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An Interlaboratory Study of Complementary Mass Spectrometric Methods for Quantifying Palytoxin-like Compounds in Seafood and Microalgae

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Abstract

Harmful algal blooms caused by *Ostreopsis* species are globally distributed and impact marine ecosystems and seafood safety. Some species produce palytoxin (PLTX)-like compounds, which are large, structurally complex molecules that are associated with respiratory irritation in bystanders, and foodborne poisoning following consumption of contaminated seafood. In Aotearoa-New Zealand, recurrent summertime blooms dominated by *Ostreopsis* cf. *siamensis* have occurred for nearly three decades and have been linked to ecological impacts, including sea urchin mortality. Robust analytical methods are required to support monitoring programs and emerging regulatory frameworks for measuring these toxins in seafood. This study aimed to develop a quantitative analytical method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) capable of detecting intact PLTXs, ovatoxins, and ostreocins in multiple biological matrices, and to evaluate its performance alongside an established oxidative cleavage analytical method. The intact toxin method achieved chromatographic separation of eight target analogues within a nine-minute run time and demonstrated acceptable sensitivity and precision, although recoveries varied across seafood matrices. Both methods were further assessed through an interlaboratory comparison involving laboratories in Aotearoa-New Zealand, the United Kingdom, Italy, and the United States of America. Mass spectrometers from three vendors were used, and the results showed good precision and accuracy for most analyte–matrix combinations. Together, the intact and oxidative cleavage approaches offer complementary capabilities for screening and confirming known PLTX-like analogues, while also identifying new analogues, thereby enhancing analytical capability to support HAB monitoring and risk assessment.

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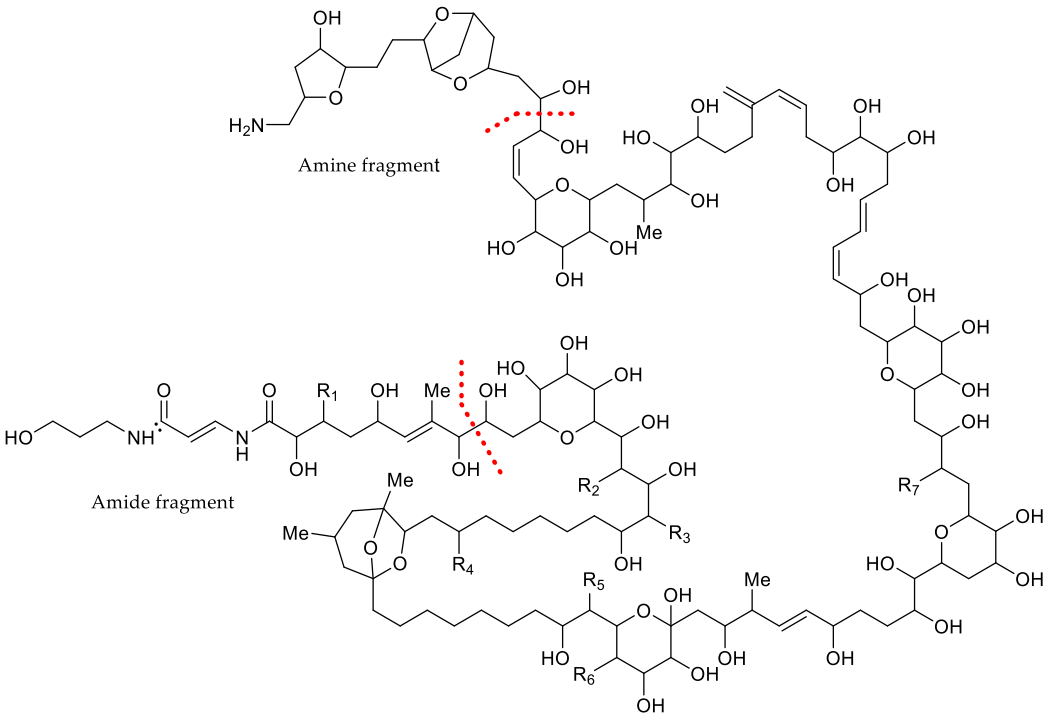
1. Introduction

Marine microalgae that cause harmful algal blooms (HABs) have been studied extensively, with a focus on those that adversely impact humans. These impacts occur through, for example, consumption of seafood contaminated with the toxins they produce or through other routes of exposure, such as direct skin contact with the microalgae or aerosolized toxins causing respiratory symptoms in bystanders. *Ostreopsis* is a genus within the family Ostreopsidaceae, with the first species, *O. siamensis,* being described from the Gulf of Siam in Thailand in 1901. With renewed research efforts, *Ostreopsis* spp. have been found to be distributed globally [1]. Intense blooms of *O.* cf. *ovata* have been recorded periodically over the last 25 years around the Mediterranean, with adverse human impacts being reported through direct contact with cells in seawater or through inhalation of aerosols created at the air–sea interface [2]. For example, in the summer of 2005, a massive *O.* cf. *ovata* bloom occurred along the Italian coastline near Genoa that resulted in negative impacts on marine life and adversely affected humans. Bystanders exposed to aerosols experienced respiratory symptoms with some requiring hospitalization [3]. It is hypothesized that *O.* cf. *ovata* blooms could increase due to favourable conditions on the French and Spanish coastlines as well as in the Adriatic Sea, although a decrease is proposed for the Tyrrhenian Sea [4]. *Ostreopsis* species have now been detected in the Northeast Atlantic, on macroalgal samples from Madeira and Canary Islands, and from both mainland Portugal and the Azores [5]. *O.* cf. *siamensis*, which has low toxicity relative to *O. ovata*, was the key species described initially, but now the more toxic *O.* cf. *ovata* has been determined to be a bloom former off the French Basque coast with respiratory disorders reported [6].

