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Non-Targeted Screening of Lipophilic Marine Biotoxins by Liquid Chromatography – High-Resolution Mass Spectrometry

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Key Words

- Exactive
- Hypersil GOLD
- Liquid Chromatography
- Marine Biotoxin
- Non-Targeted Screening
- Orbitrap Technology

Introduction

Marine biotoxins are produced by naturally occurring microalgae, whose populations can increase significantly under certain environmental conditions to form a harmful algal bloom (HAB). During the incidence of a bloom, marine biotoxins pose a significant food safety risk when bioaccumulated in shellfish that are ingested by humans. Therefore, adequate testing for biotoxins in shellfish is required to ensure public safety and long-term viability of commercial shellfish markets.

The lipophilic marine toxins class includes the dinophysistoxins, azaspiracids, pectenotoxins, and yessotoxins. The compounds are structurally diverse, as shown in Figure 1, and thus do not contain a common UV chromophore or reactive functional group for fluorescence derivatization. Therefore, LC-MS is the method of choice for their analyses and several MRM-based methods have been reported.[1-3]

In response to the need for non-targeted methods that can potentially detect unknowns, high-resolution LC-MS has been successfully implemented for screening and quantification in food safety applications.[4-6] The lower-cost, higher-mass accuracy, and ease-of-use of modern quadrupole time-of-flight (QTOF) and Thermo Scientific Orbitrap based mass spectrometers have made high-resolution systems viable alternatives to triple-quadrupole systems for routine analysis. After full-spectrum data acquisition, specificity is typically achieved by extracting narrow mass windows (ie. 2–5 ppm) centered around a list of target analytes. Using this approach, it has been demonstrated that a resolving power of 50,000 or greater is required for correct mass assignments in complex matrices.[6] This report describes the use of the Thermo Scientific Exactive benchtop LC/MS system powered by Orbitrap™ technology for screening lipophilic marine biotoxins commonly found in shellfish.[7] The method was optimized using a standard mixture of marine biotoxins, and then applied to a mussel tissue extract.

Experimental

Chemicals and Materials

Certified calibration solutions and mussel tissue reference materials were purchased from the NRC Certified Reference Materials Program (Halifax, Nova Scotia, Canada). Certified calibration solutions were used for the following biotoxins: okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2), pectenotoxin-2 (PTX2), azaspiracid-1 (AZA1), azaspiracid-2 (AZA2), azaspiracid-3 (AZA3), and yessotoxin (YTX). As a test sample, a mussel tissue containing certified levels of OA and DTX1 was used (CRM-DSP-Mus-b).

HPLC grade acetonitrile and formic acid (98%) were purchased from EMD chemicals (Gibbstown, NJ, USA). Distilled-in-glass grade methanol was acquired from Caledon Laboratories (Georgetown, ON, Canada), and ammonium formate (≥ 99.0%) was from Fluka (St. Louis, MO, USA).

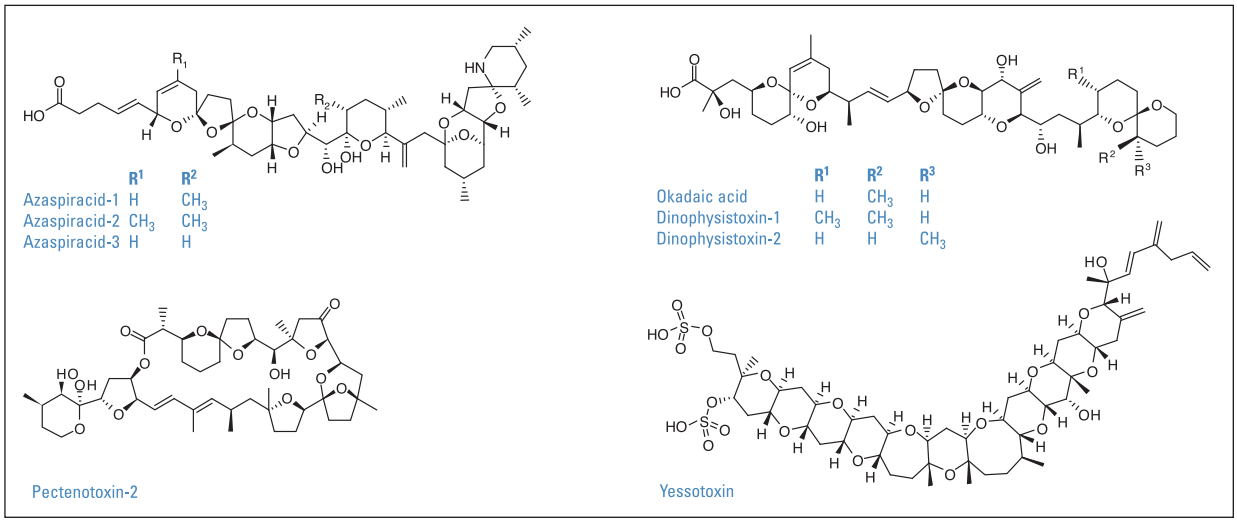

Figure 1: Chemical structures of the primary analogs of the regulated lipophilic marine biotoxins

