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High Sensitivity Analysis of Diarrhetic Shellfish Poisoning (DSP) Toxins Using Liquid Chromatography Tandem Mass Spectrometry

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Introduction

For the past decades marine toxins in shellfish have been monitored by the mouse bioassay (MBA) in many countries including Japan. Recently several alternative testing methods have been developed and a few of them have been validated. The most widely accepted method for many kinds of the marine toxins is liquid chromatography (LC) combined with mass spectrometry (MS), deemed to be the powerful tool than the MBA in sensitivity and accuracy. Diarrhetic shellfish poisoning (DSP) toxins, okadaic acid (OA) and dinophysistoxins (DTXs), are very important target for marine bio-toxin monitoring in Japan. The MBA for DSP toxin monitoring was replaced with a LC/MS/MS method on the new regulation issued in March 2015 in Japan (Notification No. 1 issued by the Dept. of Food Safety, Pharmaceutical and Food Safety Bureau, Japanese Ministry of Health, Labour and Welfare on March 6, 2015).
In this presentation, we demonstrate the developed LC/MS/MS methods for the screening of OA, dinophysistoxin1 (DTX1), pectenotoxin1, 2, 6 (PTX) and yessotoxin (YTX) as well as for the routinely quantification of OA and DTX1.

Materials & Methods

Sample Preparation (EU-RL-MB*1)

Table 1 Sample List

Sample	Food	Origin
1	The naturally contaminated midgut gland of scallops with toxins	Japanese National Research Institute of Fisheries Science
2	Midgut gland of scallops	Market
3	Oyster	Market

Step 1 : Sample Extraction

1. Homogenizing samples using a homogenizer
2. 2.00±0.05 g of homogenized sample accurately weighed
   ← Add 9 mL of methanol
3. Vortex mixing for 3 min
4. Centrifuge for 10 min. at 2000 g (at 20 °C)
5. Transfer the supernatant into 20 ml measuring flask
   ← Add 9 mL of methanol
6. Homoginize samples for 1 min again
7. Centrifuge for 10 min. at 2000 g (at 20 °C)
8. Dilute to the extract 20 ml with methanol
9. Filtrate sample with disposable filter*2 **(Extract 1)**
10. LC-MS/MS analysis for free OA and DTX

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Step 2 : Hydrolysis (In order to detect the total content of OA and DTX toxins)

1. Transfer 1 mL **Extract 1** into a 1.5 mL HPLC vial
   ↓ ← Add 125 µL of 2.5 mol/L sodium hydroxide aqueous solution
2. Vortex mixing for 0.5 min
   ↓
3. Heat the mixture at 76 °C for 40 min
   ↓
4. Cooling down to room temperature
   ↓ ← Add 125 µL HCl 2.5 M for neutralise
5. Vortex mixing for 0.5 min
   ↓
6. Filtrate sample with disposable filter*2 **(Extract 2)**
   ↓
7. LC-MS/MS analysis

*1 EU-Harmonised Standard Operating procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS Ver.4

*2 TORAST DISC 0.22 µm P/N GLCTD-PTFE1322

Standard Solutions

- The mixture of six standards solution (OA, DTX1, PTX1,PTX2,PTX6,YTX) was provided by courtesy of Dr. Toshiyuki Suzuki in the Japanese National Research Institute of Fisheries Science for the purpose of this research.
- The certified solutions for calibration of OA and DTX1 standards were purchased from a National Research Council Canada.
  [CRM-OA-c (Lot #20070328) CRM-DTX1 (Lot #20071024)]

