Application Note

Food Safety

A Total Solution for the Quantitative Analysis of Benzocaine Metabolites in Fish Tissue

A streamlined QuEChERS extraction and EMR cleanup followed by sensitive detection using the Agilent 6495D triple quadrupole LC/MS

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Abstract

A comprehensive end-to-end liquid chromatography/tandem mass spectrometry (LC/MS/MS) workflow was developed for the quantitative determination of benzocaine metabolites in fish matrices. The workflow employs QuEChERS extraction followed by Agilent Captiva EMR–Lipid passthrough cleanup, chromatographic separation on an Agilent Altura ZORBAX Eclipse Plus C18 column, and sensitive detection using an Agilent 1290 Infinity III liquid chromatographer (LC) coupled to an Agilent 6495D triple quadrupole LC/MS system (6495D LC/TQ). The entire sample preparation requires fewer than 30 minutes per batch with no evaporation or reconstitution steps, reducing per-sample hands-on time. The method achieved excellent calibration linearity (R^2 > 0.999) over a dynamic range of 0.1 to 100 µg/L, parts-per-trillion (ppt) level method detection limits (MDLs) for all analytes, and matrix-spiked recoveries within 80 to 120% with %RSD < 3% across three fish species. These results demonstrate a robust, accurate, and sensitive solution for regulatory compliance monitoring of benzocaine metabolites in aquaculture products.

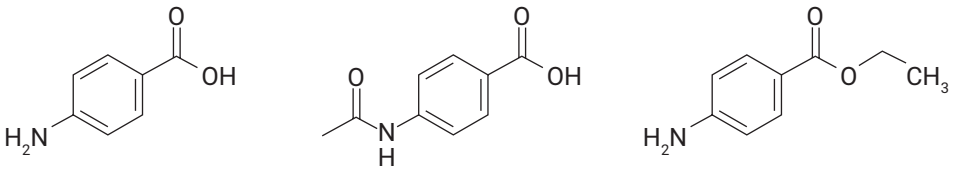
Introduction

Caine-based anesthetics such as benzocaine and tricaine methanesulfonate (MS-222) are widely used in aquaculture to reduce stress during farming, handling, and live transportation.[1] However, residues of these compounds and their metabolites may persist in edible fish tissue, raising potential food safety concerns. Importantly, metabolite monitoring is as critical as parent compound detection. From a market surveillance study, it was found that even when the parent anesthetic was absent, their metabolites were still detected in commercial fish.[2]

While some jurisdictions have approved benzocaine for aquaculture use with mandatory withdrawal periods, clear numeric maximum residue limits (MRLs) have not been universally established. This highlights the need for sensitive and reliable monitoring methods. However, the complex, high-fat matrix of fish tissue increases the difficulty of detection. Conventional HPLC-UV methods often lack the sensitivity and selectivity required for trace-level metabolite detection in lipid-rich matrices, while GC/MS approaches may require derivatization of polar metabolites such as PABA. LC/MS/MS overcomes these limitations through its high sensitivity and specificity.

This application note describes a complete workflow for the quantitative analysis of benzocaine metabolites—4-aminobenzoic acid (PABA), 4-acetamidobenzoic acid (AcPABA), and N-acetylbenzocaine (AcBZ)—in fish tissue. The method employs Agilent Bond Elut QuEChERS extraction with Captiva EMR–Lipid passthrough cleanup for effective lipid removal, chromatographic separation on an Agilent Altura ZORBAX Eclipse Plus C18 column, and sensitive detection on an Agilent 6495D triple quadrupole LC/MS system.

While recent publications have demonstrated LC/MS/MS methods for caine-class residues[2], these often require customized extraction protocols or lack comprehensive validation across multiple fish species with varying fat content. This application note addresses this gap by providing a fully integrated, commercially available workflow—from extraction consumables to data analysis—validated across low-fat (tilapia), medium-fat (seabass), and high-fat (Norwegian salmon) matrices, enabling laboratories to adopt the method directly without additional development.