Although *Ostreopsis* spp. were initially reported in subtropical to tropical regions associated with macroalgae and coral reefs, they have, in recent years, been found in more temperate waters associated with macroalgae and seagrasses [1,7]. Morphology-based identification is often difficult, and molecular approaches are usually required to determine the species. To date, the genus comprises thirteen described species, four being regarded as potentially toxic to humans (*O.* cf. *ovata*, *O. siamensis*, *O. mascarenensis*, *O. fattorussoi*) [8]. Extensive blooms of *Ostreopsis* spp. have occurred regularly during the austral summer in Aotearoa-New Zealand over the last three decades. The blooming species has been identified as *O.* cf. *siamensis*, a species which has yet to be fully described [9,10]. Blooms have been reported from the northern tip of Te Ika-a-Māui, the North Island, down to the Hauraki Gulf around Auckland. They have been predominantly found covering macroalgae beds and cells are rarely observed in the water column (New Zealand's National Marine Biotoxin monitoring program phytoplankton records) [11]. *O.* cf. *siamensis* produces palytoxin (PLTX)-like compounds and kina (sea urchins; *Evechinus chloroticus*) are vulnerable to these blooms with mass mortalities occurring [11]. Three species of *Ostreopsis* have been reported in Aotearoa-New Zealand to date, *O. ovata*, *O. lenticularis* and *O.* cf. *siamensis* [9,12], representing the earliest records of *Ostreopsis* at latitudes below 35° S.

The increased spread, frequency, and intensity of *Ostreopsis* blooms, particularly in coastal waters of the Mediterranean and surrounding Atlantic waters, have raised concern about the causes and consequences of these events. Transfer of toxins produced by *Ostreopsis* sp. through the marine food chain [13] and human illnesses associated with consumption of contaminated seafood have been documented in tropical regions with

clupeotoxism and Haff disease representing poisoning syndromes from eating clupeoid fishes like sardines, herrings or anchovies containing PLTX-like compounds [14]. Some *Ostreopsis* spp. produce a group of large, complex, and structurally related toxins known collectively as PLTX-like compounds, which comprise PLTXs, ovatoxins (OVTXs), mascarenatoxins, and ostreocins (OSTs) with example structures shown in Figure 1 [15–22]. Additional classes of bioactive compounds have also been reported from *O.* cf. *ovata*, including liguriatoxins, rivieratoxins [22], ostreol A [23] and ovataline [24]. Over the past two decades many variants have been described with many more likely to be discovered in the future.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
PLTX	Me	OH	OH	Me	H	OH	OH
OVTX-a	Me	H	OH	Me	OH	H	H
OST D	H	OH	H	H	OH	H	OH

Figure 1. Structures of palytoxin (from *Palythoa* spp.), ovatoxin-a (from *O.* cf. *ovata*) and ostreocin D (from *O.* cf. *siamensis*), showing the cleavage sites (red dotted line) to produce the diagnostic amine and amide substructures when using the oxidative cleavage method [25].

To better elucidate toxin production by *Ostreopsis* species and their transfer through marine food webs, robust and sensitive detection methodologies are required. Liquid chromatography tandem mass spectrometry (LC–MS/MS) provides a highly sensitive and selective analytical platform suitable for a broad range of marine biotoxins. LC–MS/MS supports structural characterization of novel toxin analogues and allows quantification of individual compounds produced by microalgae or accumulated in seafood. However, the analysis of PLTX-like compounds remains particularly challenging due to their large size, when compared to many other marine toxins, and their tendency to form multiple cationic adducts during electrospray ionization [26]. Despite these analytical complexities, progress has been made. Notably, the development of an oxidative cleavage protocol, followed by LC–MS/MS analyses, has enabled the targeted detection of diagnostic

substructures common to PLTX-like compounds [25]. This approach provides sensitive detection across diverse sample matrices, and in many cases yields structural information that can infer the identity of the intact parent compound. Nevertheless, there remains a need for analytical methods capable of unambiguously identifying intact PLTX-like toxins. Such confirmation is essential for integrating structural identity with compound-specific toxicological data, which together underpin risk assessments. This requirement has become increasingly important in light of potential regulatory limits for PLTX in shellfish being proposed [27] and the limited sensitivity of some established monitoring approaches.

The objective of this study was to develop a quantitative analytical method capable of detecting intact PLTX, OVTXs, and OSTs in a range of biological and environmental matrices, and to evaluate the performance of the method within our laboratory. Following satisfactory in-house validation, the performance of the newly developed intact method, together with the previously described oxidative cleavage LC–MS/MS approach, was assessed through an interlaboratory comparison involving three external laboratories using instruments from three vendors. The outcomes of these evaluations are presented and discussed herein.

2. Experimental Design

Initially, the intact method was developed, and key performance characteristics were assessed within our laboratory. The intact and oxidative cleavage methods then underwent a small interlaboratory study with collaborators in the United Kingdom (UK), Italy and the United States of America (USA), using LC-MS/MS instruments from Sciex, Waters and Agilent.

For the intact method, development involved optimizing LC conditions and MS parameters for the target compounds. Method performance characteristics, such as calibration linearity and accuracy, were assessed prior to conducting the interlaboratory study.

For the interlaboratory study, the matrices evaluated were Greenshell mussels (*Perna canaliculus*), Pacific oysters (*Magallana gigas)*, snapper (*Pargus auratus*) and *Ostreopsis* species microalgal cultures (*O.* cf. *ovata*, *O. rhodesiae*, and *O*. cf. *siamensis*). Homogenates of each blank seafood matrix and *Ostreopsis* extracts were prepared in Aotearoa-New Zealand and shipped frozen to the three overseas collaborators. A PLTX-only stock and a mixed stock that contained PLTX, OVTX-a and OST-D were also included in the shipment to fortify samples and generate calibration standards. Samples were prepared for analysis by study collaborators in their respective laboratories as per the provided standard operating procedures for the intact and oxidative cleavage methods. The method performance parameters investigated were linear range, precision, and accuracy.

For both the intact and oxidative cleavage methods, each blank seafood matrix was fortified with the mixed stock on three different days and extracted, with aliquots of the resulting supernatant used for each cleanup approach prior to analysis. For the intact method, aliquots were subjected to solid phase extraction cleanup (SPE), and a calibration curve was prepared from the mixed stock in solvent. For the oxidative cleavage method (modified from [25]), aliquots were subjected to on-column (SPE) oxidation and cleanup prior to analysis.

Samples fortified with the mixed stock prior to extraction were known as full spikes and used to assess the extractability from the matrix, and blank extracts fortified post-SPE were known as overspikes and used to assess the impact of matrix co-extractives on instrument response. For the oxidative cleavage method, overspikes were prepared using the mixed stock standards that had been pre-oxidized with periodic acid. A calibration curve was prepared by oxidizing the PLTX-only standard with periodic acid using the on-

column oxidation procedure. *Ostreopsis* extracts that contained endogenous levels of toxins were not fortified prior to analysis.