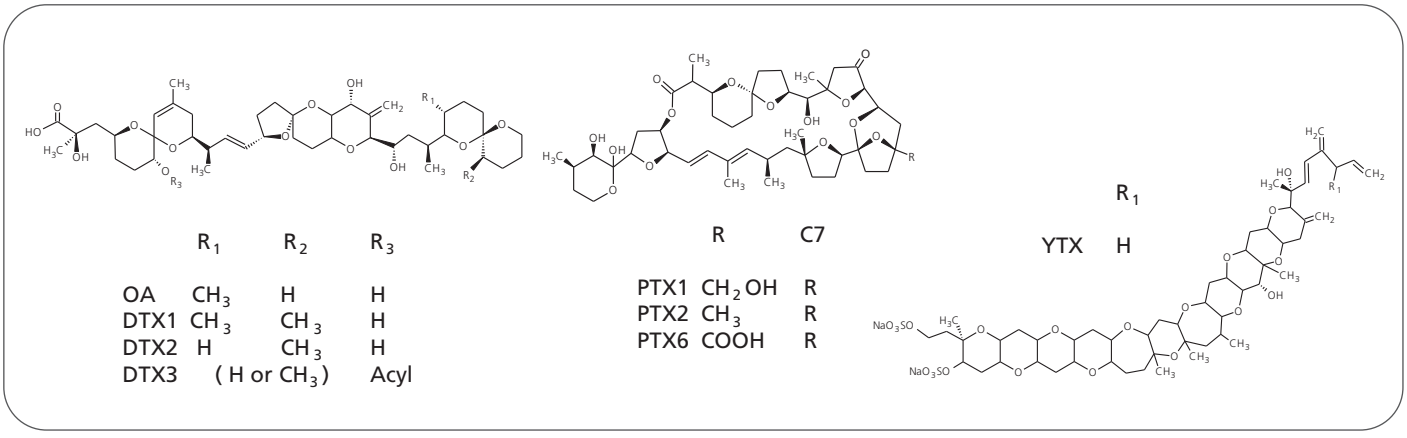

Figure 1. Structure of DSP toxins

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LC/MS/MS analysis

Table 2 Analytical Conditions

HPLC : Nexera UHPLC system	
Column	: L-column2 ODS (50 mmL. x 2.1 mm, 2 µm, CERI, Japan)
Mobile phase	: A - Aqueous solution of 2 mM ammonium formate with 50 mM formic acid B - Acetonitrile / Water : 95 / 5 (v/v) including 2 mM ammonium formate with 50 mM formic acid
Gradient program	: 30% B concentration (0 min) to 100% B concentration (5 to 10 min)
Flow rate	: 0.2 mL / min
Column temperature	: 20 °C
Injection volumn	: 5 µL
MS : LCMS-8050 Triple quadrupole mass spectrometer	
Ionization	: ESI (Negative)
Ion spray voltage	: -3.0 kV
Heating Gas Flow	: 10 L/min
Nebulizing Gas Flow	: 2 L/min
Drying Gas Flow	: 10 L/min
IF Temp.	: 350 °C
DL Temp.	: 150 °C
HB Temp.	: 450 °C
MRM	: OA : *m/z* 803.5>255.2 , *m/z* 803.5>113.0 DTX1 : *m/z* 817.5>255.2 , *m/z* 817.5>113.0 Dwell time 200 msec / Pause time 3 msec

Triple Quadrupole LC/MS/MS [LCMS-8050]

Result

Screening Analysis

- Figure 2(B) shows the MRM chromatograms of the sample1 acquired from the naturally contaminated scallops (Extract 1). Principle analytical condition is shown in Table 2 MRM transitions of PTX1,2,6 and YTX were in Figure 2. Six ingredients of sensitive screening analysis was successfully performed shown as Figure 2.

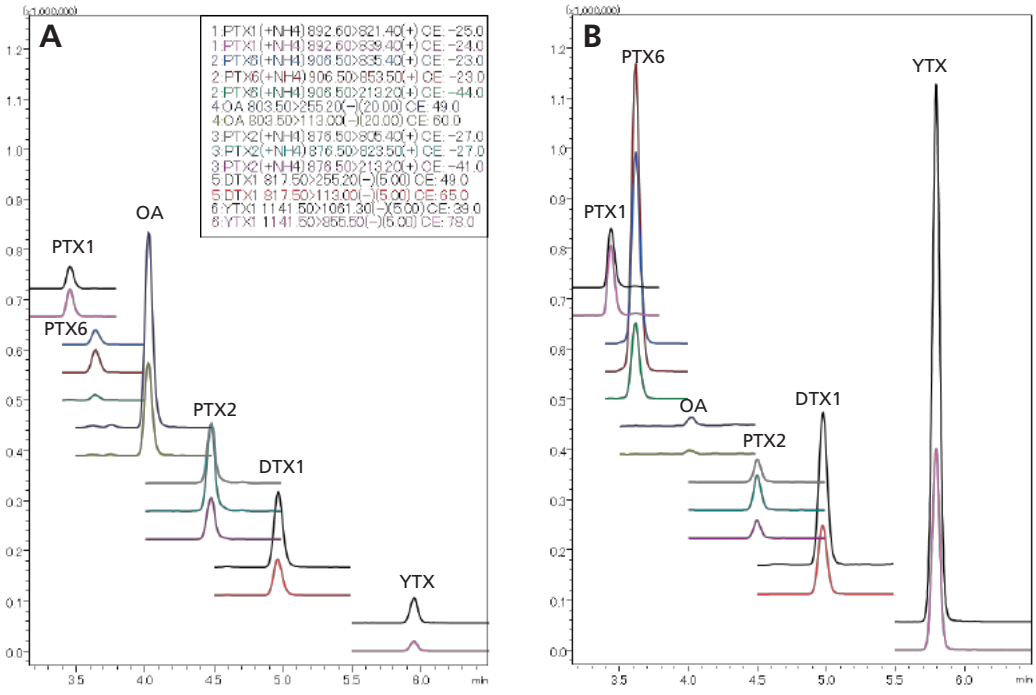

Figure 2. MRM chromatograms of DSP toxins
A) 10 ppb standard solution, B) The naturally contaminated midgut gland of scallops sample

