Determinations of lipophilic shellfish toxins in bivalve mollusks by ultra-performance liquid chromatography quadrupole linear ion trap mass spectrometry

G Y Liu¹, Z H Wang¹ ³, K Ge², L Song¹, J Du¹, J H Wu¹, K Wang and A Li¹

¹Liaoning Ocean and Fisheries Science Research Institute, No. 50 Heishijiao Street, Shahekou District, Dalian, China
²Dalian Ocean School, No.40 Lingshui Street, Shahekou District, Dalian, China
Email: 37765762@qq.com

Abstract. In the study, a method developed for the determination of lipophilic shellfish toxins in muscular tissues of Ruditapes philippinarum was presented. Lipophilic shellfish toxins contain Dinophysistoxin-1 (DTX₁), dinophysistoxin-2 (DTX₂) and homo-yessotoxins (h-YTX). We used microwave-assisted extraction (MAE) method with methanol as extractant to perform the sample preparation for muscular tissues of Ruditapes philippinarum and analyzed the crude extracts by ultra-performance liquid chromatography quadrupole linear ion trap mass spectrometry (UPLC-MS/MS). The microwave-assisted extraction operational parameters, including extraction temperature and volume, the solvent type and time, were selected detailed about extraction efficiency of lipophilic shellfish toxins from Ruditapes philippinarum muscular tissues. The method recoveries at three different spiked levels proved to be in the range from 50.47% to 94.98%. (<1%). The limits of detection (LOD) of three compounds were between 0.001–0.05μg/ kg, and it could meet the requirements for the actual samples. Then, this developed method was applied to actual samples which demonstrated that the method was a useful tool for the rapid quantitative detection lipophilic shellfish toxins. All in all, this determined method shows its good selectivity and sensitivity for the analysis of lipophilic shellfish toxins in tissues of Ruditapes philippinarum.

1. Experimental

Lipophilic shellfish toxins (LSTs) are produced by several microalage species and frequently bioaccumulated in filter-feeding molluscans shellfish. Although some toxins have no adverse
effects on shellfish themselves, severe intoxication may occur when the contaminated shellfish is consumed by human, with diarrhea, nausea and emesis as the common symptoms [1]. Dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2) and homo-yessotoxins (h-YTX) were found in bivalves or algae. They are lipophilic marine biotoxins with similarity in structure to lipid-soluble cyclic polyether compounds and are now regarded as typical LSTs. Their chemical structures are shown in Figure 1. These toxins are easy to accumulate in shellfish fat and may influence human beings by trophic transfer. It is reported that DTX1 and DTX2 are potent phosphatase inhibitors [2] and they can cause inflammation of the intestinal tract and diarrhea [3]. YTXs have been reported to modulate the calcium homeostasis in human lymphocytes through studies [4]. In addition, YTX also induces a selective disruption of the E-cadherin-catenin system in epithelial cells [5]. In this case, maximum levels of several toxins that should not be exceeded when placing shellfish products on the shelf has been established in the European Union (EU) regulation, for protecting shellfish consumers, [6]. But the mouse bioassay analysis shows some obvious drawbacks, for example, low sensitivity and reproducibility, and lack of specificity and information on toxin composition [7].

Therefore, some methods for simultaneous analysis of LSTs in different matrix were reported, for example, liquid chromatograph [8], chromatography-tandem mass spectrometry (LC-MS) [9-15], Enzyme-linked immunosorbent assay (ELISA) [16], potentiometric chemical sensors assay [17]. Some analytical determination methods for the LSTs in seawater and sediment have been developed with LC-MS [18-20].

However, DTX1, DTX2 and h-YTX used as lipophilic toxins in bivalve molluscsb y have not been detected at the same time. Because the similar potential hazards to people, simultaneous analysis of DTX1, DTX2 and h-YTX in shellfish is critical.

