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Application News

The Determination Method of the Lipophilic Marine Biotoxins in Bivalve Subject to EU Regulations Using a Triple Quadrupole Mass Spectrometer

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User Benefits

- In compliance with procedures issued by the EU Reference Laboratory for Marine Biotoxins (EU-RL-MB), simultaneous analysis of five lipophilic marine biotoxins in bivalves is possible.
- Yessotoxin (YTX) could be analyzed with high sensitivity using a basic mobile phase.
- Matrix-matched calibration curves could be automatically created using the pre-processing function of the autosampler.

■ Introduction

Shellfish poisoning mainly occurs when bivalves feed on highly toxic marine dinoflagellates and these toxins accumulate in the body of the bivalve. Eating these poisonous shellfish can cause poisoning syndromes in humans.

The EU has established limits for lipophilic marine biotoxins. Regulation (EC) 853/2004, Annex III, Section VIII, Chapter V prescribes a maximum concentration of lipophilic marine biotoxins in bivalves intended for human consumption of 160 µg/kg (okadaic acid equivalent) for okadaic acid, dinophysistoxins, and pectenotoxins, and 160 µg/kg (azaspiracid equivalent) for azaspiracids. In addition, Regulation (EU) 786/2013, Annex III reduced the permissible level of yessotoxin in live bivalves to 3.75 mg/kg (yessotoxin equivalent). Regulation (EU) No. 15/2011 recognizes an EU-RL LC-MS/MS method as the standard method for detecting lipophilic marine biotoxins in live bivalves.

In Japan, safety standards and regulatory limits for shellfish toxins are established based on the Food Sanitation Act (Shokuan Notice No. 0306-1, dated March 6, 2015). For diarrheal shellfish toxins, the amount of toxin present in the edible parts of bivalves is restricted to 0.16 mg okadaic acid equivalent/kg and below. Shokuanki Notice No. 0306-3, dated March 6, 2015, specifies an instrument-based testing method for detecting okadaic acid and dinophysistoxins that uses an acidic mobile phase, which is the testing method in current use.

This article describes an LC-MS/MS method that uses a basic mobile phase and an analysis of samples extracted from scallops spiked with known amounts of five standard toxins. The five toxins have been chosen to represent the five compounds/chemical groups of lipophilic marine biotoxins cited in EU regulations (okadaic acid, dinophysistoxins, pectenotoxins, azaspiracids, and yessotoxins). The procedures used during extraction and analysis follow the methods of the EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS Ver. 5[1]. In order to determine total amounts of okadaic acid and dinophysistoxins, a hydrolysis step is needed after sample extraction. In this study, scallop extract solutions were prepared with and without this hydrolysis step, then matrix-matched calibration curves were prepared and quantitative analysis was performed on both types of samples.

■ Compounds and Structures

Simultaneous analysis was performed on five compounds (okadaic acid [OA], dinophysistoxin-1 [DTX1], pectenotoxin-2 [PTX2], azaspiracid-1 [AZA1] and yessotoxin [YTX]).

Fig. 1 shows the structure of YTX.

Yessotoxins are a group of lipophilic sulfur-bearing polyether toxins that are related to ciguatoxins.

Yessotoxins are produced by dinoflagellates, in particular *Lingulodinium polyedrum* and *Gonyaulax spinifera*.

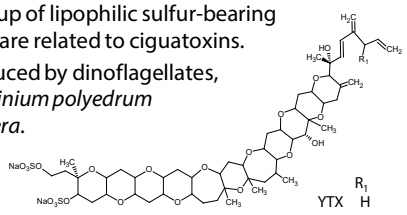


Fig. 1 YTX Structure

■ MRM Transitions

The MRM transitions used in this analysis are shown in Table 1. OA, DTX1, and YTX were detected in negative ion mode while PTX2 and AZA1 were detected in positive ion mode. The precursor ion used for PTX2 was $[M+NH_4]^+$, which provides good sensitivity.

