SHIMADZU

TP 211

# Development of Sensitive and Selective Methods for Identification of Marine Toxins by Liquid Chromatography Tandem Mass Spectrometry

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## 1.Introduction

Over many decades, marine toxins have been monitored by the mouse bioassay (MBA) for the food safety purpose in many countries. In place of MBA, liquid chromatography (LC) with mass spectrometry (MS) is expected to use for the analysis of the marine toxins, deemed superior to the MBA in the point of sensitivity and accuracy.

Our purpose is establishment of the analytical conditions using Liquid Chromatography Tandem Mass Spectrometry for three groups of marine toxins of which each structure and property is different.

The target three groups of marine toxins are diarrhetic shellfish poisoning (DSP) toxins as okadaic acid (OA) and dinophysistoxins (DTX1 and DTX2) , ciguatera fish poisoning (CFP) as ciguatoxin 3C (CTX3C) , and globefish poisoning as tetrodotoxin (TTX).

Since globefish poisoning, tetrodotoxin (TTX) have been reported to be detected from the bivalves in a certain sea area near New Zealand and European coast, it's argued internationally to add TTX to the shellfish poisoning toxin.

| Paralytic Shellfish Poisoning (PSP) | Diarrheic Shellfish Poisoning (DSP) | Ciguatera Fish Poisoning (CFP) |
|---|---|---|
| Serious effects.<br>Fatal toxic symptoms. | Diarrhea and/or vomiting.<br>Not so serious conditions. | Fatal toxic symptoms<br>(in the limited area) |
| LC-MS/MS in Japan &EU | MBA in Japan<br>Fluorescence HPLC method in addition to MBA in EU and the USA (AOAC 2005.06 & 2011.02) | Review of regulatory frameworks |
| OA:0.16 mg OA eq/ kg ※1.<br>4 MU/g as MBA<br>STX 0.8 mg STX eq /kg※1<br>(as 2 HCl) | | |



※1 CODEX STAN 292-2008.

## 2. Experimental

### 2-1. Standard Solutions

Standard solutions of OA, DTX1 and DTX2 were purchased from NRC (Canada).
CRM-OA-c (Lot #20070328), CRM-DTX1 (Lot #20071024), CRM-DTX2 (Lot #20150819)
Standard of CTX3C and TTX were purchased from Wako Chemical Industry (JAPAN).
Ciguatoxin CTX-3C 100 ng, Wako Chemical # 030-21581
Tetrodotoxin TTX 1 mg, Wako Chemical # 206-11071
Methanol including 0.1% formic acid was used for dilution of standard mixture from above.
Each structure of marine toxins compound is shown in Figure 1 as below..

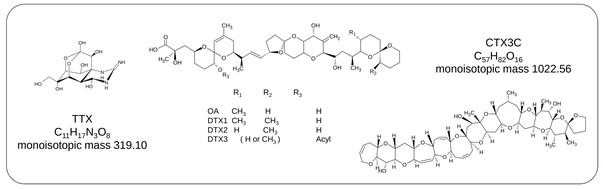


Figure 1. Structure of marine toxins

**LCMS-8050 Triple quadrupole mass spectrometer**

### 2-2. LC/MS/MS analysis

With the shift from the MBA toward to the instrumental method, the simultaneous analytical method of DSP and PSP has been eager to be utilized; however DSP is generally hydrophobic, while PSP mostly hydrophilic. It is relatively hard to analyze simultaneously both DSP and PSP with reversed phase mode.

Our purpose in this study is evaluation of potential analytical condition such as,
1) The simultaneous analytical method for DSP and PSP with a multi mode ODS column.
2) Reversed phase condition for DSP (acidic and neutral conditions)
3) Specified method for PSP, especially TTX with a HILIC mode column.