In this paper, based on the ultra-high performance liquid chromatography and microwave assisted extraction (MAE) device, we have established a method to detect DTX1 and DTX2 and hYTX simultaneously. The developed method was applied to separate and test DTX1, DTX2 and h-YTX in bivalve molluscsb y though extraction and separation. Figure 1 showed the chemical structure of the target.
1.1. **Reagents and materials**
Methanol, acetonitrile, ammonium hydroxide (absolute hypergrade for LC-MS) were bought from Fisher Scientific (Pittsburgh, U.S.A). Water was deionized and passed through a Milli-Q water purification system (Millipore, Billerica, MA, USA). DTX₁ (15.1±1.1 μg/mL), DTX₂ (7.8±0.4 μg/mL), h-YTX (5.8±0.3 μg/mL) standard were bought from the National Research Council. Institute for Marine Biosciences (Halifax, Canada). Individual DTX₁, DTX₂, h-YTX stock solutions were prepared in methanol with different concentration and they were stored at -18°C for three months. The different concentration was prepared in methanol with 1.0, 2.0, 5.0, 20.0, 50.0 μg /L. They were used to prepare calibration curves.

1.2. **Apparatuses**
The extraction of LSTs from molluscan shellfish tissue was extracted from a CEM MARS microwave sample preparation system, which equipped with 24 teflon vessels (Matthews, North Carolina). A Waters ACQUITY UPLC system (Waters, Milford, USA) was equipped with a vacuum degasser, an autosampler and empower workstation (Waters Milford, USA). The chromatographic separation was carried out on an ACQUITY UPLC BEH C18 (2.1×50mm i.d., 1.7μm, Waters, USA). A XEVO-TQD Mass Spectrometer (Waters, Milford, USA) coupled with electrospray ionization (ESI) source was tested in both positive and negative ionization mode and interfaced to a computer for running. Analyst Masslynx Version 4.1 software was applied for instrument control, data acquisition and the processing. MS/MS data acquisition was implemented in the multiple-reaction monitoring (MRM). Quantification was applied by using Targetlynx.

1.3. **Sample preparation**
*Ruditapes philippinarum* which was obtained from the food market was distinguished by Prof. Lun Song. The samples were crushed completely. The sample was divided into four parts after homogenization and stored at -18°C. One part was blank. Another three parts were used for spiking. The concentration were 2.0 μg/kg, 10 μg/kg and 20 μg/kg. They were prepared by adding an appropriate volume of spiking solution of three LSTs (DTX₁, DTX₂ and h-YTX) to the sample, then homogenize carefully. All samples were cryopreserved no more than three months.

1.4. **Extraction**
We weighed accurately 1.00±0.01 g of Ruditapes philippinarum tissue sample and placed them in a MAE extraction vessel with Methanol (15 mL). Every vessel was tightened and rocked violently for a few seconds. The vessels were put into the microwave sample preparation system. Extraction was implemented at 90 °C for 10 minutes. The time of ramp to temperature was 5 minutes. When the parameters are set, the MAE was opened. After extraction, the vessels were cooled down to the room temperature for 15 minutes. Then the vessels were taken off from the microwave sample preparation system. The residue was washed three times with 5mL methanol at each time. These aliquots of methanol were collected together with methanol extract after filtration. Subsequently, each collected solution was distilled to dry at low pressure using a rotary evaporator at 60°C.

### 1.5. Conditions of UPLC

The reversed-phase BEH C-18 column was used for the UPLC method. Temperature of the column was set at 25°C. Sample injection volume was 5μL. The flow rate was set at 0.4 mL/min. The mobile phase consisted of acetonitrile (solvent B) and water (solvent A) with 0.1% ammonium hydroxide, the initial proportion in volume of solvent B was 30%, which was increased to 80% within 1 minutes, kept constant for 3.5 minutes, and reduced to 30% in 0.5 minutes. The total run time was 5 minutes. Compared to HPLC, UPLC supplied improved resolution and sensitivity, higher efficiency, a fast run time, and reduced solvent use.

