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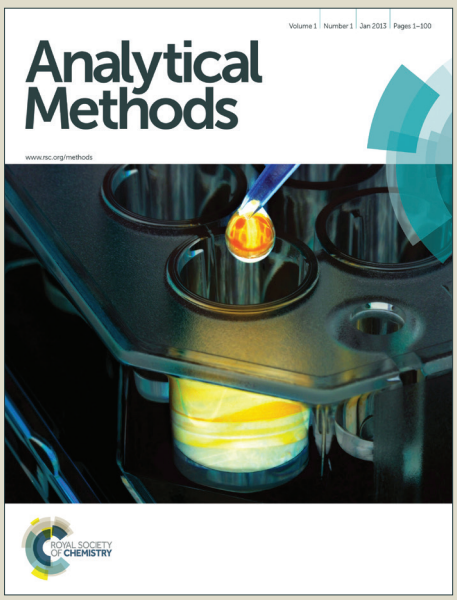

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Sensitive determination of domoic acid in mussel tissue using dansyl chloride derivatization and liquid chromatography - mass spectrometry

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Abstract

This paper describes a new method for sensitive determination of domoic acid (DA), the causative toxin of amnesic shellfish poisoning (ASP), in shellfish. The method involves extraction of tissue homogenates with 50% methanol followed by a highly selective strong anion exchange solid phase extraction and a derivatization with dansyl chloride (DNS-Cl) to form the dansyl derivative of domoic acid (DNS-DA). Reaction times were very rapid and proceeded under ambient conditions to yield stable derivatives. A study of the collision-induced dissociation of ESI-produced protonated DNS-DA was carried out to identify of the most sensitive transitions to use in development of a selected reaction monitoring detection method. Compared with un-derivatized DA, DNS-DA showed a 5-fold increase in sensitivity of MS/MS detection and improved retention on a reversed phase LC stationary phase. Resolution of DNS-DA and its isomers was achieved using isocratic elution in 15 min. A quantitative verification of the new method was carried out by analyzing a mussel tissue certified reference material (CRM) containing 49 mg/kg DA, as well as a toxin-free mussel tissue CRM spiked at levels ranging from 0.003 to 10 mg/kg levels. Results showed good recovery (83% - 107%) with a between-sample variability of $\leq$ 5% RSD. The LC-MS/MS method presented is suitable for DA analysis over a broad range of concentrations spanning from above the regulatory limit of 20 mg DA/kg tissue down to near the method detection limit of 1.1 µg DA/kg mussel tissue. The resulting method serves as a confirmatory method with alternative selectivity to existing methods. It is also suitable for quantification of low levels of DA in shellfish as an early warning sign for toxic events or in forensic applications after intoxication has occurred.

Keywords: domoic acid, dansyl derivatization, LC-MS, shellfish, algal toxin

1. Introduction

Domoic acid (DA), a secondary amino acid (Figure 1), is a naturally-occurring neurotoxin found in several species of the diatom *Pseudo-nitzschia* [1] and at low concentrations in various species of red algae [2]. Shellfish contamination with DA occurs when bivalves ingest the toxin-producing diatoms. This presents a significant risk to public health and the stability of the aquaculture and shellfish harvesting industries. The first report of human intoxication by domoic acid occurred in the fall of 1987, in Canada, when more than 150 people suffered from acute intoxication and 3 people died after eating contaminated cultured blue mussels (*Mytilus edulis*) [3, 4]. In most countries, the regulatory limit for DA in shellfish is set at 20 mg DA/kg edible tissue and routine testing is carried out by regulatory agencies to enforce this limit.

Liquid chromatography with UV detection (LC-UVD) is the most commonly used analytical technique for routine determination of DA in shellfish [5,6]. The detection limits achieved by these methods range from 0.02 to 1 mg DA/kg tissue, depending on the method of sample cleanup, the degree of pre-concentration and the sensitivity of the UV detector.

