Determination of lipophilic marine biotoxins by liquid chromatography-tandem mass spectrometry in five shellfish species from Washington State, USA

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Abstract

Low extraction efficiency (60-81%) of okadaic acid (OA) and dinophysistoxin 1 (DTX1) was obtained for 4 out of 5 shellfish species from Washington State (WA), USA, during application of a standard extraction method for determination of lipophilic marine biotoxins by LC-MS/MS as recommended by the European Union Reference Laboratory for Marine Biotoxins (EURLMB). OA and total OA including esters, DTX1, DTX2, and total DTX including esters, azaspiracid 1, 2, and 3 (AZA1, AZA2, and AZA3), pectenotoxin 2 (PTX2), and yessotoxin (YTX) were the toxins examined. Matrix-matched standards prepared from the same control samples used for spike-and-recovery tests were employed to evaluate toxin extraction efficiency and sample clean-up procedures. We adjusted the EURLMB extraction method by either using an acidified methanol extraction or pre-cooking shellfish homogenates at 70 ºC for 20 min before EURLMB extraction. Extraction efficiency was improved markedly for OA and DTX1 with both modified methods and for YTX with the pre-cooking step included. However, recoveries were lower for YTX using the acidified methanol extraction and for PTX2 in non-mussel samples with the pre-cooking step. A hexane wash was applied to clean water-diluted non-hydrolyzed samples and a hexane wash was combined with solid-phase extraction for cleaning hydrolyzed samples. Improved sample clean-up, combined with LC-MS/MS adjustments, enabled quantification of U.S. Food and Drug Administration-regulated toxins in five shellfish species from WA with acceptable accuracy using non-matrix matched calibration standards.

Keywords: Lipophilic marine biotoxins; okadaic acid; dinophysistoxins; azaspiracids; shellfish; LC-MS

1. Introduction

Phycotoxin-contaminated shellfish can cause severe intoxication of consumers and economic losses to the seafood industry. Okadaic acid (OA), dinophysistoxins (DTXs), and their esters, are referred to as diarrhetic shellfish poisoning (DSP) toxins. These toxins, along with azaspiracids (AZAs), are associated with severe gastrointestinal disorders. Yessotoxins (YTXs) and pectenotoxins (PTXs) can cause acute toxicity in mice but have not been linked to human illness [1]. Maximum permitted levels of the above toxins were established in the European Union (EU). In the USA, the Food and Drug Administration (FDA) has adopted the same action levels for DSP toxins (total DSP toxins at 160 µg OA equivalents/kg) and AZAs (160 µg AZA equivalents/kg), but no regulations for YTXs or PTXs [1]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are used widely for determination of these toxin groups and matrix effects (signal suppression or enhancement) have been commonly reported [2-5]. Due to the lack of stable isotope-labelled internal standards for obtaining precise and accurate LC-MS/MS measurements, standard addition and matrix-matched standards (MMS) are employed for correcting matrix effects [2]. Standard addition is generally avoided due to the large amounts of toxin standards, extra sample run, and longer turnaround time required [2]. MMS are normally used for routine analysis of these toxins [3, 6]. However, MMS made from one shellfish species may not work for other species [3], and MMS calibration could cause errors when matrices in MMS and samples are not truly identical, even with the same species [2,5], due to different harvest locations and times [5]. Matrix effects can be removed or mitigated by sample clean-up and instrument adjustments. Online solid phase extraction (SPE) LC-MS/MS has reduced matrix effectively [5,7], especially for removing salts produced during hydrolysis of esterified DSP toxins (DTX3) [7].

Methanol with or without water is generally used to extract lipophilic toxins, except when extraction is combined with high salt content and dispersive-SPE clean-up [8,9]. It is assumed that methanol has high extraction efficiencies for all toxins in all shellfish and spike-and-recovery validations are often done following extraction in order to reduce consumption of certified reference materials (CRM) [6]. Herein, we employed spike-and-recovery tests along with MMS prepared from the same control samples to ensure that spiked samples and MMS had truly matched matrix for evaluating toxin extraction, sample clean-up, and quantitative LC-MS measurements. We examined a wide range of commercially available toxins and multiple shellfish species. Both alkaline and acidic LC conditions are commonly used for toxin detection [10]; however, alkaline conditions showed better (e.g., OA and YTX) or comparable (e.g., AZAs) sensitivity compared to acidic conditions and enabled analysis of all non-hydrolyzed toxins in a single run without the need for MS fast polarity switching [11]. Therefore, alkaline mobile phases were employed in this study for method development and sample analysis.