### 1.6. Conditions of MS

The mass spectrometer equipped with an ESI source and operated in negative polarity. MRM experiments were carried out. Nitrogen (99.99%) was used for desolvation gas and argon (99.99%) was used for cone gas. In order to obtain maximum sensitivity for identification and detection of LSTs, the ion source temperature (TEM) was set at 150°C, and the desolvation TEM was set at 500°C. The flow rate of the desolvation gas was 800 L/hr. The flow rate of the cone gas was 50 L/hr. Table 1 showed that the analyte specific parameters (cone voltage, collision energy, etc) for three compounds.

| Compound | Ionisation | Parention | MS/MS transition (m/z) | Dwell (s) | Cone Voltage (V) | Collision energy (eV) |
|----------|------------|-----------|------------------------|----------|------------------|----------------------|
| hYTX     | ESI        | [M-H]+    | 577.3>474.2            | 0.038    | 72               | 32                   |
|          |            |           | 577.3>403.2            |          |                  |                      |
| DTX2     | ESI        | [M-H]+    | 803.3>255.1            | 0.038    | 80               | 60                   |
|          |            |           | 803.3>113.1            |          |                  |                      |
| DTX1     | ESI        | [M-H]+    | 817.3>255.0            | 0.038    | 80               | 60                   |
|          |            |           | 817.3>112.9            |          |                  | 45                   |

Table 1. The parameter of MS/MS for the tested three compounds.
2. Results and discussion

2.1. Selection of solvent
Methanol, 80% methanol, acetonitrile, 80% acetonitrile were selected as the extractants of LSTs from Ruditapes philippinarum tissue. The extractions were detected at 90°C with 15 minutes using 15 mL solvent and tissue spiked with LSTs at 10 μg/kg level. Figure 2 shows the extraction recoveries of LSTs for detected extractants. When 80% methanol was used for the extraction solvent, Three LSTs contents was lower. So under this condition, the recoveries of DTX1, DTX2 and h-YTX were lower. The recoveries nearly to 90% of three LSTs were the highest in methanol. However, the recoveries of h-YTX and DTX1 were comparatively lower, especially that of DTX1 which is only about 35%, lower than other solvent, although DTX2 is higher. The data presented in Figure 2, methanol can be used as the best solvent for extraction.

![Figure 2. The influence of solvent types (n = 3).](image)

2.2. Selection of extraction temperature
The interactions, equilibrium rate and control partition of analytes between sample and solvent could be influenced by the temperature of microwave irradiation. In order to extraction temperature, extractions were tested at 60, 80, 85, 90 and 100 °C, respectively. Figure 3. Showed the extraction recoveries. For DTX1, the recoveries increased from 60 °C to 90 °C and the recovery decreased at 100 °C, it is possible that DTX1 was decomposition and transferred into other matter. So, the recoveries of DTX1 reach at a pinnacle and the irradiation temperature hardly influenced the recoveries in 100 °C. when temperature reached 80 °C, the extraction meets its maximum and further temperature increasing to 90 °C did not ultimately influence the recovery of DTX2. At 100 °C, the recovery decreased distinctly. For h-YTX, the recovery was highest at 90 °C than other temperatures. On the basis of data indicating a different degree of thermal sensitivity for each solute presented in Figure 3, the 90 °C extraction temperature was selected for further experiment.
2.3. Selection of solvent volume

Four kinds of solvent volume were selected, Figure 4 showed the influence of solvent volume on the extraction efficiency by MAE. The volume was important. If the volume of solvent is small, the extraction of target material will be incomplete, and the volume of solvent is too large, resulting in the waste of solvent and the increase of workload. After repeated tests, the volume of the solvent is determined. The recoveries of DTX1, DTX2 and h-YTX were the highest with 15mL. So, the solvent volume was set at 15mL.