More sensitive analysis of DA in shellfish tissues, as well as unambiguous confirmation of the identity of detected LC peaks, can be achieved by using mass spectrometric detection. Analysis of DA in mussel tissue extracts by LC-MS was one of the first applications reported for early commercial ESI-MS instruments, which were capable of detection limits similar to those of LC-UV [7]. Since that time more sensitive instruments and more selective tandem mass spectrometry scan modes, such as selected reaction monitoring (SRM) with triple quadrupole

instruments or MS^3 with ion trap instruments, have been used to improve the selectivity and sensitivity of analysis [8-12]. The use of an alternative mode of liquid separation, hydrophilic interaction liquid chromatography (HILIC), in combination with MS/MS detection has also been reported as suitable for DA analysis in shellfish tissue [9]. The LODs of these LC-MS/MS methods range from 0.01 to 0.2 mg/kg tissue.

Various derivatization approaches have been employed to improve the detection limits of optical detectors for trace analysis of domoic acid in environmental samples such as seawater and phytoplankton. These include chemical derivatization with 9-fluorenylmethylchloroformate (FMOC) [13] or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [14] followed by LC determination with fluorescence detection. An alternative approach to derivatization is to use large volume LC injections of un-derivatized seawater to obtain lower limits of detection for DA (15 ng/L) [15]. Methods based on chemical derivatization are often not suited to the analysis of shellfish tissue extracts because of matrix interference with the derivatization reactions commonly encountered with crude extracts. For derivatization of shellfish tissue extracts to be feasible, a highly selective method of sample cleanup would need to be employed to isolate DA. A sample cleanup using a strong anion-exchange (SAX) solid-phase extraction (SPE) has been reported for use with LC-UV and LC-MS for the analysis of DA in complex shellfish tissue extracts [5]. The remarkable selectivity of this cleanup method could allow for chemical derivatization to be effectively used to lower detection limits for DA in shellfish tissue.

Compared to its use with optical LC detection, the use of chemical derivatization to enhance LC-MS analysis is a relatively new approach that is growing in popularity, particularly in fields

of proteomics and metabolomics [16]. Advantages of using derivatization in LC-MS analysis include increased ESI ionization efficiency, better chromatographic retention and reduced chemical interference from background ions in the low *m/z* region. To date, a limited number of applications of derivatization for LC-MS analysis of algal toxins have been reported for analysis of microcystins [17] and β-methylamino-L-alanine [18, 19].

Dansyl chloride (DNS-Cl) has long been used as a derivatization reagent for the trace detection of amino acids [20, 21]. The analysis of dansylated amino acids by HPLC is effective because of the speed, automation, and the low detection limits possible using fluorescence or chemiluminescence detection [20, 22, 23]. Dansylation has also been used to enhance the LC-MS detection of amino acids [24] and in a novel differential isotope labeling metabolomics workflow to introduce a site of isotope labeling into amine and phenol containing metabolites [25]. DNS-Cl has the additional advantage over other patented amino acid derivatization reagents of being very inexpensive.

Here, we present the development of a new method for determination of DA in shellfish tissue that combines a SAX SPE sample cleanup with dansylation of DA and LC-MS/MS analysis. Additional analytical methods are required as confirmation of existing methodology for assignment of concentration values to certified reference material (CRM) and lower detection limits are desirable for environmental monitoring and forensic studies after toxic events. We report the optimization of the derivatization of DA in tissue extracts, the MS/MS dissociation and detection of DNS-DA using SRM, and the LC-MS/MS analysis of tissue extracts. Finally, a verification of the quantitative capabilities of the developed method was carried out to determine

its suitability for analysis of DA in shellfish tissues at a wide range of concentrations relevant to both trace environmental analysis and regulatory limits.