3. Results

3.1. Intact Method Development and Performance

LC conditions were optimized to allow chromatographic separation of the eight target compounds over a 9 min run time (Figure 2), with PLTX, OVTX-a and OST D (shown in bold in Figure 2) used for the multi-laboratory study. Target compounds were monitored in both positive and negative electrospray ionization modes with doubly or triply charged precursor ions, which provided the greatest sensitivity and specificity due to monitoring three multiple reaction monitoring (MRM) channels. Although baseline separation was not achieved in all cases, the use of specific MRM transitions supported unambiguous assignment of the chromatographic peaks. The retention time of each toxin monitored was confirmed by comparison with purified PLTX, OVTX-a and OST D or from a well characterized algal extract that was used as a quality control (QC) sample [28]. On-column isomerisation of PLTX was observed, resulting in a split peak. To demonstrate this, the first peak was isolated and re-injected, resulting in the immediate formation of the split peak. Therefore, both peaks were integrated in their entirety for PLTX quantitation.

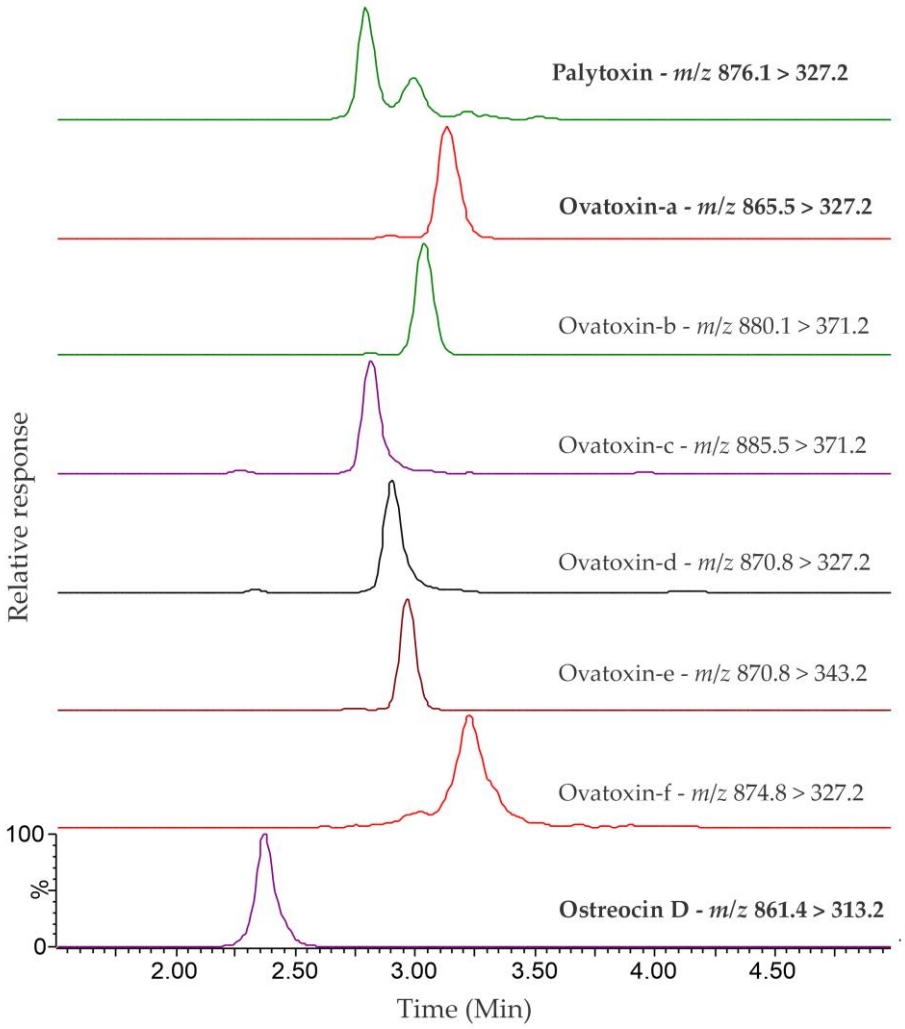


Figure 2. Multiple reaction monitoring transitions for palytoxin-like compounds present in microalgal QC samples or purified compounds when using the intact toxin method developed as part of this study. Those compounds in bold were monitored for the multi-laboratory study.

Recoveries for the three target toxins were consistent within each matrix but varied markedly between matrices (Table 1, Table S1). The highest recoveries were obtained from the 50% aqueous acetonitrile with 0.1% acetic acid solvent (95–99%), while the lowest were observed in oysters (55–59%). Mussels (70–74%) and fish flesh (60–70%) were consistently higher than oysters but still considerably lower than observed with solvent alone. Analytical repeatability was <10% RSD for all toxin–matrix combinations assessed. PLTX exhibited the greatest variability, especially in mussels.

Table 1. Intact method recovery and precision (mean ± SD, $n = 3$) of PLTX, OVTX-a and OST D from fortified solvent and seafood matrices. Single laboratory data.

	Recovery (%)		
Analyte	**PLTX**	**OVTX-a**	**OST D**
Solvent [a]	99 ± 4.4	95 ± 0.53	95 ± 1.4
Mussel	71 ± 12	74 ± 0.38	70 ± 3.5
Oyster	56 ± 8.9	59 ± 5.7	55 ± 8.6
Fish flesh	65 ± 10	60 ± 2.0	70 ± 3.2

[a] 50% aqueous acetonitrile with 0.1% acetic acid ([29]).

3.2. Intact Method Interlaboratory Comparison

Fortification experiments were performed (full spikes) on four matrices to evaluate extraction efficiency and analytical precision (Table 2). Solvent spikes gave the highest recoveries (87–93%) and lowest variability (6–7% RSD). Biological matrices showed lower and more variable recoveries for all analytes, with the trends observed in the interlaboratory study aligning with those from the single laboratory data. Recovery differences were also observed between the different laboratories, although precision remained within acceptable limits (<20%RSD), ranging from 6 to 18% RSD. Oyster were the worst-performing matrix, with recoveries consistently between 60 and 70%. Mussel and fish flesh showed intermediate performance with recoveries typically around 70%, and acceptable precision across all analytes monitored.

Table 2. Summary of intact method interlaboratory performance using samples fortified with PLTX, OVTX-a and OST D pre-extraction (known as full spikes).