MRM of OA and DTX1 standards

- Highly precise and sensitive DSP toxin analysis can be performed by LC/MS/MS.
- Linear calibration curves of both OA and DTX1 were established with a correlation coefficient (r^2) of 0.999 in the range 0.04 - 20 ppb.
- Repeatability of peak area of standard solutions were %RSD 4.2 (OA *m/z* 803.5>255.2) and 8.1 (DTX1 *m/z* 817.5>255.2) at 0.1 ppb (n=5).

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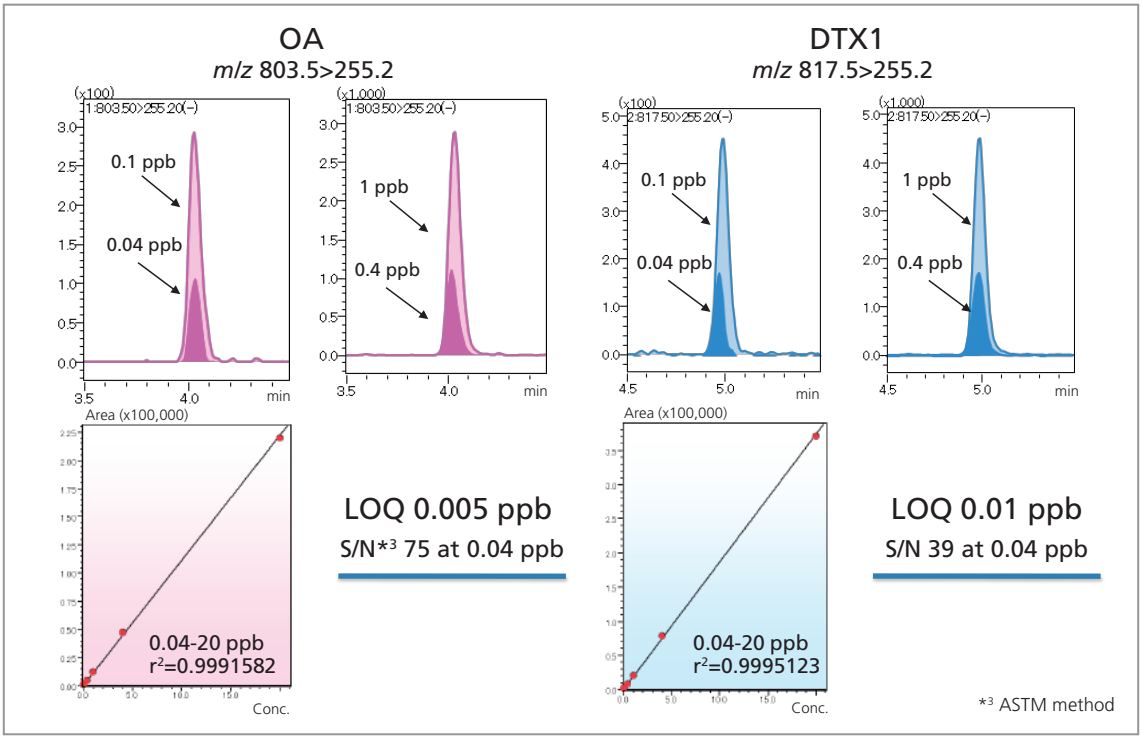

Figure 3. Calibration curves and MRM chromatograms of OA and DTX1

Recovery and Matrix Correction

- Convenient sample preparation is one of the advantage in this method.
- There is some consideration for a matrix effect.
- Correction due to the matrix effect should be estimated.
- QC parameter requires the response drift of 25% slope variation between the two sets of the calibration curve.
- In this study, the results of spiked 1 ppb of OA and DTX1 standard in the sample (2 and 3) are demonstrated with QC parameter of response drift within the 2 - 6%.
- According to the investigation, samples of 10- and 100-fold dilution can be recommended. Result of the recovery is shows in Figure 4.