2. Materials and methods

2.1. Reagents and standards

CRM of OA, DTX1-2, YTX, AZA1-3, PTX2, CRM-Zero-Mus, and CRM-AZA-Mus were purchased from National Research Council (NRC) of Canada (Halifax, Canada). HPLC grade acetonitrile and methanol were from Honeywell Burdick & Jackson (Muskegon, USA) or Fisher Chemical (Optima LC/MS grade; Frederick, USA). HPLC grade water or Milli-Q water were used to prepare LC mobile phases. HPLC grade *n*-hexane (95%) was acquired from J.T. Baker (Phillipsburg, USA). Calibration standards and mixed toxin stock solutions were prepared initially in 100% methanol and later in 85% methanol/water to reduce solvent evaporation. Formic acid (Guaranteed Reagent, minimum 98%; EMD Millipore, USA) was purchased from VWR International (USA). Ammonium hydroxide (28-30%) was from Sigma Chemicals (St. Louis, USA).

2.2. Shellfish samples

Uncooked homogenates of five shellfish species (blue mussel, California mussel, Manila clam, littleneck clam, and Pacific oyster) were obtained from Washington State (WA) Department of Health (DOH) in 2016; samples generally contained DTX1 esters with or without YTX and PTX2. Control shellfish homogenates used for spike-and-recovery tests were characterized as follows: no OA, DTX2, their esters, AZA1-3, a negligible amount of DTX1 in one sample, DTX1 esters in all samples (0.6 to 14.5 µg DTX1 equivalent/kg), PTX2 in several samples (0 - 3.5 µg/kg), and YTX in only non-clam samples (2.6 - 7.9 µg/kg). For initial evaluation of extraction efficiency calibrated against MMS, spike levels were 20 µg/kg for each DSP toxin and PTX2 and 12.8 or 20 µg/kg for each AZA toxin and YTX. Toxins present in control samples showed no or weak effects on recovery evaluations, with use of the same control samples to prepare MMS. All recovery data were calculated while accounting for toxin levels present in original control samples. For evaluation of sample preparation reproducibility and toxin quantification calibrated with standards in solvents, all toxins were spiked into triplicate homogenates of the five shellfish species. Due to high costs of methanolic CRM, CRM-AZA-Mus mussel homogenate containing certified AZA1-3 concentrations was mixed individually

with homogenates of each species to obtain an AZA1 concentration of ~112 µg/kg (~10% homogenate from CRM). Homogenates were split into 50 ml centrifuge tubes (2.0 g each) for spiking other toxins from methanolic CRM (µg/kg in homogenates: OA 160, DTX2 20, DTX1 40, PTX2 20, YTX 18.75). Blue mussel extract prepared with a homogenate from EU (source: Dr. Steve Morton, NOAA/NCCOS, Charleston, USA) containing total OA ~2.45 µg/ml with esterified OA ~47% (total DTX and PTX2, not detected) was used as a source of esterified DSP toxins for evaluating sample preparation.

2.3. Extraction and sample extract processing

2.00 ± 0.05 g of raw shellfish homogenate in a 50 ml polypropylene centrifuge tube (United Laboratory Plastics, USA) were either left unspiked or spiked with toxins before tightly capping and cooking in a water bath at 70 ºC for 20 min. Samples were cooled and extracted, according to EURLMB [10], using 9 ml methanol with 3 min vortex mixing. Supernatant was removed after centrifugation (2000 × *g*) for 10 min at ~20 ºC. Another 9 ml methanol were added to the pellet, homogenized at maximum speed for 1 min using a PRO200 homogenizer (PRO Scientific, USA), and centrifuged. Supernatants were combined into a 20 ml volumetric flask and brought to volume with methanol. Acidified methanol was examined initially for toxin extraction efficiency, whereby 9 ml methanol (original EURLMB method) were combined with 14 µl formic acid (≥ 98%), with centrifugation at 2750 × *g*. For non-hydrolyzed samples, 2 ml of methanol extract were diluted with 0.5 ml water and defatted with 5 ml hexane; at least 1 ml of extract was filtered (PTFE syringe filter; 0.2 µm) into an LC vial. For hydrolysis, 250 µl of 2.5 M aqueous NaOH were mixed with 2 ml of unfiltered methanol extract in a 15 ml polypropylene centrifuge tube (United Laboratory Plastics, USA), capped tightly, and heated in a water bath at 76 ºC for 40 min. After cooling, 250 µl 2.5 M aqueous HCl were added for neutralization [10]. The hydrolyzed extract was defatted with 5 ml hexane; 1.2 ml of extract were subjected to SPE clean-up (Strata X, 30 mg, 1 ml; Phenomenex, USA). The extract was diluted with water (2.64 ml) to ~25% methanol and acidified with formic acid (57.6 µl 20% aqueous formic acid), then loaded onto a pre-conditioned SPE cartridge (1.5 column vol. methanol; 1 column vol. water). The sample tube was washed with 3 ml 50% aqueous methanol containing 0.2% formic acid and transferred to the cartridge. The cartridge was washed with 2 column vol. 20% methanol/water. DSP toxins were eluted with 1.2 ml methanol.

