatographic separation process results in matrix effects (ME), which may increase or reduce the signal of target components. For this reason, assessing ME is essential to guarantee the precision of quantitative studies. Generally, the ME is often considered to be negligible when the value is between 0.85 and 1.15 (Zheng et al., 2018). The matrix-matched standard curve should be compensated when the ME is less than 0.85 (indicating matrix inhibition) and is greater than 1.15 (indicating matrix enhancement). The slope ratio of each compound ranged from 0.70 to 1.36, which meant that the ME of several compounds could not be ignored. Of them, MS-222, prilocaine, lidocaine, chloroprocaine hydrochloride, ropivacaine, bupivacaine, and cinchocaine showed matrix-enhancing effects (ME>1.15), while benzocaine and procainamide had matrix inhibitory effects (ME<0.85). Therefore, matrix-matched standard calibration curves were necessary for quantification. The result was similar to the previous study that the matrix-matched standard curve is needed (Wang et al., 2024; Yang et al., 2024). A calibration curve was created for each analyte by graphing the peak area versus the associated concentration. Good linearity was demonstrated by all analytes, with correlation values (R^2) $\geq$ 0.9942. The LODs and LOQs were 0.5–4.4 ng/g and 1.8–14.8 ng/g. Table 2 displayed the ME, linear range, calibration curves, LOD, and LOQ of 9 caine-based anesthetics and 3 of their metabolites.

3.3.2. Accuracy, precision, and specificity

The recoveries of all compounds at the three spike levels ranged from 71.4 % to 115.8 % at low concentrations, 72.1 % to 109.3 % at medium concentrations, and 73.1 % to 107.7 % at high concentrations (Table 3). The intraday precision ranged from 0.5 % to 8.5 %, and the interday precision ranged from 3.9 % to 9.2 %. The acceptable accuracy and precision indicated the reliability of the method for analyzing anesthetic residues in fish. The specificity results demonstrated that all target compounds in the blank fish samples had no interference peaks around the retention durations, indicating that the specificity of the approach was satisfactory.

3.4. Application to actual sample analysis

The validated method was applied to examine the residues of 9 caine-based anesthetics and 3 of their metabolites in actual freshwater fish samples. A total of 15 fish samples were collected from local markets

Table 4
Comparison with other previous methods.

Analytes (Categories, kind)	Quantitative method	Extraction solution	Purification method (amount)	LODs (ng/g)	Optimization	Recovery (%)	Ref.
3 eugenol anesthetics (Eugenol, eugenol acetate, and methyl isoeugenol)	HPLC	10.0 mL acetonitrile	Magnetic solid-phase extraction (MSPE)	2.4–3.6	Sorbent dosage, extraction time, solution pH, eluent composition, desorption time, eluent volume	80.0 %–118.8 %	(Xia et al., 2023)
Benzocaine	HPLC	15.0 mL acetonitrile	SPE column (Oasis HLB SPE)	3.3	Chromatographic conditions, extraction reagent, eluent composition, eluent volume	85.5 %–93.1 %	(Xuan et al., 2018)
5 eugenol anesthetics (Eugenol, isoeugenol, eugenol methyl eugenol, methyl isoeugenol, and acetyl isoeugenol)	Fluorescence immunoassay	10.0 mL acetonitrile	gold nanoclusters	0.1–36.0	Fluorescent gold clusters and related parameters	84.1 %–111.9 %	(Luo et al., 2024)
6 eugenol anesthetics (Eugenol, methyl eugenol, isoeugenol, methyl isoeugenol, eugenol acetate, and acetyl isoeugenol)	GC–MS	10.0 mL acetonitrile	multiplug filtration cleanup (m-PFC) SPE column	2.0–10.0	Extraction solvent, extraction method, and purification conditions	76.4 %–105.1 %	(Y. Huang et al., 2021)
11 anesthetics (4 eugenol and 7 caines)	immunochromatographic assay (ICS)	10.0 mL acetonitrile	colloidal gold-based quadruplex-ICS	0.1–83.0	Synthesis of antigens, preparation of mAbs, bioconjugation, and immunochromatographic-related parameters	72.4 %–111.3 %	(Lei et al., 2023)
MS-222	LC-MS/MS	10.0 mL acetonitrile containing 30 % acetate buffer (pH 4.0)	QuEChERS (1.0 g of NaCl, 3.0 g of $MgSO_4$, and 300 mg PSA)	1.0	Selection of the internal standard and evaluation of the matrix effect	92.1 %–97.5 %	(Xie et al., 2019)
14 anesthetics (Caine category)	UPLC-Q-TOF-MS	10.0 mL sodium acetate buffer solution	SPE column (Oasis HLB SPE)	0.3–0.7	Extraction reagent, SPE column, UPLC-Q-TOF–MS parameters,	72.4 %–111.3 %	(Li et al., 2023)
7 anesthetics (eugenols and MS-222) + 2 sedatives	Liquid chromatography coupled with quadrupole linear ion trap tandem mass spectrometry (LC-QLIT-MS/MS)	10.0 mL acetonitrile	40 mg graphitized carbon nanotubes)	0.03–0.4	Chromatographic conditions, MS parameters, extraction reagent, purification sorbents	86.3 %–111.7 %	(Q. Huang, et al., 2023)
20 benzodiazepine sedatives and 4 eugenol anesthetics	LC-MS/MS	14.0 mL Acetonitrile	QuEChERS (0.8 g of NaCl, 0.3 g of Na_2SO_4 and 0.1 g of C_{18})	0.01–2.2	Chromatographic conditions, MS parameters, and extraction solvent	70.0 %–114.9 %	(Wang et al., 2024)
10 anesthetic and sedative (mainly sedatives)	LC-MS/MS	10.0 mL 0.1 % formic acid acetonitrile	QuEChERS (250 mg C_{18} and PSA)	0.003–0.2	Chromatographic conditions, extraction solvent, and QuEChERS sorbents	80.0 %–120.0 %	(Hong et al., 2022)
9 Caine anesthetics and 3 metabolites	LC-MS/MS	10.0 mL 0.05 % (*v*/*v*) formic acid-80 % acetonitrile	QuEChERS (4 g NaCl, and 50 mg C_{18})	0.5–4.4	Chromatographic conditions, MS parameters, extraction reagent, QuEChERS salting-out and dehydrating agents, and QuEChERS sorbents	71.4 %–115.8 %	this work