PABA
$C_7H_7NO_2$
CAS 150-13-0

AcPABA
$C_9H_9NO_3$
CAS 556-08-1

AcBZ
$C_{11}H_{13}NO_3$
CAS 5338-44-3

Figure 1. Chemical structure and formula of the three benzocaine metabolites.

Experimental

Standards preparation

Standards of PABA (CAS 150-13-0, $C_7H_7NO_2$), AcPABA (CAS 556-08-1, $C_9H_9NO_3$), and AcBZ, (CAS 5338-44-3, $C_{11}H_{13}NO_3$) were purchased from Sigma-Aldrich.

Individual stock solutions were prepared at 1 mg/mL in methanol (MeOH) and stored at −20 °C in the dark. A mixed intermediate standard solution (1 µg/mL) was prepared in 80% acetonitrile (ACN)/water (H_2O) (v:v) and stored at −20 °C. Nine calibration levels from 0.1 to 100 ng/mL were prepared from the intermediate mix by serial dilution in 80% ACN/H_2O.

Sample preparation

Three species of fish (seabass, tilapia, and Norwegian salmon) were purchased from a local market. Fish tissue was manually separated from the bones and homogenized using a domestic blender. The samples were kept at −20 °C for storage.

Homogenized seafood (5 ± 0.1 g) was weighed into a 50 mL tube. After adding 10 mL of water, the mixture was vortexed for 10 to 15 minutes. Then, 10 mL of ACN and QuEChERS EN extraction salts (part number 5982-5650) were added and shaken vigorously for five minutes in the mechanical shaker.

After centrifugation at 5,000 rpm for five minutes, 4 mL of extract was mixed with 1 mL of water and passed through a Captiva EMR–Lipid HF cartridge (part number 5610-2235). The eluate was transferred to high recovery sample vial (part number 5183-2073) for LC/MS/MS analysis. The procedure is illustrated in Figure 2.

Notably, the entire extraction and cleanup procedure eliminates evaporation-to-dryness and reconstitution steps—common bottlenecks in traditional SPE workflows—reducing total hands-on time to approximately 25 minutes per batch of 10 samples.

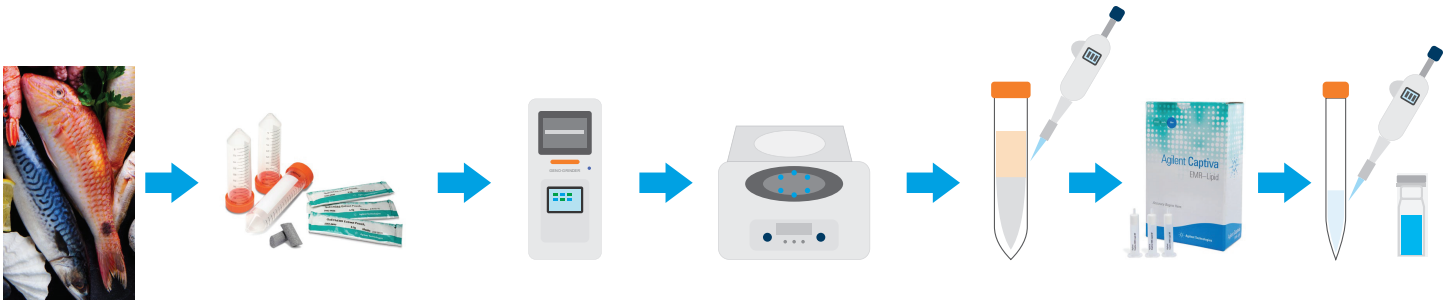


Figure 2. Sample preparation procedures. **Note:** 2.5-times dilution was involved due to the entire sample extraction process.