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Extraction of Lipophilic Toxins From Mussel Tissue

Approximately 4 g of tissue was homogenized with 4 mL of 80% methanol solution using a Polytron PT3000 mixer (Brinkmann, USA) at 10,000 rpm with ice cooling. The sample was then centrifuged at 7,000 rpm for 15 minutes and the supernatant was decanted into a flask. Another 8 mL of 80% was used to clean the mixer by running the homogenizer briefly. The rinsate was centrifuged as before and this supernatant was combined with the first supernatant. 6 mL of 80% methanol was then added to the original pellet, which was homogenized again. After centrifugation, the final supernatant was combined with the previous two. The final volume was made up to 25 mL with 80% methanol solution. Approximately 0.5 mL of this solution was filtered through a 0.45 µm spin-filter (Millipore, Billerica, MA, USA) prior to analysis.

LC-MS Instrumentation and Method

LC-MS analysis was carried out on a Thermo Scientific Accela High-Speed LC coupled to an Exactive™ mass spectrometer, equipped with an Orbitrap mass analyzer and a HESI-II probe for electrospray ionization. The instrument was mass-calibrated daily for positive and negative modes, and the capillary and tube lens voltages were optimized daily, using the automated script within the Exactive acquisition software in both cases. For positive mode, mass calibration was performed with a mixture consisting of caffeine, MRFA tetrapeptide, and Ultramark 1621, while the negative mode calibration was performed with sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621. All analyses were performed using the 'balanced' automatic gain control (AGC) setting with a 50 ms maximum inject time. Data acquisition was carried out using Thermo Scientific Xcalibur 2.1. Optimal ion source and interface conditions consisted of a spray voltage of 3 kV, sheath gas flow of 50, capillary temperature of 360 °C, and a heater temperature of 250 °C. Alternating positive and negative polarity scans were acquired at a scan rate 2 Hz (50,000 resolution) for an overall cycle time of 1.25 seconds.

Lipophilic toxins were separated on a Thermo Scientific Hypersil GOLD C18 column (2.1 × 100 mm, 1.9 µm particle size), at a flow rate 400 µL/min and using 3 µL injections. Mobile phases were prepared from a stock solution of 1% formic acid solution in water with the pH adjusted to 3.0 using concentrated ammonium hydroxide. This stock solution was then diluted 10-fold with water (A) or acetonitrile (B), resulting in 0.1% formic acid in water for mobile phase A and 0.1% formic acid in 90% acetonitrile for B. Analytes were eluted with a linear gradient from 10 to 90% B from 0 to 2 min, held for 1 min, before returning to the initial conditions of 10% B.

Results

Lipophilic toxins were separated by reversed phase chromatography coupled to the Exactive mass spectrometer. As shown in Figure 2, eight lipophilic toxin standards were baseline separated in just under 6 min and the data shown represents 5 ppm extracted mass chromatograms centered around the masses of the target analytes. As OA, DTX1, DTX2, and YTX ionize significantly better in negative mode, alternative positive and negative polarity scans were acquired to achieve maximum signal for all analytes. To maintain a sufficient number of data points across chromatographic peaks, data was collected at a scan rate of 2 Hz. The scan rate of 2 Hz generates resolution of roughly 50,000, much lower than the maximum resolution possible with the mass spectrometer, but was selected as a reasonable compromise between selectivity and quantitative performance. In addition, it has been demonstrated that a