for analysis. A single anesthetic was detected in 3 different samples. The anesthetics detected were 3-aminobenzoic acid, 4-aminobenzoic acid, and 4-acetamidobenzoic acid, with values of 6.8, 16.8, and 7.7 ng/g, respectively. Among these, 3-aminobenzoic acid is a metabolite of MS-222, while 4-aminobenzoic acid and 4-acetamidobenzoic acid are metabolites of benzocaine. The literature currently in publication contains very few investigations on the levels of caine-based anesthetics in samples. Li et al. (Li et al., 2023) did not detect any of the 14 caine-based anesthetics in aquatic products. Similarly, Huang et al. (Q. Huang et al., 2022) found no MS-222 residues in their research. In this work, 9 common caine-based anesthetics were not detected while 3 metabolites were found. The findings show that anesthetics are utilized in freshwater fish but a period of withdrawal time before marketing may have gone through. Thus, regular monitoring of anesthetics in aquatic products should be advocated.

3.5. *Comparing with existing methods*

The established method was compared with previously reported methods in determining anesthetic residues in fish (Table 4). The majority of the existing literature pertains to fish sedatives and eugenol anesthetics, or rather tends to analyze individual caine-based anesthetics, such as MS-222 or benzocaine (Xie et al., 2019; Xuan et al., 2018). A paucity of studies exists in the literature on the multi-residues of caine-based anesthetics in fish and even fewer on the detection of their metabolites. Li et al. (Li et al., 2023) developed a method for the quantification of 14 caine-based anesthetics in aquatic products, which were extracted by sodium acetate buffer solution, cleaned up via solid-

phase extraction, and analyzed using ultrahigh-performance liquid chromatography system coupled with a tandem quadrupole-time-of-flight mass spectrometer (UPLC-Q-TOF-MS). However, using sodium acetate buffer solution may result in insufficient extraction, and the solid phase extraction (SPE) column purification process is time-consuming. Additionally, TOF screening may suffer from poor sensitivity.

Compared to previously published methods, this work is the pioneering detection of 9 caine-based anesthetics and 3 of their metabolites in fish using LC-MS/MS coupled with the modified QuEChERS purification method. The results showed that the technique was simple, rapid, and sensitive. In addition, this method focuses on optimizing the types and amounts of reagents used in QuEChERS purification. By removing extra procedures like protein and fat removal, the approach increases analytical efficiency and lessens the workload of laboratory personnel, which aligns with the principles of green sample preparation. The established method offers a valuable reference to the high-throughput determination of common caine-based anesthetics and their metabolites. In subsequent studies, attempts could be made to establish a simultaneous detection method for commonly used anesthetics, extending beyond the scope of those caine-based.

4. Conclusion

This study developed a simple, rapid, and sensitive method to quantify the residues of 9 caine-based anesthetics and 3 of their metabolites in fish samples. The sample was extracted with 0.05 % (*v/v*) FA-80 % acetonitrile, and a modified QuEChERS method with 4 g NaCl and 50 mg C_{18} was used for purification. This method showed good linear coefficients with $R^2 \geq 0.9942$. The LOD and LOQ were 0.5–4.4 ng/g and 1.8–14.8 ng/g, respectively. The recoveries ranged from 71.4 %–115.8 %, and the relative standard deviation for intra-day and inter-day precision was less than 8.5 % and 9.2 %, respectively. Overall, the established method was simple, sensitive, and effective in analyzing the residues of 9 caine-based anesthetics and 3 metabolites in fish. Anesthetics with caine-based were limited in this study, so high-throughput screening techniques may be developed in future studies to detect more kinds of anesthetics simultaneously and to analyze more samples. This work provides a reference for the detection of anesthetic residues in aquatic products, thus contributing to protecting food safety.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Shouer Lin: Writing – original draft, Validation, Project administration, Data curation, Conceptualization. **Wenqian Qiu:** Methodology, Formal analysis, Data curation. **Yongyou Hua:** Validation, Data curation. **Yan Yang:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102032.

Data availability

Data will be made available on request.

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