Analyte	**Matrix**	**Recovery (%)**				**Mean**	**%RSD**
		Lab 1	**Lab 2**	**Lab 3**	**Lab 4**		
PLTX	Mussel	71	84	69	85	77	10
	Oyster	56	74	63	54	62	13
	Fish flesh	65	65	92	60	71	18
	Solvent	99	92	84	98	93	6
OVTX-a	Mussel	74	84	73	83	78	7
	Oyster	59	74	60	63	64	9
	Fish flesh	60	66	93	71	72	17
	Solvent	95	88	80	85	87	7
OST D	Mussel	70	88	69	76	76	10
	Oyster	55	81	54	75	66	18
	Fish flesh	70	70	60	65	66	6
	Solvent	95	96	82	83	89	7

To distinguish between losses during sample preparation and matrix-induced suppression, blank sample extracts were fortified with PLTX, OVTX-a and OST D post-SPE cleanup (overspikes; Table 3). Algal material was included in this assessment because its sample preparation differed from that of seafood samples (i.e., no SPE cleanup applied).

Across all toxin–matrix combinations, overspikes produced higher average recoveries than those obtained in the full spike experiments. However, matrix suppression on the LC-MS/MS signal was observed for all analogues on all matrices. Furthermore, the degree of suppression the varied, meaning results were reported as quantified using the LC-MS/MS method and not recovery-adjusted. Algal extracts showed excellent performance, with recoveries ranging from 95% to 101%. Overall, interlaboratory variability for the seafood samples was reduced with precision consistently <10%RSD. Data was most similar between Lab 1 and Lab 3, and Lab 2 and Lab 4.

Table 3. Summary of intact method interlaboratory performance using samples fortified with PLTX, OVTX-a and OST D post-SPE cleanup (known as overspikes).

Analyte	Matrix	Recovery (%)				Mean	%RSD
		Lab 1	Lab 2	Lab 3	Lab 4		
PLTX	Mussel	88	99	77	92	89	9
	Oyster	84	90	72	92	84	9
	Fish flesh	77	89	106	86	90	12
	Algae (CAWD257)	98	105	103	96	101	4
	Algae (CAWD208)	89	103	101	113	101	9
OVTX-a	Mussel	83	93	75	86	84	8
	Oyster	79	93	77	90	85	8
	Fish flesh	66	91	105	86	87	16
	Algae (CAWD257)	93	102	99	106	100	5
	Algae (CAWD208)	89	98	95	97	95	4
OST D	Mussel	78	101	73	111	91	17
	Oyster	76	94	69	95	84	14
	Fish flesh	74	96	70	82	80	12
	Algae (CAWD257)	94	105	97	99	99	4
	Algae (CAWD208)	85	108	98	98	97	9

An algal extract previously shown to contain PLTX-like compounds was included in the interlaboratory comparison to assess method performance on naturally contaminated material (Table 4). Labs 1 and 2 detected an isomer of PLTX in the three PLTX MRM transitions monitored, which aligned with the initial publication of this isolate [28]. The isomer eluted earlier than PLTX produced by *Palythoa* sp. and did not have the correct daughter fragment for isobaric PLTX produced by Mediterranean *O*. cf. *ovata* isolates [30]. All four labs detected OVTX-a in the sample. OST D was not produced by the strain of algae that was included.

Table 4. Summary of the microalgae extract (S0752) cell quota analysis for the intact method [28].

Analyte	Level Observed (pg/Cell)				Mean	%RSD
	Lab 1	Lab 2	Lab 3	Lab 4		
PLTX isomer	0.24	0.20	n/o	n/o	-	-
OVTX-a	5.6	5.2	6.0	5.4	5.6	4
OST D	n/o	n/o	n/o	n/o	-	-

n/o = not observed.

3.3. Oxidative Cleavage Method Pre-Study Evaluation

The oxidative cleavage method monitors diagnostic fragments produced during the periodate oxidation of PLTX-like compounds, as described by Selwood et al. [25]. For the three toxins included in this interlaboratory study, the reaction generates a shared amine fragment, referred to as the common amine fragment (Figure 1). Therefore, when the three

toxins monitored as part of this interlaboratory validation were present in a mixed standard at a concentration of 5 ng/mL each (PLTX/OVTX-a/OST D), the expected concentration of the common amine fragment was 3 × 5 ng/mL = 15 ng/mL. Oxidative cleavage at a different part of the molecule produces fragments that contain an amide moiety. In the case of PLTX and OVTX-a, the same amide fragment is formed, giving an expected concentration of 10 ng/mL from the mixed stock. OST D, however, produces a distinct amide fragment, resulting in an expected concentration of 5 ng/mL when the mixed stock is used. Experimental data generated at Cawthron prior to the interlaboratory study confirmed these expectations (Table 5). Recoveries of the common amine fragment ranged from 100 to 117%, while the amide fragment shared by PLTX and OVTX-a showed recovery of 98–126%. The OST D-specific amide fragment exhibited recoveries of 89–101%. In all cases, precision was ≤12% RSD, with repeatability in solvent being particularly tight (<2% RSD; n = 3 replicates). Greater variability was observed in biological matrices, with fish flesh showing higher variability than mussel or oyster extracts.

Table 5. Summary of the 'oxidative cleavage' method performance based on the analyses of samples fortified with a mixed stock containing PLTX, OVTX-a and OST D. Single laboratory data.

Analyte	Recovery (%)		
	Common Amine	PLTX/OVTX-a Amide	OST D Amide
Solvent	120	130	90
Mussel	113	100	98
Oyster	100	110	100
Fish flesh	113	120	102

3.4. Oxidative Cleavage Method Interlaboratory Comparison

Results from the interlaboratory study demonstrated good performance for all analytes monitored, independent of the matrix analyzed (Table 6). For the common amine fragment, accuracy ranged from 78 to 114%, with inter-precision consistently <14%RSD. Comparable performance was observed for the amide fragments: the PLTX/OVTX-a amide fragment recovery ranged from 87 to 129% with precision < 14%RSD, while the OST D amide fragment recovery ranged from 80 to 116% and precision < 13%RSD. Laboratory 3 consistently reported lower recoveries, contributing a large proportion of the variability seen across the different laboratories.

Table 6. Summary of performance characteristics of the 'oxidative cleavage' method for palytoxin (PLTX), ovatoxin-a (OVTX-a) and ostreocin D (OST D) in mussels, oysters and fish flesh.