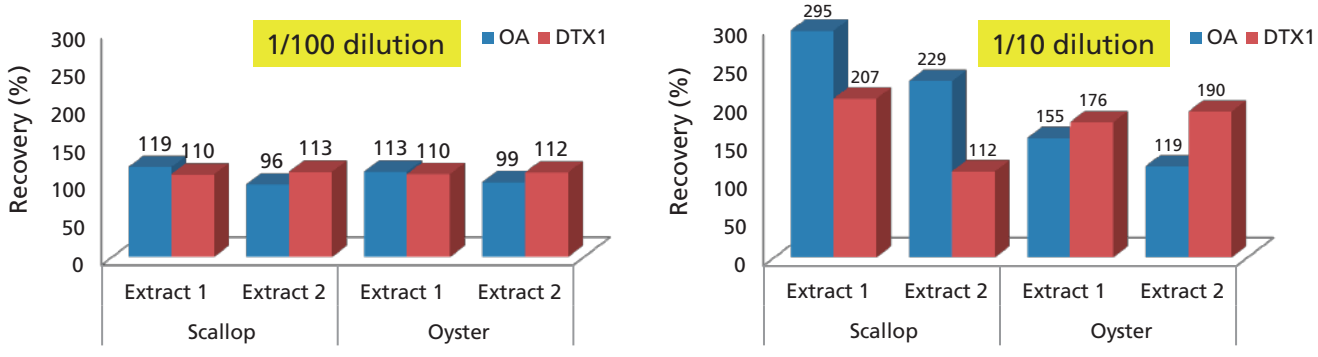

Figure 4. Recovery (1 ppb spiked)

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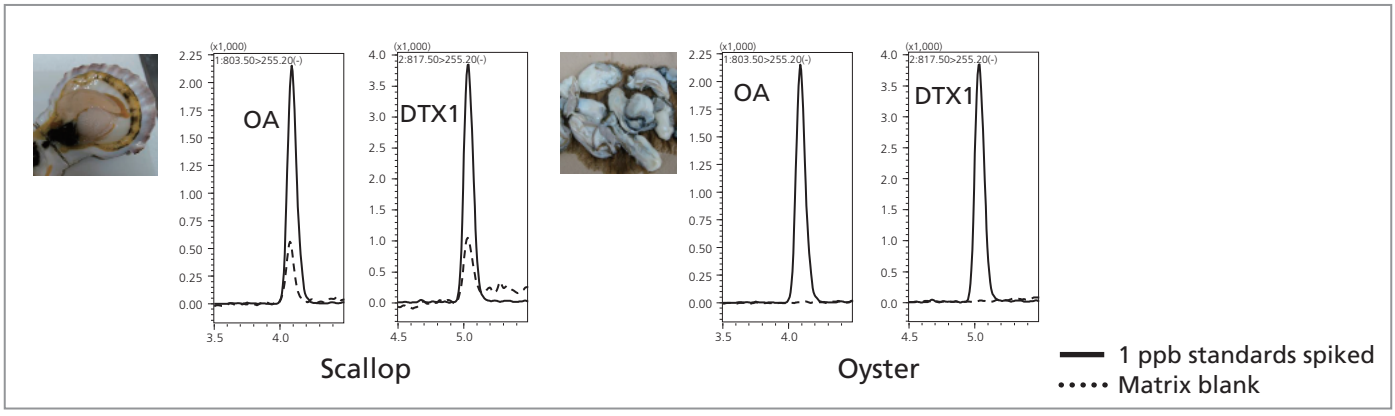

Figure 5. MRM chromatograms of 10 times dilution matrix sample (Extract 2)

Table 3 Quantity Results of Sample2 and Sample3 (µg/Kg)

Toxin	Sample 2 Scallop	Sample 3 Oyster
OA (ester)	17.4	N.D.
DTX1 (ester)	32.8	N.D.
DTX1 (free)	6.0	N.D.
	56.2 µg OA equivalent/Kg	-

N.D.:Not Detected

Good scallop dish!!

Conclusion

- LC/MS/MS is a powerful methodology for an analysis of DSP toxins.
- LCMS-8050 has highly precise and sensitivity features.
- LCMS-8050 can be applicable for the SOP of EU-RL-MB.
- OA and DTX1 was detected in the midgut gland of scallop bought at the market, but it was lower enough than the regulated value in Japan (160 µg OA equivalent/Kg).
- Further study, we would like to investigate the matrix removal by solid phase extraction.

Reference

- EU-Harmonized Standard Operating procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS Ver.4
- Toshiyuki SUZUKI and Michael A. QUILLIAM, *Anal. Sci.* 27 (2011) 571-584

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