2.4. Selection of extraction time

Figure 4. The influence of extractant volume (n = 3).
The speed of extraction time affects the whole analysis process. The analysis time is short and the analysis efficiency is high. It is an important part of the analysis work. According to the microwave extraction device and the actual sample requirements, we chose 5, 10, 15 and 20 minutes. So, extractions were operated at 90 °C for 5, 10, 15 and 20 min with using 15 mL methanol as the extraction solvent for optimizing extraction time. The results were suggested in Figure 5 under different extraction time. From 5 minutes to 20 minutes, the recoveries of all LSTs increased obviously. The recovery of LSTs 5 minutes was lower than 10 minutes. However, the recovery of LSTs did not changed significantly after the extraction time which was prolonged from 10 to 20 minutes. Further increase in extraction time did not lead to the significant recovery improvement. In generally, the optimum extraction time was 10 minutes for giving to its comparatively better performance.

![Figure 5. The influence of extraction time (n = 3).](image)

### 2.5. Determination

From table 2, the relationships between the analyte concentration and measured signal for three LSTs was showed. The linearity of the calibration curves was verified by the correlation coefficients. Calibration curves were set up at concentration range from 1.0 μg/L to 50.0 μg/L by UPLC-MS. Table 2 illustrated the results. The LODs of DTX₁, DTX₂ and h-YTX were

| LSTs  | Calibration curve | Linear coefficient (r) |
|-------|-------------------|------------------------|
| DTX₁  | Y=155.23X+103.93   | 0.9942                 |
| DTX₂  | Y=198.42X+136.6    | 0.9942                 |
| h-YTX | Y=63.65X-38.20     | 0.9995                 |
0.05 μg/kg, 0.01 μg/kg and 0.01 μg/kg. The LOQs of DTX\(_1\), DTX\(_2\) and h-YTX were 0.16 μg/kg, 0.04 μg/kg and 0.03 μg/kg.

2.6. Accuracy and precision

Three level spiked samples (2 μg/kg, 10 μg/kg and 20 μg/kg) were selected for continuous injection 5 times, respectively. The method validation studies for spiked samples suggested that the developed method provides good accuracy and high sensitivity for LSTs of three levels (2 μg/kg, 10 μg/kg and 20 μg/kg). The recoveries were 50.47–94.98% and the relative standard deviation was between 3.45%–7.86% in table 3. Figure 6 showed the TIC chromatograms of LSTs (A) and linear ion trap MS spectra of LSTs (B). The results showed that the method can separate the three LSTs well. In addition, these results illustrated the applicability of the method to extract DTX\(_1\), DTX\(_2\) and h-YTX from Ruditapes philippinarum tissue.

![Figure 6](image_url)

**Figure 6.** The TIC chromatograms of LSTs (A) and Linear ion trap MS spectra of LSTs (B).
Table 3. Recoveries and precision of extracted procedure of the samples (n = 5).

| LSTs | Added (μg/kg) | Average Recovery (%) | R. S. D. (%) |
|------|---------------|----------------------|-------------|
|      | 2.0           | 50.47                | 4.19        |
| DTX1 | 10.0          | 86.09                | 3.45        |
|      | 20.0          | 94.98                | 5.62        |
|      | 2.0           | 52.2                 | 7.86        |
| DTX2 | 10.0          | 79.34                | 5.78        |
|      | 20.0          | 82.69                | 3.56        |
|      | 2.0           | 57.62                | 6.89        |
| h-YTX| 10.0          | 75.76                | 4.35        |
|      | 20.0          | 64.11                | 5.23        |

2.7. Application of the method

We applied the method to detect the LSTs in the different *Ruditapes philippinarum* samples. Table 4 presented the result. DTX1, DTX2 and h-YTX were analysed in the visceral mass and muscle tissue, but none was detected in the muscle tissue. Because the internal viscera have detoxification function, the toxin can be detected. People try not to eat internal organs of bivalve mollusks.

