# Determination of Azaspiracids in Edible Shellfish by a Modified QuEChERS Method Coupled with Ultra High Performance Liquid Chromatography Triple Quadrupole Mass Spectrometry*

Application Note


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## Abstract

A method has been developed for the detection of azaspiracids in shellfish. This method uses modified QuEChERS for sample preparation. The separation and quantitation was accomplished with an Agilent 1290 Infinity LC system coupled with an Agilent 6460 Triple Quadrupole LC/MS. The study outlined in this application note demonstrates that the method is easy to operate, rapid, and reliable, providing separation and detection at levels that are well below those defined by regulation.



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## Introduction

Marine biotoxins are one of the major focuses in food safety globally, particularly in coastal countries. Marine food products in China are subject to contamination of marine biotoxins. In recent years, phytoplankton blooms have often been observed in inner sea of China caused by the industrialization and eutrophication of bodies of water. Large-scale mariculture of aqueous species, especially shellfish, which accounts for 40.7% of total yields of maricultural products in China, has further worsened the phytoplankton boom. Protoperidinium, one type of phytoplankton, is widely distributed with various subtypes in China seas, and some sub-types of protoperidinium can excrete azaspiracids (AZAs), a class of marine biotoxins which were selected as the target compounds on which to research. Bioaccumulation, transformation, and metabolization of these marine toxins within aquatic species and the consumption of edible shellfish from contaminated waters may pose severe threats to human health due to their very high toxicity. The provisional reference dose for AZAs is as low as 0.04 µg/kg BW [1]. Currently, the European Union regulates total AZAs with a maximum residual level of 160 µg/kg in shellfish [2]. China is on the way to establish its own regulation of marine biotoxins, especially for those urgently demanded ones such as AZAs. Commonly used methods to detect AZAs are a mouse bioassay and LC/MS/MS techniques. However, LC/MS/MS has shown superior selectivity, sensitivity, and accuracy over the mouse bioassay, and many consider LC/MS/MS approaches to be less complex and time consuming to implement as well as less prone to producing false negative results. The objective of this study is to establish an easy, sensitive, and rapid method using LC/MS/MS for routine monitoring of the three most toxic AZAs (AZA1, AZA2, AZA3) in various shellfish such as mussels, oysters, clams and scallops, to support reference method development in China.

# Experimental

## Sample preparation

Samples were prepared according to the process described in Figure 1 [3]. The samples were homogenized and extracted with 85% acetonitrile in water in the presence of 5 g of $MgSO_4$ and 2 g of NaCl. The resultant extracts were cleaned up by C18 sorbent followed by rotary evaporation for drying. The residues were further dissolved in 80% acetonitrile/water and filtered through a membrane sequentially. Separation was performed by gradient elution using acetonitrile/water. AZAs containing carbonyl groups and ether oxygen ligands (Figure 2) were then detected using positive electrospray ionization (ESI+) followed by multiple reaction monitoring (MRM).

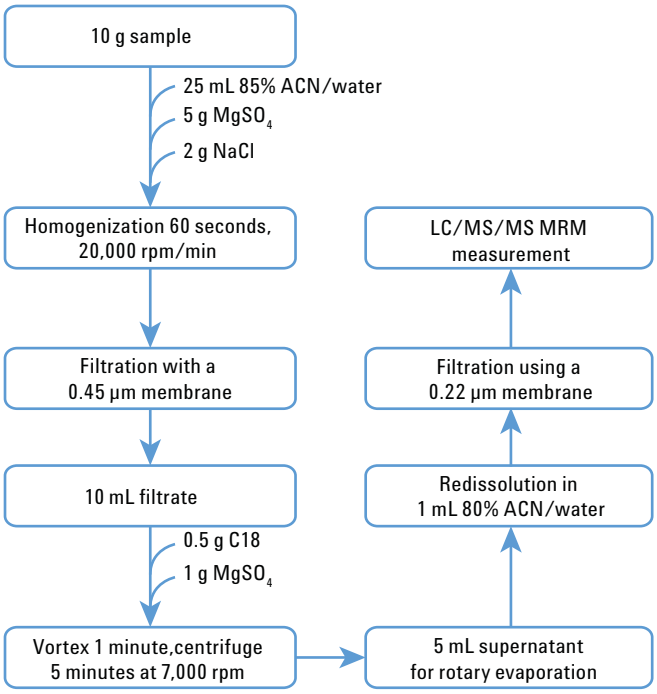
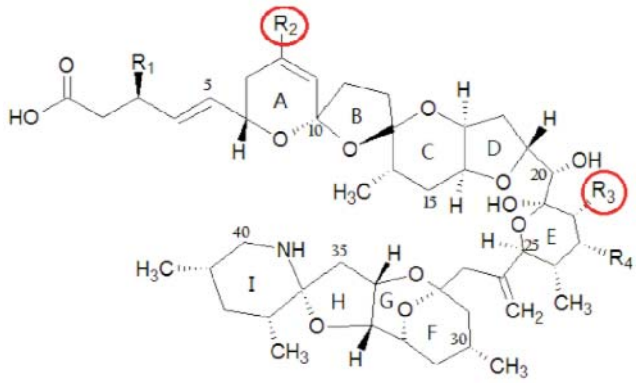