Fragment	Matrix	Recovery (%)				Mean	%RSD
		Lab 1	Lab 2	Lab 3	Lab 4		
Amine—common	Mussels	111	106	91	109	104	8
	Oysters	100	102	78	112	98	13
	Fish flesh	114	101	89	112	104	10
Amide—PLTX/OVTX-a	Mussels	98	112	87	90	97	10
	Oysters	110	129	90	101	107	13
	Fish flesh	119	120	93	91	106	13
Amide—OST D	Mussels	99	104	80	95	94	9
	Oysters	99	116	82	102	102	12
	Fish flesh	101	116	89	97	100	10

As with the intact method assessment, samples were fortified with the mixed standard after SPE to evaluate the impact of matrix co-extractives on the analytical measurement (Table S2). Again, recovery was good, ranging from 101 to 111%, with results from

Laboratory 3 aligning more closely with those observed in the other laboratories. Interlaboratory precision was typically <10%RSD, and in all cases ≤ 12%RSD.

An algal extract previously shown to contain PLTX-like compounds was included in the interlaboratory comparison (Table 7). In this material the common amine fragment and amide fragment associated with PLTX/OVTX-a were detected. The amine fragment exhibited good repeatability (8%RSD), whereas the amide fragment showed greater variability (18%RSD). The amide fragment produced through oxidative cleavage of OST D was not present in this extract.

Table 7. Summary of the microalgae extract (S0752) cell quota analysis.

Fragment Monitored	(pg/Cell)				Mean	%RSD
	Lab 1	Lab 2	Lab 3	Lab 4		
Amine—common	2.0	2.4	2.5	2.1	2.3	8
Amide—PLTX/OVTX-a	2.9	2.6	1.8	2.1	2.4	18
Amide—OST D	n/o	n/o	n/o	n/o	-	-

n/o = not observed.

4. Discussion

We successfully developed a rapid LC-MS/MS method for the detection and quantitation of intact PLTX-like compounds. The performance of this method and that of a previously developed oxidative cleavage method were assessed through an interlaboratory study. The development of the intact method addresses a long-standing gap in the ability to detect and quantify these compounds in seafood and microalgal cultures. They are among the largest and most structurally complex marine biotoxins known, factors that make their analysis particularly challenging. Method development focused on sample preparation, chromatographic separation and overcoming issues arising from mass spectrometry (MS) analysis of high-molecular-weight compounds, including the formation of multiple cationic adducts during electrospray ionization [31]. The resulting method can quantify eight known intact PLTX-like compounds across a range of seafood matrices using a straightforward sample preparation procedure comprising extraction in aqueous methanol, solid-phase extraction cleanup, and analysis on a standard reversed-phase UHPLC column. Additional compounds can be added to the method as purified samples and eventually reference material for individual congeners become available and/or their MS characteristics are defined. By carefully selecting doubly and triply charged precursor ions and compound-specific MRM transitions, sufficient chromatographic resolution and confident identification of the target analytes were achieved within a nine-minute analytical runtime, enabling rapid sample throughput. The shorter chromatographic run time represents a significant improvement over the intact method proposed by Suzuki et al. [28], with a run time of 20 min, by Ciminiello et al. [32], with run times ranging from 15 to 31 min depending on the column and gradient conditions employed, and by Klijnstra and Gerssen [33], who used lithium in the mobile phases to separate the OVTXs within a runtime of 15 min.

Chromatographic peak shapes were generally acceptable, although minor additional peaks were occasionally observed near the target retention times, likely reflecting limitations in mass resolution on the LC-MS/MS systems. For example, closely related precursor ions such as triply charged PLTX with four water losses (*m*/*z* 870.2) and OVTX-d with three water losses can overlap (*m*/*z* 870.8), and both produce the same product ion (*m*/*z* 327.2), which cannot be fully distinguished on a triple quadrupole instrument. Similarly, the $[M+3H-4H_2O]^{3+}$ ion of isoPLTX and $[M+3H-3H_2O]^{3+}$ ion of OVTX-e both produce the same product ion at *m*/*z* 343 and thus could interfere with MRM transitions if not

chromatographically separated. While complete baseline separation was not achieved for all analytes, consistent retention times were observed, and when combined with the use of quantitative and confirmatory multiple reaction monitoring transitions, provided sufficient specificity for confident identification and quantification. The ability to directly quantify intact PLTXs, OVTXs, and OSTs in a range of seafood matrices represents an important advance in understanding what compounds are present and provides a basis to estimate the toxicity of contaminated samples.

Findings from the interlaboratory method comparison demonstrated that both the intact and oxidative cleavage methods perform satisfactorily across laboratories operating different analytical instrumentation (Figure 3). For the intact method, recovery trends were similar to those observed during the single-laboratory validation, with solvent controls consistently yielding the highest recoveries and seafood matrices exhibiting lower, matrix-dependent performance. Oyster tissue was identified as the most challenging matrix, with reduced recoveries attributable primarily to poor extraction efficiency rather than matrix-induced ion suppression, as confirmed by the pre- and post-SPE fortification experiments. Despite interlaboratory differences in absolute recovery, precision remained within generally accepted performance criteria (<20% RSD) for all toxin–matrix combinations, indicating satisfactory reproducibility. The oxidative cleavage method performed very well, showing consistently high recoveries and low interlaboratory variability across all matrices evaluated, reflecting its reduced susceptibility to matrix effects and strong analytical robustness. The consistently high recoveries observed for both methods in algal extracts further emphasize their suitability for direct toxin profiling from *Ostreopsis* culture material.

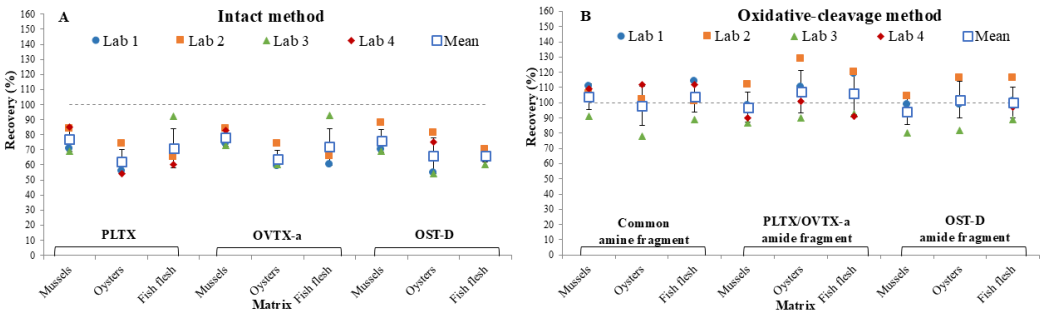


Figure 3. Summary of recoveries obtained by the four laboratories for the intact and oxidative cleavage methods across the matrices investigated. Plot (**A**) represents recoveries when using the intact method (Table 2), and plot (**B**) represents the oxidative cleavage method (Table 6).