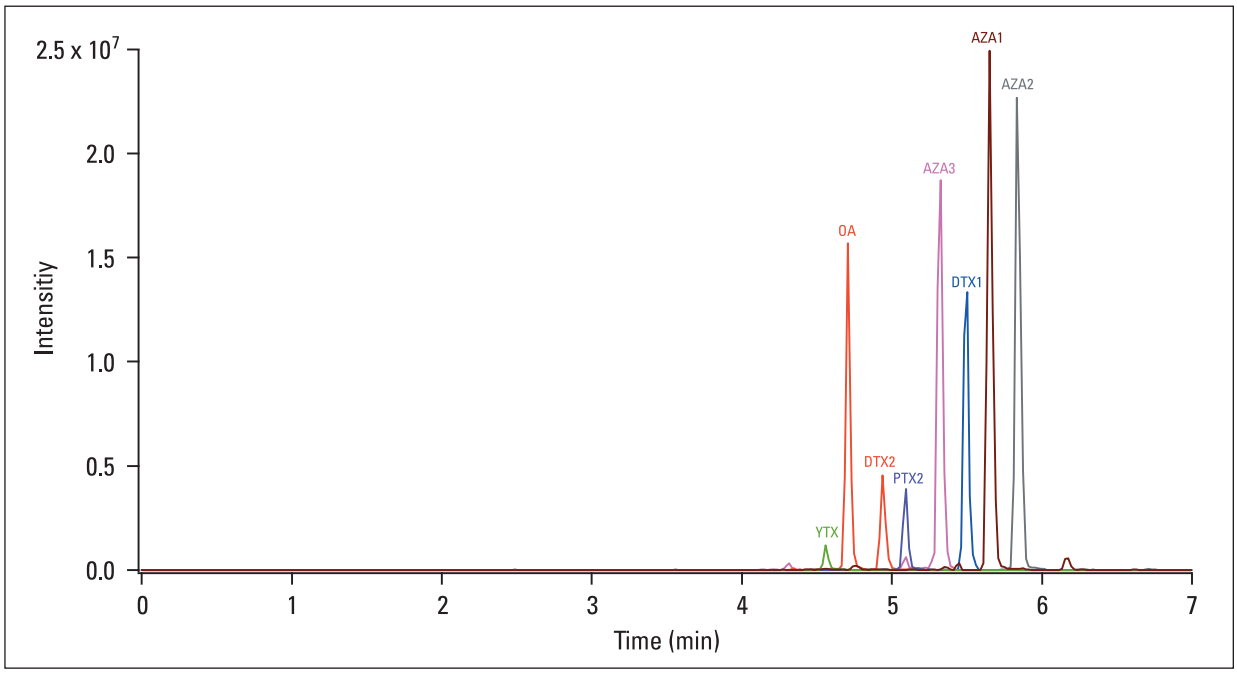

Figure 2: LC-MS chromatograms of eight lipophilic biotoxin standards acquired with alternating positive (PTX2, AZA1,-2,-3) and negative (YTX, OA, DTX1,-2) scans at 2 Hz. Data shown represents 5 ppm mass windows centered around the analyte mass.

resolving power of 50,000 provides sufficient specificity in complex matrices.[6] The ability to rapidly scan both positive and negative polarities allows data collection in a true non-targeted fashion and permits independent optimization of the LC method without consideration of the retention time of positive and negative analytes.

Listed in Table 1 are accurate masses and limits of detection for the lipophilic toxins using external calibration exclusively, without any mass correction on an internal standard or a background ion. In general, accurate masses are below 1 ppm error for analytes detected in positive mode, while those detected in negative mode range between 1–3 ppm error. Similarly, limits of detection ranged from 0.052–0.10 µg/L (ppb) for the positive ions, while those detected in negative mode were distinctly higher at 1.6–5.1 µg/L.

The utility of the screening method for lipophilic toxins was evaluated by analyzing a mussel tissue reference material containing certified levels of okadaic acid and DTX1, as shown in Figure 3. The top trace of Figure 3 represents the total ion chromatogram (TIC), revealing the complex matrix of the mussel tissue. Excellent specificity was demonstrated by the minimal background peaks detected in the 5 ppm mass windows associated with OA and DTX1 (lower trace), and OA and DTX1 are clearly discriminated from the complex matrix. Quantification against calibration with toxin standards in methanol yielded levels of OA and DTX1 of 4.1 µg/g and 0.58 µg/g, respectively, with precision of roughly 10% RSD for both analytes. These concentrations represent roughly half of the certified values for OA and DTX1, with ion suppression by the matrix being the likely cause for these discrepancies. Ion suppression effects are generally observed for all types of mass spectrometers employing electrospray ionization, and can be mitigated with the use of matrix-matched standards if accurate quantification is desired.[8]