For spike-and-recovery tests, two extracts were prepared concurrently from each shellfish sample - one with homogenate receiving toxin spikes and the other unspiked for MMS preparation, ensuring an identical matrix for MMS and toxin-spiked samples. When toxic mussel extract containing free and esterified OA was used for testing, 80 µl of extract containing 196.1 ng total OA were spiked into 2 g homogenate. For non-hydrolyzed MMS, toxins were spiked into PTFE-filtered extracts. For hydrolyzed MMS, pure toxins (or 8 µl toxic extract with 19.61 ng total OA) were spiked into unfiltered extracts, hydrolyzed, and filtered or cleaned with SPE. For the latter, MMS were prepared by spiking pure toxins into SPE-cleaned extracts. Each toxin concentration in MMS was the same as, or 1.25-fold, those in corresponding sample extracts.

2.4. LC-MS/MS analysis

LC separation was performed on an HP1100 system (Agilent Technologies, USA) equipped with degasser and binary pump with static mixer, using an Xbridge C18 column (150 × 3 mm, 5

µm; Waters, USA) at 30 ºC. Mobile phase comprised water (A) and 90% acetonitrile/water (B), both containing 6.7 mM ammonium hydroxide, at 0.4 ml/min flow rate [11]. Injection volume (final protocol) was 2 µl. Different LC gradients were evaluated. Final protocol for non-hydrolyzed samples: 3.5 min 10% B, linear gradient to 26% B at 4 min, to 62% B at 22 min, to 75% B at 22.5 min, to 85% B at 25.5 min, then to 90% B at 26 min and hold for 3 min, return to 10% B at 30 min and hold for 4 min. Final protocol for hydrolyzed samples: 5 min 10% B, linear gradient to 30% B in 0.5 min and hold at 30% B for 2 min, linear gradient to 45% B at 15 min, then to 90% B at 17.5 min and hold for 3.5 min, return to 10% B at 23 min and hold for 4 min. MS detection employed multiple reaction monitoring (MRM) scan mode on an API4000 mass spectrometer (AB Sciex, USA). Two MRM channels were applied to each toxin; the MRM channel with the strongest signal was used as the quantification channel and labeled below as a 'quantifier'. DSP toxins and YTX were detected in negative ion mode using these MRM transitions: *m/z* 803.5 → 113.1 and 255.1 (quantifier) for OA and DTX2, *m/z* 817.5 → 113.1 and 255.1 (quantifier) for DTX1, *m/z* 570.2 → 467.2 (quantifier) and 502.2 for YTX (*m/z* 570.2 → 396.1 for YTX [11] was not used due to sample matrix interference). AZA and PTX were detected in positive ion mode using these MRM transitions: *m/z* 842.5 → 672.4 and 824.5 (quantifier) for AZA1, *m/z* 856.5 → 672.4 and 838.5 (quantifier) for AZA2, *m/z* 828.5 → 658.4 and 810.5 (quantifier) for AZA3, *m/z* 876.5 → 213.1 and 823.5 (quantifier) for PTX2. Ion spray voltage (IS) was -3.5 and 5 kV for negative and positive ion mode, respectively. Turbo gas temperature was 430 ºC. MS scans for an LC-MS run were divided into periods in combination with a 2-position diverter valve (VICI, Valco Instruments Co. Inc., USA) as described previously [12]. For non-hydrolyzed samples, the second period was for detecting DSP toxins and YTX, the third period for AZA, and the fourth period for PTX. For hydrolyzed samples, the second period was for detecting DSP toxins. LC eluent for periods not used for toxin detection was directed to waste (IS 0 kV). Toxin peak shapes, relative retention times (RT), and the clustering of toxins in RT windows associated with MS scan periods for their detection were similar to those reported previously [11], except individual RTs were higher due to the use of slower LC gradients. LC-MS was controlled by Analyst 1.4.1 software (AB Sciex).