*Figure 1. Process for the analysis of AZAs in edible shellfish.*



| AZAs | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| AZA-1 | H | H | $CH_3$ | H |
| AZA-2 | H | $CH_3$ | $CH_3$ | H |
| AZA-3 | H | H | H | H |

*Figure 2. Chemical structures of AZAs.*

The detailed LC and MS conditions are listed in Table 1.

*Table 1. Instrument Conditions*

| | |
|---|---|
| **LC conditions** | |
| Instrument | Agilent 1290 Infinity LC System with built-in degasser |
| Autosampler | Agilent 1290 Infinity Autosampler with temperature control |
| Column temperature | 1290 Infinity Thermostatted Column Compartment |
| Column | Agilent ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm |
| Column temperature | 40 °C |
| Mobile phase | Solvent A) 0.1% formic acid/5 mM ammonium acetate in water; Solvent B) acetonitrile |
| Flow rate | 0.4 mL/min |
| Injection volume | 5 µL |
| Post time | 1 min |
| Gradient elution profile | 0–1 minutes, %B increasing from 20% to 50%<br>1–6 minutes, %B increasing from 50% to 90%<br>6–7 minutes, %B maintained at 90%<br>7–7.5 minutes, %B decreasing from 90% to 20% |
| **ESI-MS/MS conditions** | |
| Instrument | Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream electrospray ionization source |
| Drying gas temperature | 300 °C |
| Drying gas flow rate | 6 L/min |
| Nebulizer gas pressure | 45 psi |
| Sheath gas temperature | 300 °C |
| Sheath gas flow rate | 11 L/min |
| Capillary voltage | 3,500 V (positive) |
| Nozzle voltage | 400 V (positive) |

# Results and Discussion

## Separation of three AZAs

The MRM transition parameters listed in Table 2 were initially optimized for each compound to achieve high detection sensitivity. Using an acetonitrile/water mobile phase with 0.1% formic acid and 5 mM ammonium acetate as a modifier in the aqueous phase, the resulting chromatogram demonstrates baseline separation of the three compounds (Figure 3).

*Table 2. MRM Parameters for Monitoring AZAs*

| Compound name | Precursor ion | Product ion | Fragmentor vol. (V) | Collision energy (V) | Ret. time (min) |
|---|---|---|---|---|---|
| AZA-1 | 842.5 | 824.5* | 190 | 45 | 3.753 |
| | | 806.6 | 190 | 50 | |
| AZA-2 | 856.5 | 838.5* | 210 | 45 | 4.019 |
| | | 820.4 | 210 | 50 | |
| AZA-3 | 828.4 | 810.4* | 200 | 40 | 3.301 |
| | | 792.4 | 200 | 45 | |

*Quantification ion

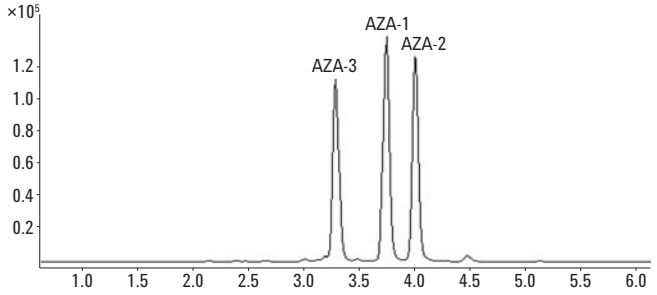



*Figure 3. Typical TIC MRM chromatogram of AZAs.*

## Effect of extraction conditions on the recovery

The conditions for extraction of AZAs were investigated, including the extraction solution, extraction method, extraction time, and temperature. It was found that the highest recovery is achieved by using an 85% acetonitrile and water solution to extract the sample by homogeneous mixing for 60 seconds at room temperature (Table 3). Therefore, such conditions were selected as optimal for the extraction of target compounds.