The inclusion of a naturally contaminated algal extract in the interlaboratory comparison provided an opportunity to directly assess method performance on real-world samples. Both approaches showed good agreement, with the oxidative cleavage method providing supportive structural information and the intact method enabling compound-specific confirmation. Collectively, these results highlight the complementary strengths of the two approaches, with oxidative cleavage providing a highly reliable and sensitive screening tool across complex matrices, and the intact method delivering compound-specific confirmation essential for definitive toxin identification.

When combined with the oxidative cleavage LC–MS/MS approach, the intact method forms part of an effective tiered analytical strategy. The oxidative cleavage method provides sensitive, matrix-tolerant screening through the detection of diagnostic amine and amide fragments common to PLTX-like compounds, as demonstrated by its strong interlaboratory performance across all matrices evaluated. This approach enables rapid identification of samples containing PLTX-related compounds, even in complex biological

matrices. Subsequent confirmatory analysis using the intact method then allows definitive assignment of specific analogues, resolving structural ambiguities inherent to fragment-based detection alone. Together, these complementary methodologies balance analytical efficiency and structural certainty, making them well suited for routine monitoring, outbreak investigation, and research applications where both sensitivity and specificity are required.

This approach also provides an opportunity for the discovery of previously uncharacterized PLTX-like compounds. The detection of oxidative cleavage fragments without corresponding intact toxin matches suggests the presence of novel, or structurally modified, analogues beyond those described to date. Further details on using the intact and oxidative cleavage methods for compound discovery can be found in Varra et al. 2026 [34]. This is particularly relevant given the growing number of PLTX-like variants reported from *Ostreopsis* species. Therefore, the oxidative cleavage method can serve as an initial flag for the presence of PLTX-related chemistry, while the intact method can provide further structural elucidation. Complementary high-resolution mass spectrometric techniques will further provide diagnostic information to support structural elucidation of previously undescribed PLTX-like compounds.

5. Materials and Methods

5.1. Chemicals, Reagents and Materials

LC-MS grade methanol and acetonitrile were used for sample preparation and LC-MS/MS analysis, with Milli-Q water (Merck, Darmstadt, Germany) used throughout. Glacial acetic acid and periodic acid were used for preparation of extraction and mobile-phase solvents and for the oxidative cleavage procedure, respectively. Phenomenex Strata-X solid-phase extraction (SPE) cartridges were used for sample cleanup and on-column oxidation, as described below. Palytoxin (PLTX) reference material was obtained from Fujifilm Wako Pure Chemical Corporation, while purified ovatoxin-a (OVTX-a) and ostreocin-D (OST-D) were gifted by Professor Carmela Dell'Aversano and Professor Masayuki Suzuki, respectively. Specific instrumentation and instrument-dependent parameters used by each participating laboratory are detailed in the relevant sections and supplementary tables.

5.2. Study Samples and Purified Toxins

Study materials for the interlaboratory study were prepared at Cawthron and distributed to collaborating laboratories in Italy, the UK and the US, providing datasets from four laboratories (including Cawthron). Greenshell mussel (Mussel; *Perna canaliculus*), Pacific oyster (Oyster; *Magallana gigas*), and snapper (Fish flesh; *Pargus auratus*) flesh was homogenized in a plastic pottle using a stick blender. Approximately 25 g of each homogenate was transferred into plastic pottles and frozen prior to shipping. Microalgal cultures of known cell concentration and volume were centrifuged at 3200× *g* for 10 min to pellet intact cells. The supernatant was decanted to afford a 'dry' pellet, which was then extracted twice with 50% aqueous methanol containing 0.1% acetic acid aided with sonication for 10 min at 59 Hz. The final target extract concentration was 100,000 cells/mL.

A PLTX stock solution was prepared by dissolving 100 µg of commercially available Wako (Fujifilm Wako Pure Chemical Corp, Osaka, Japan.) standard with 3.866 mL of 50% aqueous acetonitrile (calculated by weight using a 4 d.p balance) to give a concentration of 25.9 µg/mL. The molar extinction coefficient of PLTX was determined by spectrophotometry at 233 nm (41,082 $M^{-1}.cm^{-1}$) and 262 nm (23,697 $M^{-1}.cm^{-1}$). OVTX-a (94% pure) was obtained from *O.* cf. *ovata* NIES3351 isolate according to Miele et al. [35]. The concentrations of the OVTX-a and OST D reference materials were determined by HPLC-UVD

monitoring at 233 nm using PLTX as a calibrant. From these materials a PLTX-only stock and mixed stock containing PLTX, OVTX-a, and OST D were prepared at a concentration of 2.5 μg/mL in 50% aqueous acetonitrile. A 375 μL aliquot of each stock was provided to each laboratory participating in the study to prepare calibration curves and to fortify samples. The stock solutions were included in the shipment containing the seafood homogenates and algal extracts for each of the participating laboratories.

5.3. Intact Method Sample Preparation

PLTX-like compounds were extracted (single dispersive) from 2 g of fish/shellfish homogenate using 18 mL of 50% aqueous methanol. Samples were homogenized using an Ultra-Turrax (IKA, Guangzhou, China), or similar, and then centrifuged at 3200× *g* for 10 min to pellet insoluble debris. The extract was cleaned up using Phenomenex (Torrance, CA, USA) Strata-X 60 mg/6 mL SPE cartridges, which were conditioned with 3 mL of methanol followed by 3 mL of Milli-Q water, loaded with 6 mL of sample, washed with 3 mL of 40% aqueous methanol and then eluted with 3 mL of 80% aqueous methanol containing 0.1% acetic acid. The eluant was collected and transferred into polypropylene autosampler vials for LC-MS/MS analysis. Microalgal extracts were analyzed for PLTX-like compounds by direct injection by LC-MS/MS. These extracts were centrifuged prior to analysis to remove any insoluble material that had precipitated during frozen storage. To generate calibration curves for PLTX, OVTX-a and OST D, calibration standards ranging from 0 to 50 ng/mL were prepared from a 250 ng/mL intermediate mixed stock in 80% aqueous methanol with 0.1% acetic acid, to match the SPE eluent solvent, and analyzed. To assess method performance, samples were fortified with the mixed stock solution either prior to solvent extraction (full spike) or after SPE cleanup (overspike) to achieve an expected concentration of 5 ng/mL (equivalent to 25 μg/kg). Full spikes were prepared by adding 20 μL of the 2.5 μg/mL mixed stock, while overspikes were prepared by fortifying blank post-SPE extracts with 10 μL of a 250 ng/mL intermediate stock.