3. Results and discussion

3.1. Unexpected results with EU extraction method

EU extraction method [10] was first applied to control blue mussel homogenate for extraction efficiency testing without sample clean-up. Recoveries of AZAs, PTX2, and YTX ranged from 85 to 105%. However, recovery was generally below 75% for OA and DTX1, and above 80% for DTX2, for non-hydrolyzed and hydrolyzed samples (esterified DSP toxins were not spiked into homogenates). Other extraction methods were evaluated. OA and DTX1 recoveries were not improved using 90% methanol, and increased to ~80% for OA and above 80% for DTX1 for non-hydrolyzed samples using 3 ml methanol (3x) per 1 g of tissue (second and third extractions with vortex mixing instead of high speed homogenizer as in EU method) [6]. Table 1 shows low extraction efficiency of OA and DTX1 from four of five WA shellfish species using EU extraction method with specified LC gradient; different LC gradients were evaluated for certain samples, but yielded similar results.

3.2. Acidified methanol extraction

For non-hydrolyzed samples, extracts were diluted with water, washed with hexane, and cleaned via SPE using the procedure for hydrolyzed samples (final protocol), except that methanol containing 0.1-0.3% ammonia was used as elution solvent instead of pure methanol. OA and DTX1 recoveries were improved significantly, with most near 100% (Table 2). YTX recoveries were much lower than with EU protocol. Also, acidified methanol extraction produced more precipitate when mixing first and second extracts, as formic acid level increased.

3.3. Incorporation of pre-cooking step into EU extraction protocol

The spike-and-recovery test was applied to certified zero toxin mussel homogenate using EU extraction method without clean-up. Spiked CRM-Zero-Mus was run several times on different days with various LC gradients. Recoveries for non-hydrolyzed material were all within 91 to 112% for DSP toxins, AZAs, and PTX2 (except 121% once for DTX2), and 86 to 90% for YTX (except 77% once). LC-MS showed more stability across different days for this sample than for WA shellfish. Notably, CRM-Zero-Mus comprised pre-cooked material, whereas homogenates of WA shellfish were not cooked. Cooking improved OA and DTX1 extraction efficiencies and stability of LC-MS runs, which may reflect cooking-induced protein denaturation [13]. Although OA and DTX2 are isomeric congeners, DTX2 recovery was high (90 to 100%; Table 1) using EU extraction method. Investigating why OA or DTX1 quantification is underestimated with EU extraction protocol is outside the scope of this study, since shellfish species, harvest locations and times must also be considered.

We tested adding a cooking step prior to original EU extraction protocol. Pre-cooking sealed shellfish homogenates in a 70 ºC water bath for 20 min was adopted from extracting ciguatoxins from fish flesh [13]. For non-hydrolyzed samples, 0.5 ml water was added to 2 ml extracts to reduce methanol to ~80% and move more hydrophobic matrix into the hexane, as evidenced by sample-dependent color and its saturation in the hexane layer, in contrast with direct hexane wash without water dilution [5]. SPE clean-up was not applied to non-hydrolyzed samples because methanol containing ammonia was required to elute all toxins from cartridges, which caused inconsistent YTX recovery across shellfish species and unstable LC-MS runs. The hexane wash and SPE clean-up were applied to hydrolyzed samples, since SPE was also used for salt removal. The SPE procedure was adopted and modified from previous reports [4,12]. Samples were loaded onto cartridges in acidified solution to neutralize the negative charge on DSP toxin molecules and improve their retention, which prompted using 50% (vs. 40%) methanol/water containing 0.2% formic acid [12] to enhance washing. Recoveries of all toxins ranged from 89 to 117%, except for YTX (85 to 91%), for non-clams and below 65% for clams (Table 3), which exceeded or was comparable to the original EU extraction (DOH1661 replaced by DOH1209 due to sample exhaustion). Different water bath temperatures for the added cooking step (60, 70, or 76 ºC for 20 min) were evaluated for Manila clam (DOH1338); however, no obvious variation in YTX extraction efficiency at the three cooking temperatures was observed (OA recovery was < 80% with cooking at 60 ºC). The YTX extraction efficiency for DOH1209 (littleneck clam) with cooking at 70 ºC was slightly below 45% (based on two additional extraction pairs with YTX spiked into the raw homogenate and into its control extract as a MMS for each pair). DSP ester extraction efficiencies with pre-cooking were examined by spiking the toxic mussel extract into homogenates of DOH966 (blue mussel), DOH1209 (littleneck clam), and DOH2056 (Pacific oyster); total OA recoveries for these samples ranged from 90 to 103%.