Toxin	Tret (min)	Chemical Formula	Ion Detected	Calculated (*m/z*)	Observed (*m/z*)	Error (ppm)	LOD (µg/L)
YTX	4.63	$C_{55}H_{82}O_{21}S_2$	$[M-H]^-$	1141.47172	1141.47433	2.3	5.1
OA	4.81	$C_{44}H_{68}O_{13}$	$[M-H]^-$	803.45872	803.45963	1.1	2.8
DTX2	5.04	$C_{44}H_{68}O_{13}$	$[M-H]^-$	803.45872	803.46002	1.6	1.6
PTX2	5.19	$C_{47}H_{70}O_{14}$	$[M+NH4]^+$	876.51038	876.51067	0.33	0.10
AZA3	5.45	$C_{46}H_{69}NO_{12}$	$[M+H]^+$	828.48925	828.48973	0.58	0.062
DTX1	5.59	$C_{45}H_{70}O_{13}$	$[M-H]^-$	817.47427	817.47639	2.6	2.0
AZA1	5.78	$C_{47}H_{71}NO_{12}$	$[M+H]^+$	842.50490	842.50477	0.15	0.052
AZA2	5.96	$C_{48}H_{73}NO_{12}$	$[M+H]^+$	856.52055	856.52062	0.080	0.064

Table 1: Accurate masses and LODs for the lipophilic marine biotoxins

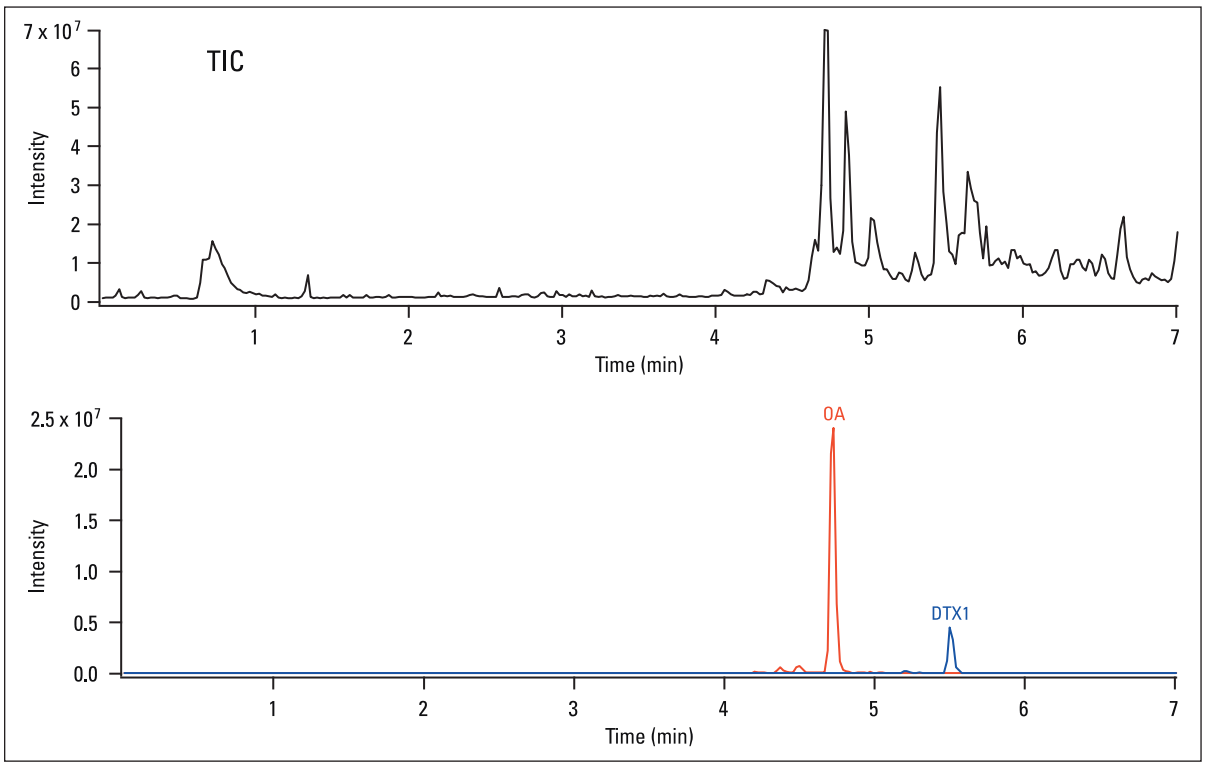

Figure 3: Exactive analysis of a mussel tissue extract showing the total ion chromatogram (TIC; top trace) and 5 ppm mass chromatograms for okadaic acid and DTX1 (lower trace)

Conclusions

The Exactive benchtop LC-MS system was successfully applied to the screening of lipophilic marine biotoxins commonly found in shellfish. This non-targeted approach provides high-resolution data over the entire chromatographic separation, allowing detection of new or unknown compounds in addition to those of interest. Furthermore, the approach requires little method development, as settings are not tuned for individual analytes. Although the results described above were limited to a relatively small subset of biotoxins for which calibration standards are available, extending the approach to other toxins or toxin analogues can be simply accomplished by expanding on the target list of analyte masses during data processing.

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