Preparation of matrix-spiked quality control samples

Matrix-spiked QC samples were prepared at three concentration levels—QC1 (1 µg/kg), QC2 (5 µg/kg), and QC3 (10 µg/kg)—in each of the three fish matrices. These correspond to ready-to-inject concentrations of approximately 0.4, 2, and 4 µg/L in the final extract. Two technical preparations per QC level with two injections per preparation (n = 4) were analyzed.

Instrumentation

Chromatographic separation was performed on an Altura ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm column (part number 204210-308), installed on a 1290 Infinity III LC system. Detection was performed using an Agilent 6495D triple quadrupole LC/MS system with a Jet Stream (AJS) heated ESI source operated in positive-ion dynamic MRM (dMRM) mode. All data acquisition and processing were performed using Agilent MassHunter software (version 12.1).

Table 1. LC parameters.

Agilent 1290 Infinity III LC System	
Parameter	**Value**
Column	– Altura ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 204210-308) – ZORBAX RRHD Eclipse Plus C18, 2.1 × 5 mm, 1.8 µm (p/n 821725-901)
Mobile Phase A	0.05% Formic acid in water
Mobile Phase B	Acetonitrile
Flow Rate	0.25 mL/min
Gradient	Time (min) %B 0 5 3.5 30 7.0 60 8.5 90 9.0 5 10.0 5
Stop Time	2 min (post-run)
Injection Volume	2 µL
Autosampler Temperature	10 °C
Needle Wash	Wash 2: isopropanol/methanol/acetonitrile/H_2O (1:1:1:1) for 5 s; Wash 1: 50% methanol for 15 s at flush port
Column Temperature	30 °C

Table 2. LC/MS/MS parameters.

Agilent 6495D Triple Quadrupole MS	
Parameter	**Value**
Source	Agilent Jet Stream
Polarity	Positive
Gas Temperature	290 °C
Gas Flow	15 L/min
Nebulizer	30 psi
Sheath Gas Temperature	330 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	1,500 V
Acquisition Mode	Dynamic MRM
Time Event	0 min → waste; 0.5 min → MS; 9 min → waste

Table 3. MRM transition parameters.

Compound	RT (min)	Precursor (*m/z*)	Product (*m/z*)	CE (V)	iFunnel Mode	CAV (V)	Polarity
PABA	3.02	138	**65**, 77, 39	27, 21, 54	Standard	4	Positive
AcPABA	3.76	180.1	**94**, 77	17, 38			
AcBZ	6.48	208.1	**94**, 77, 43	21, 46, 60			

Note: Quantifier ions are bolded.

Results and discussion

Robust chromatographic profile

With the Altura ZORBAX Eclipse Plus C18 column and the optimized gradient, excellent chromatographic separation of PABA, AcPABA, and AcBZ was achieved within 10 minutes. As illustrated in Figure 3, symmetric, sharp peaks and a robust elution profile were obtained over triplicate injections of 100 ppb of targets. The Altura Ultra Inert surface technology minimizes secondary metal-ion interactions with polar analytes such as PABA, yielding symmetric peak shapes and eliminating the peak tailing commonly observed on conventional C18 phases. This directly translates to improved integration accuracy and lower detection limits.

Linearity and accuracy

Calibration curves in neat solvent were generated over a range of 0.1 to 100 µg/L using nine calibration points (Figure 4). Excellent linearity $R^2 > 0.999$ was achieved for all three analytes over three orders of magnitude.

Good accuracy (80 to 120%) was observed across all calibration levels, and no carryover was detected even after injection of the highest standard (100 µg/L).