Cooking shellfish homogenates prior to executing EU extraction was selected as the final sample preparation protocol. There are advantages to pre-cooking before extraction: stabilization of shellfish tissues enzymatically, improved comparability between shellfish CRM (pre-cooked materials) and samples analyzed [14], and enhanced reproducibility of toxin detection by LC-MS. Some countries pre-cook shellfish for toxin determination [14,15].

3.4. Quantification with solvent-based standards for calibration

Reproducibility of sample preparation was assessed via spiking experiments (section 2.2). Seven calibration standards ranging from 0.5 to 24 ng/ml were prepared for all toxins. MMS and solvent standards were used to optimize LC conditions with the aim of employing solvent standards for LC-MS calibration. In addition to sample clean-up, a small injection volume (2 µl) and slow LC gradients were adopted to reduce matrix effects. In the Certificate of Analysis for CRM-AZA-Mus, AZAs were reported as certified values and other toxins identified at uncertified low/trace levels. We examined toxins in this CRM using the final protocol (without heating due to use of pre-cooked material) with calibration by solvent standards along with standard additions to correct for possible matrix effects; values determined for non-AZA toxins were added to spike levels for recovery calculations. Repeatability and reproducibility of LC-MS analyses were evaluated by running samples on different days using LC column 1 and 2 for a total of five LC-MS sequences for non-hydrolyzed samples (Table 4) and using LC column 1 with three sequences for hydrolyzed samples (Table 5). Column 1 was employed for method development, whereas column 2 was used for the initial LC-MS sequence and, after six months of intermittent use, for the second sequence. Average recoveries ranged from 82 to 114% for free DSP and from 84 to 104% for total DSP toxins, which exceeds that obtained for OA and DTX1 with original EU extraction (Table 1). Average YTX recovery varied from 87 to 103% for non-clams and below 80% for clams, which was better than or similar to original EU method. Average AZA1, AZA2, and AZA3 recoveries were 91-109%, 99-124%, and 112-132%, respectively. Recoveries of AZA2 and AZA3 in MMS samples, calibrated against solvent standards, ranged from 99 to 113%, indicating slight matrix enhancement; however, this effect could not account for their overestimates. The AZA1 mean value determined for CRM-AZA-Mus agreed with the certified mean value (0.8% relative error), but mean values for AZA2 and AZA3 were slightly higher than certified ranges (4.2% and 5.6%, respectively). If extreme AZA2 and AZA3 values from certified ranges were used, recoveries would vary from 90 to 120%. Average PTX2 recovery was low for clams and oyster; PTX2 in clams and oyster was likely degraded during pre-cooking and overestimated for LC column 2, especially on the second sequence, as indicated by MMS accompanying the run.

For all toxins (except PTX2) in each shellfish type, total precision (RSD_R) was < 11% for DSP, < 6% for AZAs, < 15% for YTX (except 20% for YTX in DOH1209). The HorRat value [16] ranged from 0.1 to 0.4 for DSP, 0.04 to 0.2 for AZAs, 0.2 to 0.4 for YTX (except 0.7 for YTX in DOH1209). Most HorRat values were < 0.5, better than predicted (based on intra-laboratory data); even the highest YTX values remained within the acceptable between-laboratory reproducibility range (0.5 to 2.0). Limits of detection (LOD; µg/kg) for all toxins, defined by signal-to-noise ratios (S/N) ≥ 3 for both MRM confirmation and quantitation channels, were: OA 6.3, DTX2 3.5, DTX1 4.6, YTX 3.6, AZA1 2.3, AZA2 1.4, AZA3 2.8, total OA 5.5, total DTX2 3.4, and total DTX1 4.0 (varied slightly with shellfish type and day-to-day runs; values are highest observed). Limits of quantitation (LOQ; µg/kg) for all toxins, defined by S/N ≥ 10 for MRM quantitation channels and S/N ≥ 3 for the corresponding MRM confirmation

channels, were: OA 9.6, DTX2 7.5, DTX1 5.6, YTX 3.6, AZA1 2.5, AZA2 3.4, AZA3 5.0, total OA 7.9, total DTX2 7.3, and total DTX1 6.3 (variation similar to LOD; values are highest observed).