*Table 3. Comparison of Recoveries Under Various Extraction Conditions*

| | | Oysters spiked 50 µg/kg AZAs | | |
|---|---|---|---|---|
| **Sample compound** | **Solvents** | **AZA-1** | **AZA-2** | **AZA-3** |
| Extraction solution | Acetic ether | 43.5 | 30.9 | 34.4 |
| | Methanol | 37.2 | 33.1 | 32.9 |
| | Acetonitrile | 44.5 | 40.9 | 39.3 |
| | **ACN-Water** | **44.9** | **42.1** | **38.5** |
| Extraction method | Shaking | 32.2 | 29.8 | 27.3 |
| | Ultrasonic | 29.5 | 22.3 | 19.8 |
| | **Homogenization/ Ultraturrax/ Dispersing** | **44.2** | **45.1** | **40.0** |
| Extraction time (sec) | 20 | 22.5 | 26.9 | 25.1 |
| | **60** | **43.2** | **40.9** | **42.6** |
| | 120 | 44.0 | 41.5 | 41.9 |
| Extraction temperature (°C) | **RT** | **41.5** | **40.9** | **40.2** |
| | 40 | 41.4 | 41.2 | 40.5 |
| | 70 | 40.2 | 39.9 | 41.5 |

## Effect of salt on the extraction efficiency using QuECheERS method

Magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) are commonly added to an extraction solution in the QuEChERS method to improve the extraction efficiency. Sodium chloride can decrease the target compound's distribution in the aqueous phase, while $MgSO_4$ can absorb water efficiently. As a result, the addition of both can enhance the distribution of analytes in the organic phase, and improve extraction efficiency. As shown in Figures 4A and 4B, 5 g of $MgSO_4$ and 2 g of NaCl can provide highest extraction efficiency for the four types of shellfish examined, including mussels, oysters, clams, and scallops.

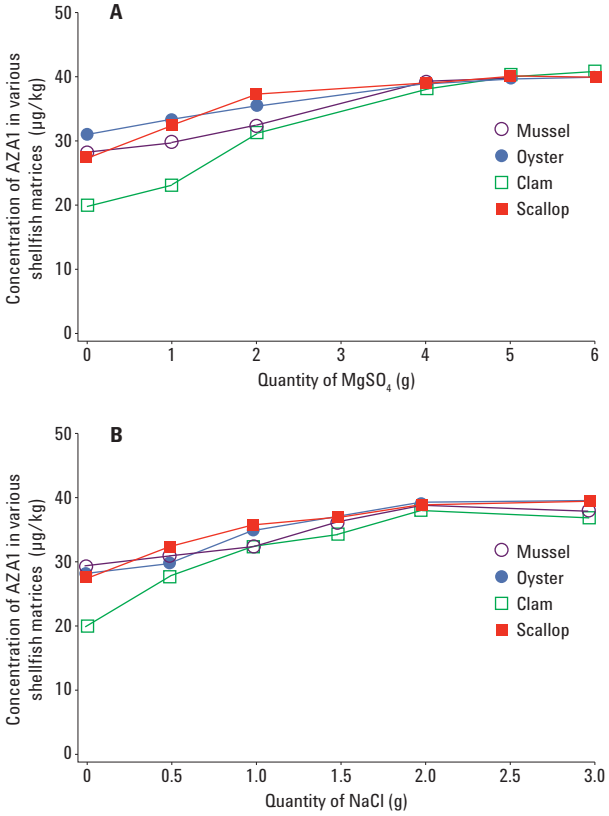



*Figure 4. Effect of $MgSO_4$ (A) and NaCl (B) on extraction recovery of AZA1.*

## Selection of cleaning sorbents

Florisil, C18, PSA, and GCB were tested as sorbents to clean up the extractants. It was found that florisil, as a polar sorbent, has difficulty adsorbing the lipid components from the matrices and leads to lower recovery of AZAs in the shellfish matrices. GCB, due to its strong adsorbance to the compounds with planar rings, strongly adsorbs AZAs and resulted in low recovery. PSA as a basic sorbent, can interact with the acidic compounds of AZAs and lead to low recovery.
In comparison, C18 was found to remove lipid and carbohydrates efficiently, however, too much C18 decreases the recovery. After several experimental trials, the best recovery was achieved when 1 g of C18 per 20 g of sample in the presence of 1 g of $MgSO_4$ was used as the cleanup sorbent.