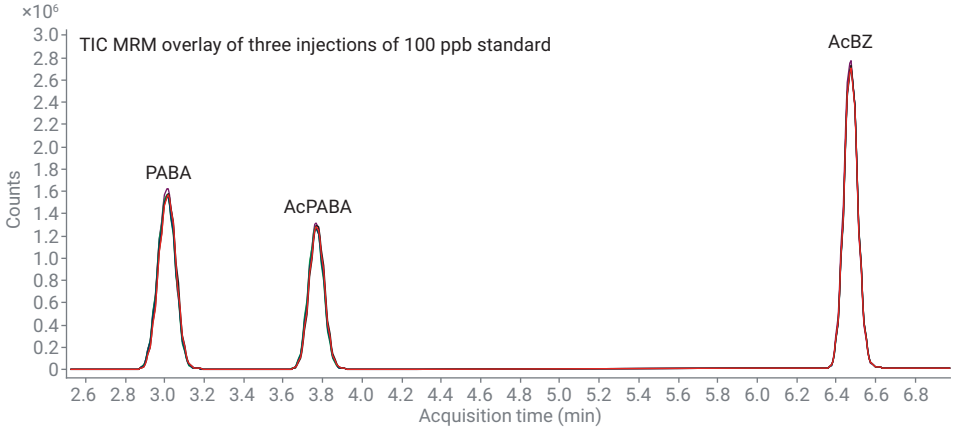


Figure 3. Chromatogram overlay of triplicate injections of the concentration of all targets at 100 ppb on an Agilent Altura ZORBAX Eclipse Plus C18 column.

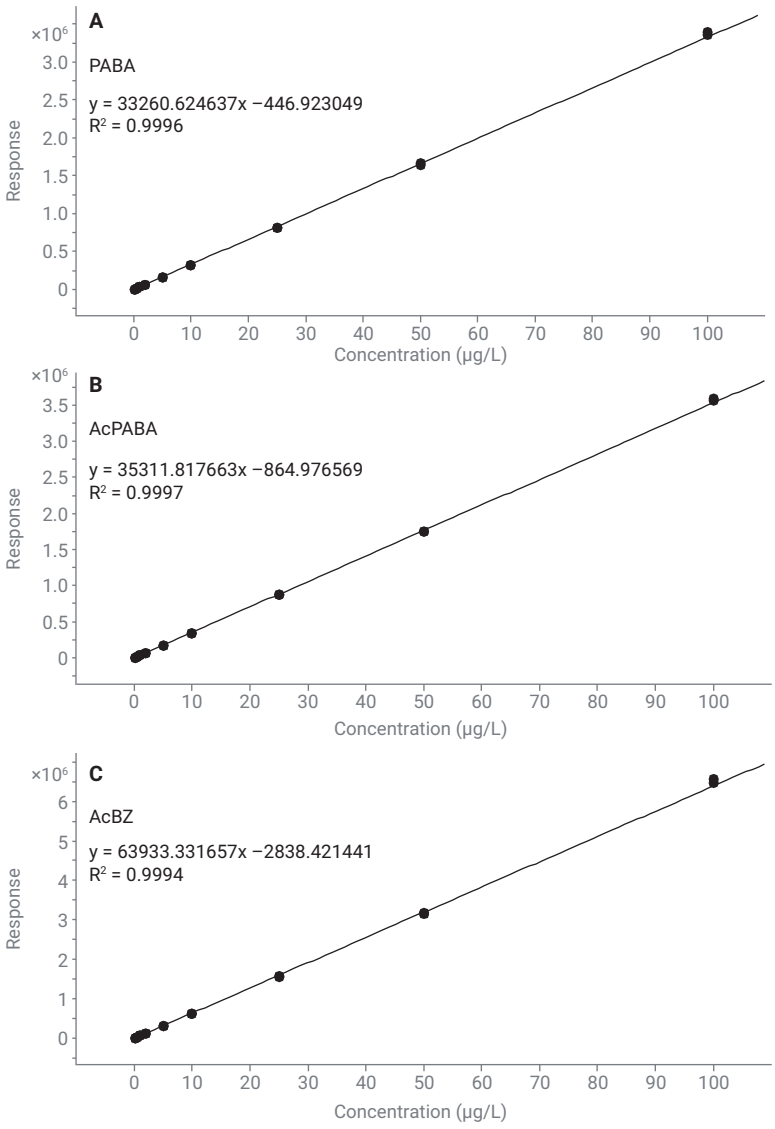


Figure 4. Calibration curves of PABA, AcPABA, and AcBZ from 0.1 to 100 µg/L.