Table 1 Analytical Conditions

| | Condition | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| HPLC | Instrument | UHPLC Nexera X2 (Shimadzu) | | | |
| | Target compounds | TTX<br>OA, DTX1, DTX2, CTX3C | OA, DTX1, DTX2, CTX3C | OA, DTX1, DTX2, CTX3C | TTX |
| | Total run time (min) | 40 | 17.5 | 17.5 | 15 |
| | Column | Scherzo SM-C18<br>(150×2 mm, 3 µm)<br>Imtakt | L-column2 ODS<br>(75×2.1 mm, 2 µm )<br>CERI | L-column2 ODS<br>(75×2.1 mm, 2 µm )<br>CERI | InertSutain Amide PEEK<br>(150×2.1 mm, 3 µm )<br>GL Sciences |
| | Mobile phase A | 0.05% formic acid water | 2 mM ammonium formate<br>with 50 mM formic acid | 2 mM ammonium formate | 0.1% formic acid water |
| | Mobile phase B | Acetonitrile<br>with 0.05% formic acid | Acetonitrile / Water : 95 / 5 (v/v)<br>including 2 mM ammonium formate<br>with 50 mM formic acid | Acetonitrile / Water : 95 / 5 (v/v)<br>including 2 mM ammonium formate | Acetonitrile<br>with 0.1% formic acid |
| | Time program | B conc. 0%（0-2 min）→ 100%（30-35min）→ 0%（35.01 – 40 min） | B conc. 40%（0-2.5 min）→ 100%（7.5-12.5 min）→ 40%（12.51 – 17.5 min） | B conc. 40%（0-2.5 min）→ 100%（7.5-12.5 min）→ 40%（12.51 – 17.5 min） | B conc. 100%（0-3 min）→ 5%（10 min）→ 100%（10.01 – 15 min） |
| | Flow rate (mL/min) | 0.2 | 0.2 | 0.2 | 0.4 |
| | Column Temp. (℃) | 25 | 30 | 30 | 30 |
| | Injection Volume | 5 µL | | | |
| MS | Instrument | LCMS-8050 (Shimadzu) | | | |
| | Ionization | Heated ESI(+/-) | | | Heated ESI(+) |
| | Mode | MRM | | | |
| | CID gas pressure | 330 kPa | | | |
| | Temperatures | HESI:350℃ / Desolvation line:200℃ / Heat block:400℃ | | | |
| | Gas flow | Nebulizing gas ($N_2$) : 2.5 L/min<br>Heating gas (Air) : 15 L/min<br>Drying gas (N2) : 5 L/min | | | |

## 3. Result and discussion

### 3-1. Mass Spectra of CTX3C

With electro spray ionization (ESI) on LC-MS/MS, since DSP and CFP are lipophilic toxins, various ions represented by sodium adduct and dehydrated ion are observed at positive mode (as $[M+Na]^+$ , $[M+K]^+$ , $[M+H-H_2O]^+$). While, OA and DTX1, DTX2 are monitored as simple mass peak at negative mode. Under this observation, precursor ion of OA and DTX1, DTX2 were selected as deprotonated molecule at negative mode.

Since deprotonated molecule of CTX are weak signal at negative mode, its precursor ion were evaluated as protonated molecule or sodium adducted ion depending on the mobile phase constitutions at positive mode.
It was observed that sodium adducted ion decreased with acetonitrile rather than methanol as mobile phase solvent.

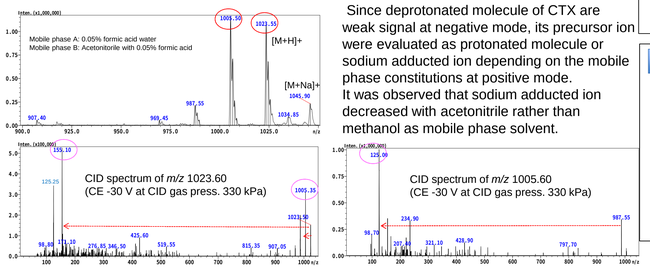


Figure 2. Mass spectra of CTX3C

### 3-2. MRM chromatograms of standard solution

With multi mode ODS column, both of the separation and sensitivity of 5 compounds (TTX, OA, DTX1, DTX2 and CTX) was successfully optimized.

TTX is hard to retain in reversed phase mode due to its hydrophilicity. Thus, hydrophilic interaction (HILIC) mode is alternative choice in comparison with another condition in the point of separation and sensitivity. As a result of evaluation using several types of columns, we found the InertSustain Amide column gave the better result of TTX analysis than other columns.