Table 1 MRM Transitions

Compound	Polarity	Q1	Q3	CE
		m/z	*m/z*	(V)
OA	-	803.5	255.2	48
OA	-	803.5	113.0	55
DTX1	-	817.5	255.2	49
DTX1	-	817.5	113.0	55
YTX	-	1141.5	1061.3	33
YTX	-	1141.5	855.5	54
PTX2	+	876.5	823.5	-26
PTX2	+	876.5	805.4	-27
PTX2	+	876.5	213.2	-40
AZA1	+	842.5	824.4	-31
AZA1	+	842.5	806.4	-40

■ Analytical Conditions

LC and MS analytical conditions are shown in Table 2.

Table 2 Analytical Conditions

[HPLC conditions] (Nexera™)	
Column	: ODS column resistant to high pH *1 (100 mm × 2.1 mm, 3.5 µm)
Mobile Phases	: A) 2 mmol/L ammonium bicarbonate aqueous solution (pH 11) B) Acetonitrile: 2 mmol/L ammonium bicarbonate aqueous solution (pH 11) = 9:1
Flowrate	: 0.3 mL/min
Column Temp.	: 40 °C
Injection Volume	: 5 µL
Time Program	: B Conc. 25 % (0 - 1 min) – B Conc. 100 % (11.4 - 16.7 min) – B Conc. 25 % (16.71 - 22 min)
The flow was loaded into the MS detector between 2.5 to 11 min using a flow switching valve.	
[MS conditions] (LCMS™-8060)	
Ionization	: ESI Positive & Negative
Interface Voltage	: +4 kV & -3 kV
Nebulizing Gas Flow	: 2.5 L/min
Heating Gas Flow	: 5 L/min
Drying Gas Flow	: 15 L/min
IF/DL/HB Temp.	: 350/200/450 °C
CID Gas Pressure	: 270 kPa
ESI Probe Position	: +2 mm

*1 See Reference 1

■ Chromatograms

Using the sample preparation function on the autosampler, 5 µL of scallop extract solution was aspirated as the matrix solution, the same volume (5 µL) of mixed standard solution was aspirated at various concentrations, and both solutions were injected. For actual samples (standard-spiked scallop extract solution), methanol solvent was aspirated in place of the mixed standard solution. Chromatograms of the minimum calibration curve point for each toxin are shown in Fig. 2. Scallop extract solutions without hydrolysis were used as the matrix solution for AZA1, PTX2, and YTX, and scallop extract solutions after hydrolysis were used as the matrix solution for OA and DTX1.

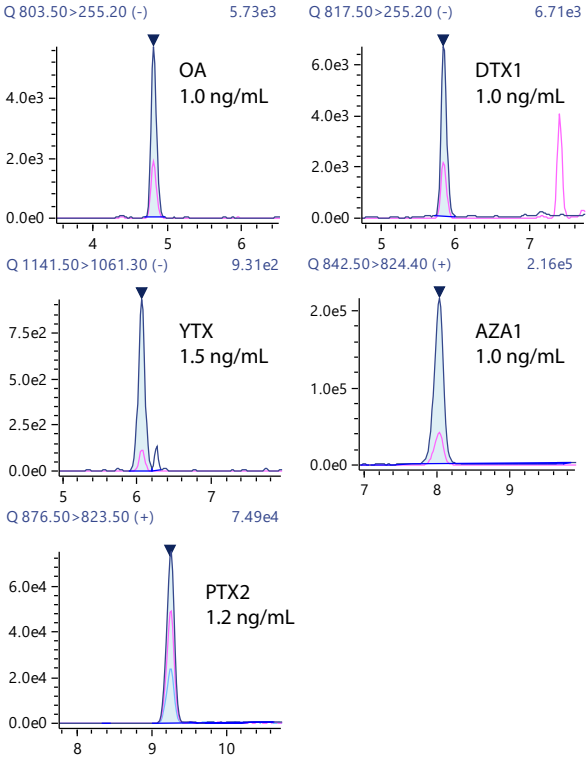
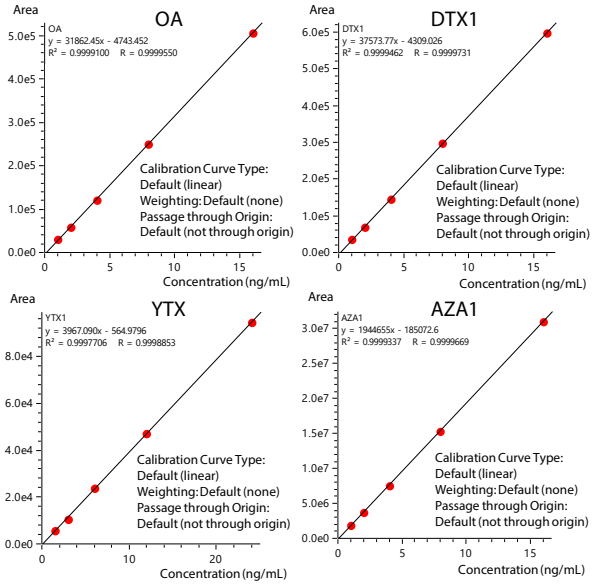