5.4. Oxidative Cleavage Sample Preparation

The oxidative cleavage method was the same as that described in [36]. The principle of this method is that PLTX-like compounds are oxidized with periodic acid as part of an on-column SPE cleanup procedure, which produces specific oxidation fragments that can be monitored in the eluant to provide insight into the presence of PLTX-like compounds. PLTX-like compounds were extracted (single dispersive) from 2 g of fish/shellfish homogenate using 18 mL of 50% aqueous methanol. Samples were homogenized using an Ultra-Turrax, or similar, and then centrifuged at 3200× *g* for 10 min to pellet insoluble debris. This represents the same sample extraction approach as used for the intact method. For the SPE cleanup and on-column oxidation, 6 mL of the supernatant was loaded onto a Phenomenex Strata-X 33 μm 60 mg/3 mL cartridge, washed with 3 mL of 40% aqueous methanol and 3 mL of Milli-Q water prior to on-column oxidation with 2 mL of 50 mM periodic acid. The SPE cartridges were then washed with 2 mL of Milli-Q water. The compounds of interest were eluted with 3 mL of 60% aqueous methanol containing 0.1% acetic acid and transferred to a glass autosampler vial for LC-MS/MS analysis. For algal samples and for generation of a calibration curve for the common amine fragment present in PLTXs, OVTXs, and OSTs, calibration standards (0–50 ng/mL) were prepared by on-column oxidation of PLTX. A 1 mL aliquot of algal extract or serial dilutions of the 2.5 μg/mL or 250 ng/mL PLTX-only stock in 50% aqueous methanol to align with the extraction solvent (accounting for the three-fold dilution during SPE) were loaded onto conditioned cartridges and oxidized on-column in the same manner as the study samples.

5.5. Instrumentation

The single-laboratory validation of the intact and oxidative cleavage toxin method was performed using a Waters Acquity I-Class UPLC system coupled with a Waters Xevo TQ Absolute tandem mass spectrometer (Waters, Milford, MA, USA). Interlaboratory validation was subsequently undertaken across participating laboratories using LC–MS/MS platforms from three instrument vendors to assess method transferability and robustness. These systems included an AB Sciex (Marlborough, MA, USA) 6500 Qtrap triple quadrupole mass spectrometer coupled to a Shimadzu (Kyoto, Japan) Nexera LC-30 UHPLC system, and an Agilent (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent 6495B triple quadrupole mass spectrometer, in addition to the Waters platform that was used for the single-laboratory validation. Instrument-specific source and compound-dependent parameters were optimized at each laboratory while maintaining the prescribed chromatographic conditions, MRM transitions, calibration approach, and sample preparation procedures to support comparability of results across platforms.

5.6. Instrument Conditions

Chromatographic separation for both the intact and oxidative cleavage methods was performed using a Waters Acquity UHPLC BEH C18 column (1.7 µm, 50 × 1 mm) with key method/instrument settings detailed in Table 8. For the intact method, the initial solvent composition was set at 5% B1, with a linear gradient increasing from 25% to 35% B1 between 1 and 6 min, before ramping up to 90% B1 by 6.2 min, holding until 7 min and returning to 5% B1 at 7.2 min. The column was re-equilibrated with 5% B1 for a total run time of 8.5 min. The column temperature was set to 25 °C to help reduce the on-column isomerisation observed with PLTX. For the oxidative cleavage method, the initial solvent composition of 0% B1 was held for 0.5 min, before a linear increase to 60% B1 over 2.5 min, ramping to 100% B1 by 2.6 min. Between 2.6 and 3 min the gradient was held at 100% B1 before rapidly returning to 0% B1 at 3.1 min and re-equilibrating for a total run time of 4 min. The column temperature was set at 40 °C to reduce backpressure and improve peak shape. Study participants were expected to use LC-MS/MS grade solvents for the preparation of samples, calibrants and instrument solvents.

Table 8. Summary of LC instrument conditions used for both analytical methods on a Waters TQ Absolute LC-MS/MS system.

Parameter	Intact Method	Oxidative Cleavage Method
Solvent Line A1	Milli-Q water + 0.1% glacial acetic acid	
Solvent Line B1	Acetonitrile + 0.1% glacial acetic acid	
Solvent Line A2	Not used	
Solvent Line B2	Methanol	
Seal wash	70% aq. Acetonitrile	
Needle wash	10% aq. Acetonitrile	
Injection volume	2 µL	3 µL
Run time	8.5 min	4 min
Flow rate	0.20 mL/min	0.25 mL/min
Column temp	25 °C	40 °C
Autosampler temp	10 °C	10 °C

The methods were established using specific multiple reaction monitoring (MRM) transitions as summarized in Tables 9 and 10. The Waters TQ Absolute tune settings are summarized in Table 11, with the Sciex and Agilent settings in Tables S3–S6 and Tables S7–S10, respectively. Targeted compounds in the intact method were monitored in both

positive and negative electrospray ionization modes with a dwell time of 0.015 s. For the oxidative cleavage method, only positive electrospray ionization transitions were used, with a dwell time of 0.05 s applied to all transitions.

Table 9. MRM transitions used for the intact method and collision energies (CE) for the Waters TQ Absolute mass spectrometer.