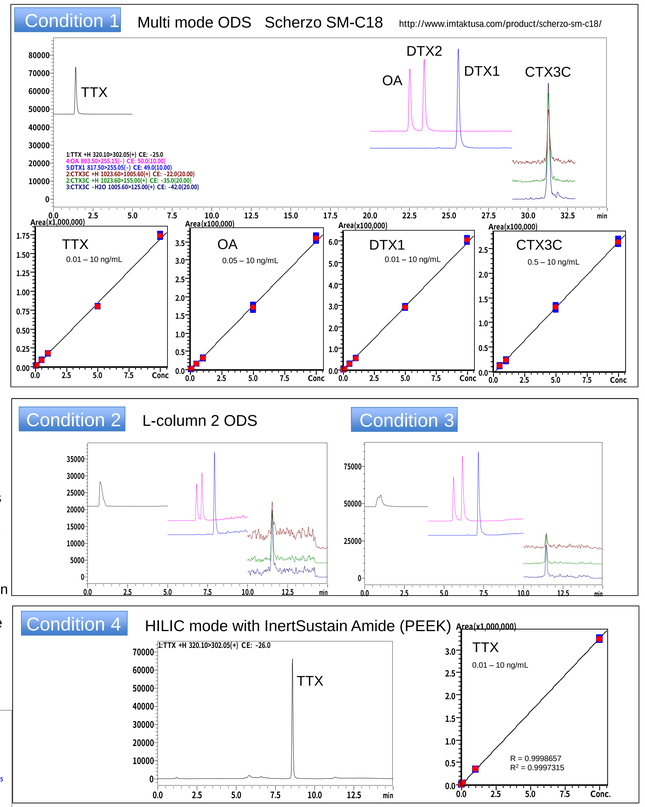


Figure 3. MRM Chromatograms (each.1 ng/mL) and Calibration curves

### 3-3. Evaluation of SPE pretreatment (Collaborated with Biotage®)

Preliminary evaluation of recovery with two different type of SPE cartridges were performed for sample preparation for two major toxic compounds (OA,DTX1).
Schematic pretreatment protocol for each SPE is illustrated in figure 4.
The recovery of representative two compound above is shown in Table 2.
The 66～83% of recovery was achieved with OA and DTX1 in the fraction of Elution 1 to 2, respectively, using ISOLUTE® C18 (EC) and EVOLUTE® EXPRESS ABN SPE.

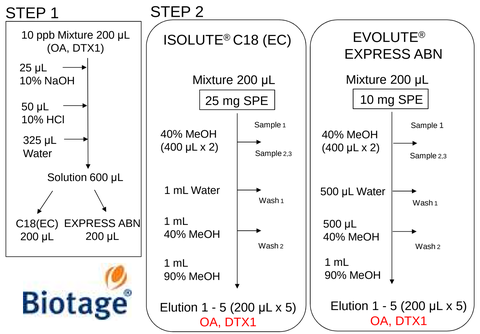


Figure 4. Protocol of SPE

Table 2 Recovery (%) with each SPE cartridge

| | C18(EC) | EXPRESS ABN |
|---|---|---|
| OA | 66 | 74 |
| DTX1 | 72 | 83 |

## 4. Summary and Conclusion

- Survey of analytical conditions indicates that the Multi mode ODS column gives better result of separation for 5 representative toxins (TTX, OA, DTX1, DTX2 and CTX3C) than other mode. The Summary is shown in Table 3.
- HILIC mode is alternative selection for TTX due to its good peak shape and retention.
- These results suggest Multi mode or HILIC are ways to achieve general condition including additional toxin, especially PSP.
- Pre-treatment with Biotage® SPEs were evaluated and good recovery was obtained.
- Development of sample clean up protocol as well as the evaluation of matrix effect has been continuously investigated with Biotage®.

Table 3 LOD (ppb) of each toxin with four condition

| | +/- | Transition (m/z) | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| TTX | + | 320.10>302.05 | 0.01 | - | - | 0.01 |
| OA | - | 803.50>255.15 | 0.05 | 0.05 | 0.05 | - |
| DTX2 | - | 803.50>255.15 | 0.05 | 0.05 | 0.05 | - |
| DTX1 | - | 817.50>255.05 | 0.01 | 0.05 | 0.01 | - |
| CTX3C | + | 1023.60>1005.60 | 0.5 | 1 | 0.5 | - |
| | + | 1023.60>155.00 | 0.5 | 0.5 | 0.5 | - |
| | + | 1005.60>125.00 | 0.5 | 0.5 | 0.5 | - |

Acknowledgement: Authors appreciate collaboration and great discussion with Dr Kato and Mrs. Kaneko, Biotage® Japan.