Sensitivity

Instrument detection limits (IDLs) and method detection limits (MDLs) were evaluated based on seven consecutive injections of the lowest calibration standard (Cal 1 = 0.1 µg/L).

Parts-per-trillion (ppt) level IDLs and MDLs were achieved for all three analytes (Table 4). Response RSD values ≤ 2.7% at the lowest calibration level confirm outstanding instrument precision (Figure 5). These MDLs correspond to tissue-equivalent concentrations of approximately 0.02 to 0.05 µg/kg, providing laboratories with substantial sensitivity headroom for unambiguous compliance determination.

Matrix effect evaluation

Matrix effects (ME%) were evaluated by comparing the response of post-extraction spiked samples to that of neat standards at equivalent concentrations.

Equation 1.

$$ME\% = 100 \times \frac{\text{Response in post-extraction spike}}{\text{Response in neat standard}}$$

All ME% values fell within the 80 to 120% threshold, indicating minimal matrix effect and confirming the effectiveness of the Captiva EMR Lipid cleanup for selective removal of lipids and other hydrophobic interferences from raw fish extracts.

Table 4. Summary of compound sensitivity based on seven replicate injections of the calibration standard at level 1 (0.1 µg/L).

Compound	RT (min)	IDL (µg/L)	MDL (µg/L)	S/N	Response RSD (%) (n = 7)
AcBZ	6.48	0.004	0.01	372.3	1.3
AcPABA	3.78	0.008	0.02	120.4	2.7
PABA	3.02	0.008	0.02	117.2	2.5