Fig. 2 Chromatograms of Each Toxin

■ Calibration Curves

Fig. 3 shows the calibration curve for each toxin.



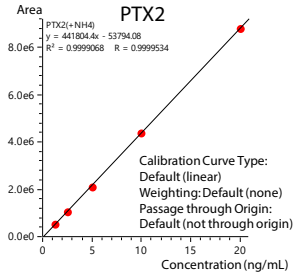


Fig. 3 Calibration Curves for Each Toxin

■ Quantitative Results

Mixed standard solution was added so the concentration of OA, DTX1, and AZA1 in the final scallop extract solution was 2 ng/mL, the concentration of YTX was 3 ng/mL, and the concentration of PTX2 was 2.5 ng/mL. The scallop extract solution was treated with hydrolysis to convert the acylated esters of OA and DTXs naturally present in shellfish into their free forms. This hydrolysis is known to break down PTX and AZA toxin groups.

Table 3 shows the coefficient of determination for matrix-matched calibration curves prepared using extract solutions with and without hydrolysis, and the quantitative results and recovery rates for standard-spiked scallop extract solutions quantified using these calibration curves. PTX2 and AZA1 were detected without hydrolysis but were made non-detectable (ND) by performing hydrolysis. Apart from PTX2 and AZA1, recovery rates were very good at 95 to 105 % whether with or without hydrolysis.

Table 3 Quantitative Results (ng/mL) and Recovery Rates (%)

Compounds	No Hydrolysis			With Hydrolysis		
	R^2	Quantitative Results (ng/mL)	Recovery Rate (%)	R^2	Quantitative Results (ng/mL)	Recovery Rate (%)
OA	0.99977	2.06	103	0.99991	1.94	97
DTX1	0.99995	1.89	95	0.99995	1.99	100
YTX	0.99977	3.09	103	0.99980	3.13	104
PTX2	0.99991	2.44	98	0.99987	N.D.	-
AZA1	0.99993	1.96	98	0.99970	N.D.	-

■ Conclusion

Simultaneous analysis is presented for five compounds (okadaic acid [OA], dinophysistoxin-1 [DTX1], pectenotoxin-2 [PTX2], azaspiracid-1 [AZA1] and yessotoxin [YTX]), which represent each of the five compounds/chemical groups of toxins cited by EU regulations: okadaic acid, dinophysistoxins, pectenotoxins, azaspiracids, and yessotoxins.

Analytical sensitivity, calibration curve linearity, and recovery of toxins from scallop samples were all confirmed to be excellent. Matrix-matched calibration curves were also created automatically and accurately by configuring the sample preparation function of the autosampler in the analysis method.

References

1) EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS, Version 5, January 2015

Acknowledgments

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