3.5. Issues with alkaline mobile phases

For charged toxin molecules, RT increased gradually as alkaline mobile phases aged [12] and RT slowly decreased as LC columns were used repeatedly, which was more significant with slow vs. fast LC gradients used in the authors' lab for an inter-laboratory validation study [17]. RT shift within each LC-MS sequence met the EU RT drift criterion of < 3% [10]. One-year of using alkaline mobile phases caused instability of LC binary pumps: PTX2 RT increased slowly during overnight runs, whereas no increase occurred (RT fluctuation ≤ 0.1 min for PTX2) when formic acid replaced ammonia additive in mobile phases. Replacing the static mixer with a capillary (Agilent) and bypassing solvent selection valves with adaptors indicated the likely cause was contamination of active inlet valve cartridges. Contamination could be removed by flushing both pumps with Milli-Q water and 40% acetonitrile/water, and then methanol for A pump (if Milli-Q water instead of HPLC water was used for mobile phase A), enabling proper operation of the next overnight run with alkaline mobile phases. Alkaline mobile phases were usually prepared fresh, since binary pump instability was worse if organic mobile phase B was several days old. Such issues have not been observed with Agilent's quaternary pump [12], which has one pump for all mobile phases; flushing the LC system after runs to remove alkaline solvents is required to maximize life span of consumables. However, with a quaternary pump, DSP toxin RTs were less stable versus the binary pump for extremely slow LC gradients of ammonia-containing mobile phases, which may reflect suppression of bubble formation with its high pressure mixing. LC and MS interface should be cleaned with MS under vacuum when mobile phases containing acid and salt additives are used before introducing alkaline mobile phases. This ensures ≤ 25% variation of calibration slopes bracketing samples based on EU criterion [10]. Without the static mixer, the LC gradient was re-adjusted for non-hydrolyzed samples only (3.5 min 10% B, linear gradient to 26% B at 5.5 min, to 57% B at 21 min, to 75% B at 22.5 min) to maintain similar toxin recoveries achieved previously.

3.6. Toxin stability during heating and storage

PTX2 was partially degraded at 70 ºC for clams and oyster spiked with certified AZA mussel homogenate and original methanolic CRM (Table 4). However, degradation was not observed for shellfish homogenates spiked initially with diluted CRM solutions (Table 3). Examination of spiked and unspiked DOH1209 (2 pairs) indicated that degradation was not due to hydrolysis, since PTX2 seco acid quantities were equivalent in PTX2-spiked and unspiked samples and peak areas of PTX2 added and PTX2 seco acid originally present were within the same order of magnitude. Steaming mussels at 100 ºC caused partial conversion of DTX3 to free DSP toxins via hydrolysis without reduction in toxicity [18]. Determination of free DSP toxins is not required by US FDA [1], but is mandated by EU along with assessing total DSP toxins [19]. DTX3 stability with pre-cooking was examined by spiking clam homogenate (DOH1209) with PTX2 (22 ng/g) from original CRM solution and the toxic mussel extract, along with their unspiked controls, with heating at 70 ºC (20 min) or 76 ºC (40 min) before extraction. No obvious degradation of total or free OA occurred at either temperature, whereas PTX2 was

partially degraded (data not shown). Heat treatment (90 ºC) of shellfish homogenates has not shown significant effects on AZAs [20].

Trace levels of DSP toxin isomeric impurities (2, 1, and 2 isomers with RT less than parent toxins for OA, DTX2, and DTX1, respectively) from methanolic CRM (Certificate of Analyses) were observed for ~32 pg injections (less with enhanced MS sensitivity achieved later) of each toxin, with either fast (e.g., 10% to 80% B in 11 min) or slow LC gradients. Small amounts of DSP toxins degraded to their isomers with RT closer to parent toxins during heating and storage; for spike-and-recovery samples calibrated with solvent standards, conversion percentages were < 2% and < 4% for non-hydrolyzed and hydrolyzed samples, respectively, at ~ 2 months after sample preparation, and < 5% for non-hydrolyzed samples ~2.5 years afterwards. Under most alkaline LC conditions, OA and DTX2 did not resolve with their degraded isomers, allowing quantification to remain virtually unaffected by isomerization.