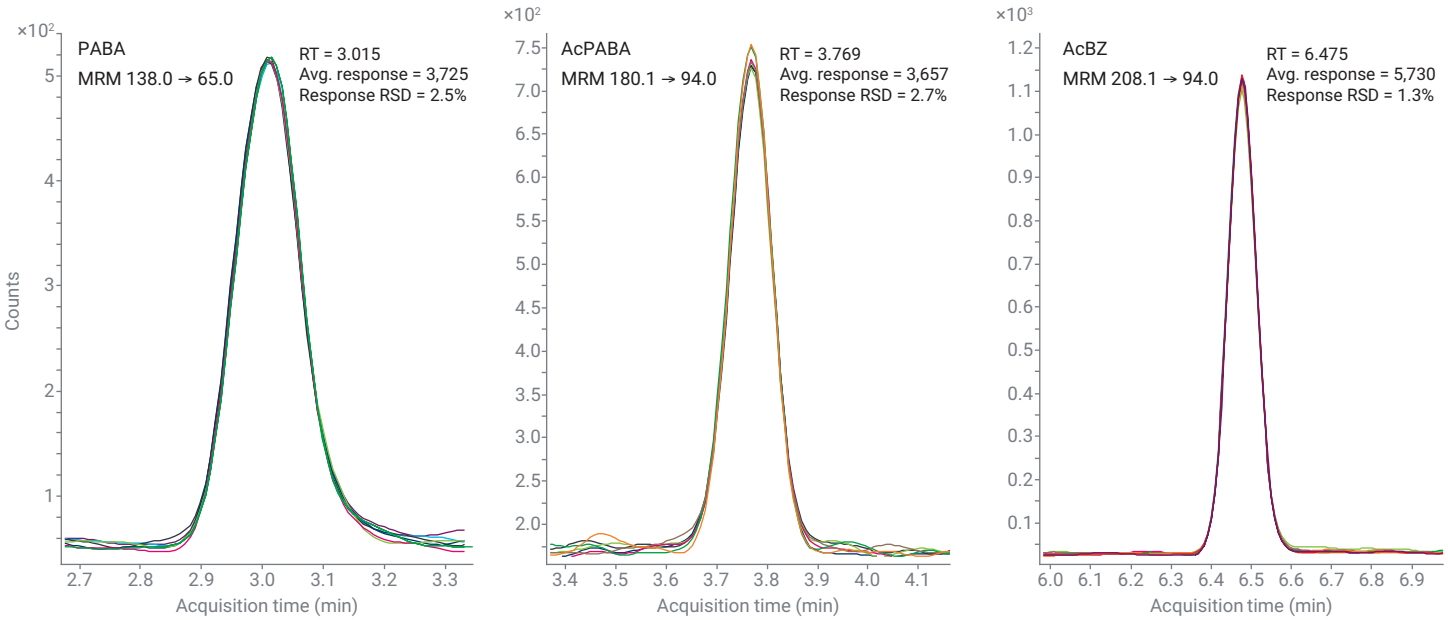


Figure 5. MRM chromatogram overlay of seven consecutive injections at a concentration of 0.1 µg/L.

Matrix-spiked recovery and repeatability

Recovery was evaluated at three QC levels (1, 5, and 10 µg/kg) in seabass, tilapia, and salmon (n = 4 per level per matrix). Recoveries within 80 to 120% (Figure 6) were achieved across all QC levels and in all three fish matrices, confirming method accuracy and the effectiveness of the QuEChERS plus EMR–Lipid workflow.

Repeatability was assessed as the %RSD of recoveries across replicate preparations and injections (n = 4). Excellent repeatability (< 3% RSD) was achieved for all QC samples (Figure 7), with the exception of PABA at QC1 (1 µg/kg) in salmon (7%), which is still well within the CODEX ≤ 20% criterion and likely reflects the challenge of quantifying the most polar analyte at the lowest spike level in the highest-fat matrix.

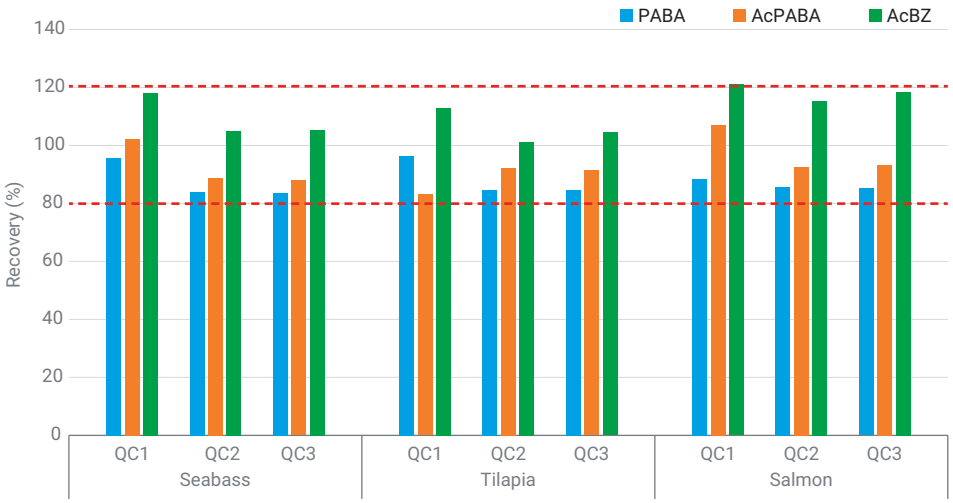


Figure 6. Matrix-spiked recoveries for PABA, AcPABA, and AcBZ in seabass, tilapia, and salmon at three QC levels.