Table 4. Results of the LSTs in the different *Ruditapes philippinarum* samples.

| LSTs | Muscle tissue (μg/kg) | Visceral mass (μg/kg) |
|------|-----------------------|-----------------------|
| DTX1 | -                     | 1.2                   |
| DTX2 | -                     | -                     |
| h-YTX| -                     | 1.3                   |

“-” not detected.

3. Conclusion

In this study, a method developed for the analysis of lipophilic shellfish toxins in muscular tissues from *Ruditapes philippinarum* was presented. The parameters of microwave-assisted extraction were determined by recovery rate, including solvent types and volume, extraction temperature and time. So, it is optimization that methanol was used as extractant at 90 °C for 10 min in 15mL. This study demonstrates that the microwave-assisted extraction with the UPLC-MS/MS method is applicable to determine DTX1, DTX2 and h-YTX in shellfish simultaneously. Compared with the reported method [21], the results presented that this extraction procedure was efficient and accurate. In the process of extraction, we only used small amounts of organic solvent, which was greee and was harmful to operators, under final optimized conditions, the total analysis time was only 20 minutes, including sample extraction, injection, chromatographic separation and data acquisition. The method has the advantages of simple operation, fast, high efficiency, accuracy and reliability. So, the validated method can be applied successfully to determine the presence of DTX1, DTX2 and
h-YTX in *Ruditapes philippinarum* tissue. It can be concluded that the determined method provided good selectivity and reproducibility for the quantitative analysis of LSTs in bivalve molluscs.

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**References**

[1] Pfannkuchen M, Godrijan J and Pfannkuchen D 2012 *Environ. Sci. Technol.* **46**(10): 5574-82

[2] Lucia S, Francese X and Jorge D 2015 *Toxicol. in Vitro*, **29**: 59-62

[3] Miguel C, Zhong T and Ricardo T 2017 *Toxicon.* 126: 23-31

[4] De la Rosa L A, Alfonso A, Vilarino N, et al. 2001 *Biochem. Pharmacol.*, **61**: 827-33

[5] Ronzitti G, Callegari F, Malaguti C, et al. 2004 *Br. J. Cancer*, **90**: 1100-7

[6] European Commission 2004 Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official J. Europ. Commun.*, L226, 22-82

[7] Hess P. *Anal. 2010 Bioanal. Chem*. **397**: 1683-94

[8] Lee J S, Yanagi T, Kenma R, et al. 1987 *Agric. Biol. Chem.*, **51**: 877-81

[9] Shen H H, Chen J H, Xu X L, et al. 2018 *Chin. J. Analyt. Chem.*, **46**(6): 985-92

[10] Wu H Y, Guo M M and Tan Z J 2014 *J. Chromatogr. A*. **1358**: 172-80

[11] Gabriel O, Lieven V and Maarten D 2017 *Harmful Algae*, **64**: 30-41

[12] Liu Y, Yu R and Kong F 2017 Chemosphere, **183**: 380-8

[13] Liu Y, Yu R and Kong F 2019 *Environ. Pollut.*, **249**: 171-80

[14] Wang L, Shi X, Zhao Q, et al, *Food Chem.*, 2019 **272**: 427-33

[15] Choonshik S J,Yun H J, Hong Y S, et al, 2018 Food Contr., **91**: 365-71

[16] Zhao R, Liu L and Liu L, 2015 *Chin. J. Anal. Lab.*, **34**(8): 882-5

[17] Ferreira N, Cruz M and Gomes M. 2018 *Toxins*, **181**: 380-4

[18] Li X, Li Z Y and Chen J H 2014 *Chemosphere*, **111**: 560-7

[19] Lane J Q, Roddam C, Langlois GW and Kudela R M 2010 *Limnol Oceanogr. Meth.* **8**: 645-60

[20] Li Z, Guo M, Yang S, et al, 2010 *Mar. Drugs.* **8**: 1263-72

[21] Yao J, Tan Z and Zhou D, 2010 *Chin. J. Chromatogr.* **28**(4):363-7