4. Conclusions

Low extraction efficiency of OA and DTX1 was obtained for four of five shellfish species from WA, USA, using standard EURLMB-recommended extraction method. Pre-cooking raw shellfish homogenates (70 ºC, 20 min) yielded extraction efficiencies for most lipophilic toxins comparable to or better than original EU protocol - except for PTX, which is not regulated in US. Extraction efficiency of YTX, which is also not regulated in US, was less than 80% for clams using original or modified (i.e., use of acidified methanol or with pre-cooking step) EU protocols, with acidified methanol yielding the lowest YTX recovery for all shellfish species examined. Further investigation of YTX extraction from clams should be pursued if regulatory action levels are established for this toxin in US. Sample clean-up improved upon published methods and, in combination with LC-MS adjustments, mitigated matrix effects and permitted quantification of US FDA-regulated toxins with acceptable accuracy in five WA shellfish species using solvent-based calibration standards and alkaline mobile phases.

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Tables; Wang & Doucette_JCA-20-1808

Table 1

Recovery results (%) using EU extraction method.[a]

Sample ID	Shellfish type	OA	DTX2	DTX1	YTX	AZA1	AZA2	AZA3	PTX2
Control	blue mussel	67	97	64	89	96	90	95	103
DOH886	California mussel	72	95	81	64	106	103	104	103
DOH966	blue mussel	71	96	61	88	104	100	104	106
DOH1338	Manila clam	70	93	60	59	99	111	110	96
DOH1661	littleneck clam	88	92	86	74	105	99	93	103
DOH2056	Pacific oyster	73	99	78	84	100	102	103	106

[a]LC gradient: 3.5 min of 10% B, linear gradient to 90% B at 19.5 min and held for 3 min, returned to 10% B at 24.5 min and held for 4 min. Injection volume was 4 µl.

Table 2

Recovery results (%) using acidified methanol extraction method.[a]

Sample ID	Shellfish type	OA	DTX2	DTX1	YTX	AZA1	AZA2	AZA3	PTX2
DOH886	California mussel	102	117	99	61	104	105	104	105
DOH966	blue mussel	107	100	99	66	118	112	111	107
DOH1338	Manila clam	104	103	100	29	108	108	106	96
DOH1661	littleneck clam	97	110	96	49	109	103	108	97
DOH2056	Pacific oyster	89	98	95	42	106	102	104	92

[a]Different LC gradients were used. All samples were 1.2 ml (except 1.5 ml for DOH966) of hexane-washed extracts for SPE clean-up. Injection volume was 4 µl.

Table 3

Recovery results (%) for samples using method incorporating cooking prior to EU extraction protocol.

Sample ID	Shellfish type	OA/ total OA	DTX2/ total DTX2	DTX1/ total DTX1	YTX	AZA1	AZA2	AZA3	PTX2
DOH886	California mussel	97/105	96/93	93/100	91	107	105	104	94
DOH966	blue mussel	93/108	93/91	91/94	85	106	111	110	96
DOH1338	Manila clam	91/106	97/113	102/104	61	94	96	89	102
DOH1209	littleneck clam	111/104	105/100	109/93	42	92	93	95	95
DOH2056	Pacific oyster	103/98	100/100	117/100	88	98	101	101	108

Table 4

Recovery (Rec.; %) and Relative Standard Deviation (RSD; %) of toxins in non-hydrolyzed samples calibrated with solvent-based standards using the final LC-MS protocol.[a]