## Confirmation of compounds with the ratio of qual/quant MRM transitions

Both qualitative MRM and quantitative MRM transitions were compared for 1 µg/kg AZAs spiked in scallop matrix. As shown in Figure 5, the ratio of qualitative over quantitative MRM transitions ranged from 100.4% to 104.4%, suggesting the correct identification of the analytes.

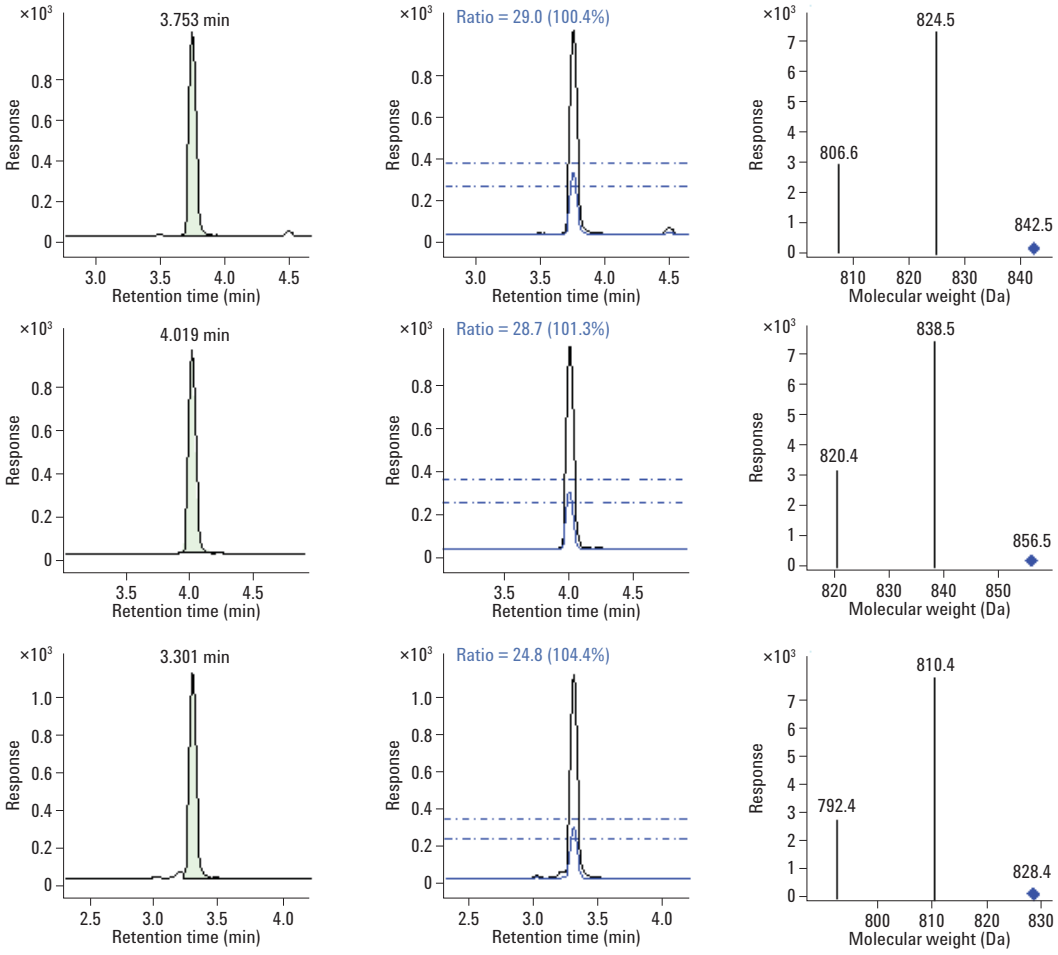



*Figure 5. Comparison of qualitative and quantitative MRM transitions for the AZAs spiked in scallop matrix at a level of 1 µg /kg.*

## Method performance

Matrix-matched calibration curves were established for AZAs in each matrix respectively. Excellent linearity was achieved in the spiked concentration range with correlation coefficients of 0.996 or above. The LOQ for each compound was determined at 1.0 µg/kg. Representative performance in scallop matrix is shown in Table 4. Spiking of AZAs in the mixed shellfish matrices at the level of 10, 20, and 50 µg/kg demonstrated that the overall recovery ranged from 71–108%, with RSD of 4.69–7.81%, suggesting that the method is accurate and precise (Table 5).