Compound	MRM	Precursor Ion	ESI Mode	CE (eV)
Palytoxin	**876.1 > 327.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Palytoxin	*876.1 > 76.1*	*$[M+3H-3H_2O]^{3+}$*	*+ve*	*40*
Palytoxin	*1338.7 > 199.1*	*$[M-2H]^{2-}$*	*–ve*	*70*
Ovatoxin-a	**865.5 > 327.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Ovatoxin-a	*865.5 > 76.1*	*$[M+3H-3H_2O]^{3+}$*	*+ve*	*40*
Ovatoxin-a	*1322.7 > 199.1*	*$[M-2H]^{2-}$*	*–ve*	*70*
Ovatoxin-b	**880.1 > 371.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Ovatoxin-b	*880.1 > 120.1*	*$[M+3H-3H_2O]^{3+}$*	*+ve*	*40*
Ovatoxin-b	*1344.8 > 199.1*	*$[M-2H]^{2-}$*	*–ve*	*70*
Ovatoxin-c	**885.5 > 371.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Ovatoxin-c	*885.5 > 120.1*	*$[M+3H-3H_2O]^{3+}$*	*+ve*	*40*
Ovatoxin-c	*1352.7 > 199.1*	*$[M-2H]^{2-}$*	*–ve*	*70*
Ovatoxin-d,	**870.8 > 327.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Ovatoxin-e	**870.8 > 343.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Ovatoxin-d, Ovatoxin-e	*870.8 > 76.1*	*$[M+3H-3H_2O]^{3+}$*	*+ve*	*40*
Ovatoxin-d, Ovatoxin-e	*1330.7 > 199.1*	*$[M-2H]^{2-}$*	*–ve*	*70*
Ovatoxin-f	**874.8 > 327.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Ovatoxin-f	*874.8 > 76.1*	*$[M+3H-3H_2O]^{3+}$*	*+ve*	*40*
Ovatoxin-f	*1336.8 > 199.1*	*$[M-2H]^{2-}$*	*–ve*	*70*
Ostreocin-d	**861.4 > 313.2**	**$[M+3H-3H_2O]^{3+}$**	**+ve**	**20**
Ostreocin-d	*861.4 > 76.1*	*$[M+3H-3H_2O]^{3+}$*	*+ve*	*40*
Ostreocin-d	*1316.7 > 185.1*	*$[M-2H]^{2-}$*	*–ve*	*70*

Bolded rows indicate quantitation MRM transitions and italicized rows indicate qualifier MRM transitions. Additional MRM transitions are included that were not part of the multi-laboratory validation study.

Table 10. MRM transitions used for the oxidative cleavage method and collision energies (CE) for Waters TQ Absolute mass spectrometer.

Fragment Monitored	MRM	ESI Mode	CE (eV)
Common * amine	300.2 > 107.1	+ve	25
Neopalytoxin amide	325.2 > 76.1	+ve	13
Ostreocin amide	329.2 > 76.1	+ve	13
Palytoxin, ovatoxin-a, -d & -f amide	343.2 > 76.1	+ve	13
Homopalytoxin amide	357.2 > 90.1	+ve	13
Ovatoxin-e amide	359.2 > 76.1	+ve	13
Bishomopalytoxin amide	371.2 > 104.1	+ve	13
Ovatoxin-b & -c amide	387.2 > 120.1	+ve	13

* Amine fragment is common to all described palytoxins, ovatoxins and ostreocins.

Table 11. Waters Xevo TQ Absolute mass spectrometer tune parameters.

Parameter	Positive Ionization	Negative Ionization
Capillary (kV)	4	2.5
Cone (V)	30	30
Source temperature (°C)	150	150
Desolvation temperature (°C)	500	500
Desolvation gas flow (L/hr)	1000	1000
Nebuliser gas flow (Bar)	7	7
Cone gas flow (L/hr)	150	150
Collision gas flow (mL/min)	0.18	0.18
Q1 low mass resolution	2.8	2.8
Q1 high mass resolution	15	15
Q1 Ion Energy	0.1	0.6
Q2 low mass resolution	2.8	2.8
Q2 high mass resolution	15	15
Q2 Ion Energy	0.7	1.0
Gain	1	1

Linear calibration curves (0–50 ng/mL) were generated using purified PLTX, OVTX-a and OST D. Other PLTX-like compounds were quantified using calibration curves from the most structurally related toxin, assuming a similar ionization response under the applied experimental conditions. Method accuracy (recovery) was evaluated by fortifying a range of seafood matrices with PLTX, OVTX-a and OST D prior to sample preparation and analysis within our laboratory. For quantitation, the peak area response of the target analytes was measured using instrument quantitative data processing software following injections of calibration standards, quality controls, and samples. Calibration data was generated to determine the linear regression curve (5-point, forced through zero) for peak area response against toxin concentration, with an $R^2 > 0.98$ regarded as acceptable. Concentrations of analytes in samples were then calculated following integration of the peak areas determined from each primary MRM, using the linear regression of the appropriate calibration curve. Where purified toxins were unavailable, compounds were calibrated off the most structurally related compound for which pure toxin was available. For example, OVTX-b-f were quantified off OVTX-a. In all cases an assumed relative response factor of 1 was applied. For the intact method, retention times of the target analytes were confirmed using a well-characterized algal extract. Toxin concentrations were then determined in terms of micrograms per kilogram of tissue by application of the method dilution factors. A similar approach was taken with the oxidative cleavage method, where purified toxins were not available, quantitation was performed using the closest structurally related analogue with an assumed relative response factor of 1. For example, OVTX-b-f were quantified using OVTX-a, while homoPLTX and bishomoPLTX were quantified using PLTX. *Ostreopsis* algal cultures were used for the confirmation of retention times of compound-specific amide fragments where purified material was unavailable.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md24120428/s1, Table S1. Intact method performance and precision (mean ± SD, n=3) of PLTX, OVTX-a and OST D from fortified solvent and seafood matrices. Single laboratory data. Table S2. Overspike summary for Oxidative Cleavage. Table S3. Summary of instrument conditions used for both analytical methods on the Sciex 6500 instrument. Table S4. MRM transitions used for the intact method on the Sciex 6500 instrument. Table S5. MRM transitions used for the oxidative cleavage method on the Sciex 6500 instrument. Table S6. AB Sciex 6500 instrument source parameters for both methods. Table S7. Summary of instrument conditions used

for both analytical methods on an Agilent 6495B instrument. Table S8. MRM transitions used for the intact method and parameters for Agilent 6495B instrument in Positive ESI mode only. Table S9. MRM transitions used for the oxidative cleavage method for Agilent 6495B instrument. Table S10. Agilent 6495B instrument source parameters for both methods.

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