Sample ID; shellfish type (n =3)	LC-MS sample sequence	OA		DTX2		DTX1		YTX		AZA1		AZA2		AZA3		PTX2	
		Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD
DOH886; California mussel	1-1	90	1.5	102	3.8	92	1.9	94	1.4	101	1.2	119	2.7	127	1.5	101	3.3
	1-2	102	5.4	99	3.2	95	5.2	100	1.8	101	2.5	121	2.0	128	2.2	107	6.7
	1-3	94	3.6	93	3.0	92	4.2	92	7.4	100	1.2	120	0.6	126	2.3	102	2.2
	2-1	94	6.5	100	2.2	96	8.9	87	6.4	98	1.9	111	2.2	126	0.9	110	3.2
	2-2	96	7.3	104	3.2	92	2.4	95	2.3	103	1.3	120	3.0	129	1.0	129	3.0
DOH966; blue mussel	1-1	92	6.0	89	2.8	93	2.8	94	2.0	103	3.1	123	3.4	131	3.5	97	5.2
	1-2	96	2.0	94	2.0	92	6.7	103	5.4	103	3.1	120	2.0	127	1.4	99	3.2
	1-3	93	0.5	92	10	94	9.2	95	3.2	104	2.3	119	4.6	128	1.9	105	2.7
	2-1	91	3.4	94	5.6	92	0.8	93	8.0	99	1.1	113	1.8	127	2.0	106	2.3
	2-2	89	2.3	98	8.4	95	4.9	102	4.2	109	2.6	124	3.7	132	1.0	119	3.3
DOH1209; littleneck clam	1-1	94	8.0	99	15	106	4.8	64	8.1	93	0.6	111	1.6	118	1.7	24	5.5
	1-2	94	4.1	95	7.6	97	3.3	56	4.8	93	1.1	107	2.4	117	0.5	26	7.0
	1-3	95	4.0	98	5.5	99	2.2	52	1.8	93	0.9	102	7.3	115	1.5	26	1.8
	2-1	90	4.0	103	7.4	100	8.8	40	6.4	90	1.1	99	1.6	116	1.3	36	2.1
	2-2	98	5.7	114	0.7	104	5.1	42	2.5	95	2.2	108	3.0	116	1.8	39	2.8
DOH1942; Manila clam	1-1	85	4.6	84	6.8	91	6.8	76	5.3	96	2.5	114	3.1	119	2.6	54	4.8
	1-2	87	3.7	91	5.0	90	5.4	77	5.6	93	3.8	107	4.4	117	5.1	55	4.6
	1-3	88	3.1	89	5.5	94	3.5	71	9.2	94	5.3	108	3.6	115	4.0	57	6.5
	2-1	84	1.8	99	3.9	96	6.2	63	14	91	4.3	102	6.1	112	2.7	68	6.1
	2-2	89	2.5	101	1.2	89	3.6	74	11	100	3.6	113	3.2	120	3.4	77	5.7
DOH2056; Pacific oyster	1-1	86	2.9	94	6.3	94	1.9	94	5.9	99	2.3	120	1.5	125	1.2	67	4.8
	1-2	90	1.5	95	1.8	96	3.3	99	4.8	96	0.9	112	1.8	120	1.0	67	2.1
	1-3	90	1.5	100	3.7	94	2.6	98	1.5	94	0.8	111	1.0	120	1.0	65	4.7
	2-1	82	1.0	96	2.4	92	2.9	90	5.3	92	3.3	106	4.1	122	2.9	82	1.1
	2-2	92	0.4	111	3.8	101	3.3	103	8.2	102	1.5	120	1.9	124	1.1	94	1.0

[a]Two LC columns were tested and their usage is described in the text. "1-2" refers to LC column 1 and LC-MS sequence 2.

Table 5

Recovery (%) and RSD (%) of toxins in hydrolyzed samples calibrated with solvent-based using the final LC-MS protocol.

Sample ID; shellfish type (n =3)	LC-MS Sample sequence	OA		DTX2		DTX1	
		Rec.	RSD	Rec.	RSD	Rec.	RSD
DOH886; California mussel	1	98	2.7	98	1.4	92	1.9
	2	97	2.4	98	1.4	91	5.2
	3	100	7.9	104	3.1	97	8.3
DOH966; blue mussel	1	91	2.3	95	4.6	90	7.7
	2	93	2.5	94	6.1	93	3.1
	3	97	5.7	102	8.7	96	4.4
DOH1209; littleneck clam	1	93	0.4	96	0.7	90	3.1
	2	93	4.3	100	3.9	94	0.5
	3	97	1.0	98	3.0	94	1.6
DOH1942; Manila clam	1	88	8.9	100	7.3	90	8.0
	2	84	1.6	96	4.7	97	2.3
	3	85	5.0	99	8.2	92	3.3
DOH2056; Pacific oyster	1	85	4.3	93	2.0	88	1.2
	2	91	6.8	98	4.5	95	3.7
	3	91	0.4	100	7.5	96	4.6