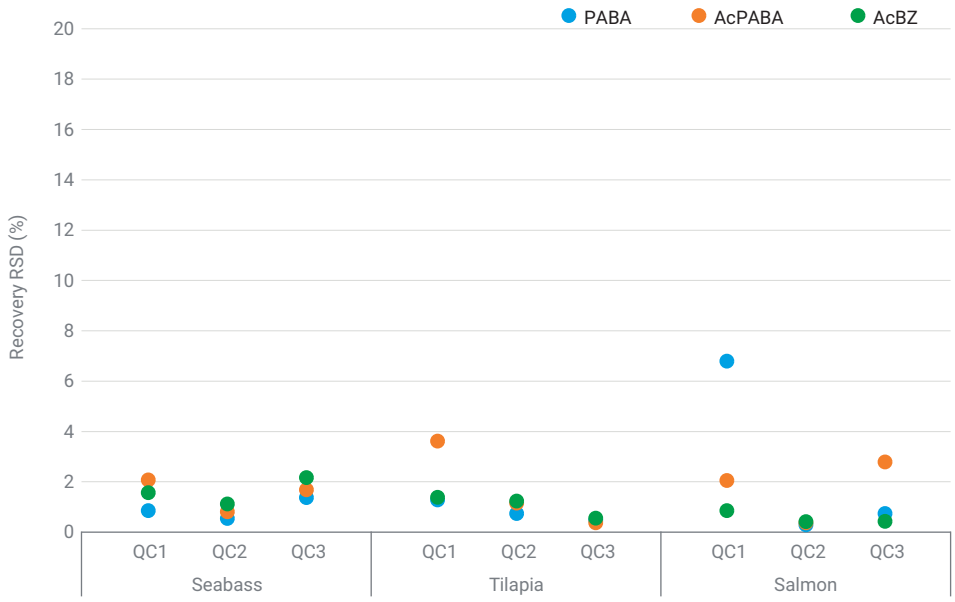


Figure 7. Recovery repeatability of PABA, AcPABA, and AcBZ in seabass, tilapia, and salmon at three QC levels.

Conclusion

This study demonstrates the successful development and validation of an end-to-end Agilent workflow for the quantitative determination of benzocaine metabolites (PABA, AcPABA, and AcBZ) in fish tissue using an Agilent 6495D triple quadrupole LC/MS system with an Agilent 1290 Infinity III LC.

Key findings include

- **Streamlined sample preparation extraction:** 30 minutes per batch with no evaporation or reconstitution, saving time and reducing labor.
- **Highly effective matrix removal:** ME% values of 93 to 110% confirm minimal ion suppression or enhancement after EMR cleanup of fatty fish matrices.
- **Excellent calibration linearity:** $R^2 > 0.999$ for all analytes over three orders of magnitude (0.1 to 100 µg/L).
- **Outstanding sensitivity:** ppt-level MDLs (0.01 to 0.02 µg/L)
- **High accuracy:** Matrix-spiked recoveries within 80 to120% across three fish species at three QC levels.
- **Robust workflow:** %RSD of recoveries < 3% for sample-to-sample preparation, meeting CODEX validation criteria.

The combination of Agilent Bond Elut QuEChERS extraction kits, Captiva EMR–Lipid cartridges, an Altura ZORBAX Eclipse Plus C18 column, and the 6495D LC/TQ system provides a robust, production-ready analytical solution for routine monitoring of caine-based anesthetics in aquaculture products.

References

1. Priborsky, J.; Velisek, J. A Review of Three Commonly Used Fish Anesthetics. *Reviews in Fisheries Science & Aquaculture* **2018**, *26(4)*, 417–442. DOI: 10.1080/23308249.2018.1442812
2. Lin, S.; Qiu, W.; Hua, Y.; Yang, Y. Rapid Determination of Caine-Based Anesthetics and Their Metabolite Residues in Fish Using a Modified QuEChERS Method Coupled with UPLC-MS/MS. *Food Chemistry: X* **2024**, *24*, 102032. DOI: 10.1016/j.fochx.2024.102032

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