## Repeatability, reproducibility, and recovery

The recovery and precision were also examined within the same day and between days. As shown in Table 6, this method provides excellent recovery and precision.

*Table 4. Linearity and LOQ of AZAs in Scallop Matrix*

| Compound | Spiked range (µg/kg) | Calibration equation | Correlation coef. ($R^2$) | LOQ (µg/kg) |
|---|---|---|---|---|
| AZA-1 | 1–100 | Y = 5,108.97x + 2,086.41 | 0.996 | 1.0 |
| AZA-2 | 1–100 | Y = 4,601.08x – 1,459.56 | 0.997 | 1.0 |
| AZA-3 | 1–100 | Y = 4,478.17x + 1,185.58 | 0.996 | 1.0 |

*Table 5. Spiking Recovery and Precision of AZAs in Mixed Shellfish Matrices with Six Replicates*

| Compound | Spiked (µg/kg) | Recovery (%) | Max. RSD (%) |
|---|---|---|---|
| AZA-1 | 10, 20, 50 | 74–108 | 5.77 |
| AZA-2 | 10, 20, 50 | 71–102 | 7.81 |
| AZA-3 | 10, 20, 50 | 78–107 | 4.69 |

*Table 6. Recovery and Precision for Intra-Day and Inter-Day Analysis by Spiking 20 µg/kg in the Mixed Matrices with Six Replicates*

| | | Day 1 10:00 | | Day 1 18:00 | | Day 2 10:00 | |
|---|---|---|---|---|---|---|---|
| Analyte | Conc. (µg/kg) | Rec (%) | RSD (%) | Rec (%) | RSD (%) | Rec (%) | RSD (%) |
| AZA-1 | 20 | 96.3 | 8.6 | 89.6 | 7.2 | 92.4 | 7.5 |
| AZA-2 | 20 | 86.5 | 6.4 | 88.2 | 9.4 | 93.5 | 5.4 |
| AZA-3 | 20 | 95.5 | 7.0 | 90.8 | 6.1 | 88.3 | 7.9 |

## Real sample screening

Seventeen samples including scallop, mussel, oyster, and geoduck were tested. AZAs were detected in seven of the samples (Table 7). Among them, three samples contained AZAs level higher than 10 µg/kg, which is still lower than the current regulated level of 160 µg/kg.

# Conclusions

This study describes a method for the detection of three azaspiracids in various shellfish using optimized QuEChERS sample preparation in combination with LC/MS/MS in MRM mode. The established method is proven to be highly sensitive, with an LOQ of 1 µg/kg for each AZA. It demonstrated excellent linearity using matrix-matched calibration curves at the examined spiking range of 1–100 µg/kg with $R^2 > 0.99$. The recovery and precision were within 71–108% and below 10%, respectively. The method is easy to operate, rapid, and reliable, and thus was sufficient for screening AZAs in real samples such as blue mussels, oysters, geoducks, and scallops.

*Table 7. Levels of AZAs in Seventeen Samples from the Local Market*

| Sample | AZA1 (µg/kg) | AZA2 (µg/kg) | AZA3 (µg/kg) |
|---|---|---|---|
| Scallop-1 | 11.2 | 1.8 | – |
| Scallop-2 | – | – | – |
| Scallop-3 | 2.5 | – | – |
| Mussel-1 | – | – | – |
| Mussel-2 | 4.1 | 1.5 | – |
| Mussel-3 (imported) | 2.8 | – | – |
| Oyster-1 | – | – | – |
| Oyster-2 | – | – | – |
| Oyster-3 (imported) | 15.2 | 4.5 | 3.1 |
| Geoduck-1 (imported) | – | – | – |
| Geoduck-2 (imported) | 2.6 | – | – |
| Geoduck-3 (imported) | – | – | – |
| Mussel canned products | – | – | – |
| Oyster canned products | – | – | – |
| Dried scallop-1 | – | – | – |
| Dried scallop-2 | 18.3 | 10.5 | – |
| Dried scallop-3 | – | – | – |

## References


1. H. Toyofuku *Marine Pollution Bull*., 2006, **52**(12):1735.
2. European Commission, Commission Decision 2002/225/EC *J. Eur.Commun*, 2002, **62**.
3. K. Ofuji, M. Satake, T. McMahon, *et al*. *Biosci. Biotechnol. Biochem.*, 2001, **65**:740.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