2. Materials and Methods

2.1. Chemicals and reagents

HPLC grade acetonitrile, methanol and hexanes-200 were obtained from Caledon (Georgetown, ON, Canada). Distilled water was deionized using a Milli-Q system equipped with ion-exchange and carbon filters (Millipore, Bedford, MA, USA). Dansyl chloride (DNS-Cl) and formic acid (ACS grade, 98%) were purchased from Sigma Aldrich (St. Louis, MO, USA) and di-sodium tetraborate was purchased from BDH laboratory (Poole, England). The National Research Council Canada (Halifax, NS, Canada) provided the domoic acid calibration solution, certified reference material (CRM) (CRM-DA-f, 327 ± 7 μM), as well as the DA-containing mussel tissue matrix CRM (CRM-ASP-Mus-d, 49 ± 3 mg DA/kg tissue) and the toxin-free control mussel tissue matrix CRM (CRM-Zero-Mus) used in quantitative method verification.

2.2. Sample extraction and clean up

Sample extraction and cleanup was carried out according to the method of Quilliam *et al*. [6] with minor modifications. Briefly, 4.0 g samples of tissue homogenate were combined with 16 mL of methanol:water (1:1, v/v), vortex mixed for 3 min and centrifuged at 6700 × *g* for 10 min. The supernatant was decanted into a storage bottle and stored at 4 °C. A strong anion exchange SPE cartridge (3 mL, 200 mg Supelco SAX, Sigma Aldrich) was conditioned with 6 mL of methanol followed by 3 mL of water and then by 3 mL of methanol:water (1:1, v/v). Crude extract was filtered to 0.45 μm and 5.0 mL was loaded drop wise onto the SPE cartridge.

It was then washed with 3 mL of methano1:water (1:1, v/v) and eluted with 3 mL of 1 M formic acid in acetonitrile. The eluate was then evaporated to dryness using a gentle stream of nitrogen at 50 °C and reconstituted in 2.0 mL acetonitrile:water (1:1, v/v).

2.3. Domoic acid derivatization with dansyl chloride

The derivatization reaction of domoic acid with dansyl chloride is summarized in Figure 1. A stock solution of 5.5 mM DNS-Cl in acetonitrile was prepared fresh daily. Dansylation of standards and samples was performed using a 5 min reaction time at ambient temperature by mixing 100 mM borate buffer, the DNS-Cl solution, the sample and acetonitrile to maintain 50% acetonitrile and a minimum molar ratio for DNS-Cl:DA of 20:1 for standards and 2000:1 for tissue samples. For quantitative LC-MS method verification, 100 µL of SAX eluate was mixed in an amber vial with 1.2 mL of borate buffer and 1.1 mL acetonitrile followed by addition of 100 µL of DNS-Cl stock solution. After capping the tube and shaking, the mixture was allowed to react at room temperature for 5-10 min. Excess DNS-Cl was removed by liquid-liquid extraction using two aliquots of hexane equal in volume to the reaction mixture. The upper hexane layers with the excess DNS-Cl were removed and discarded, leaving DNS-DA in the bottom aqueous layer, which was analyzed directly.

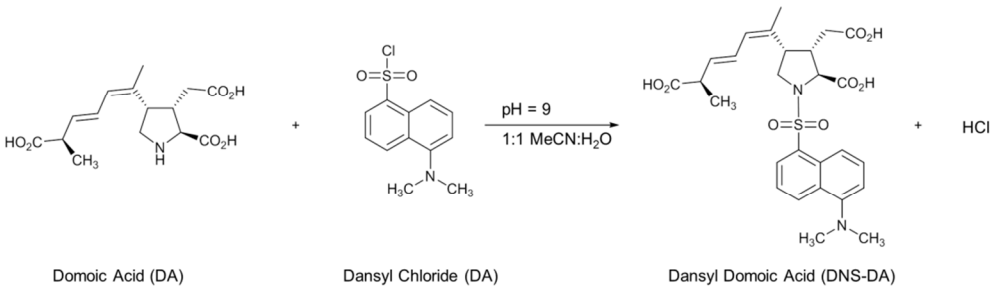

Fig. 1: Reaction of domoic acid with dansyl chloride to form dansyl domoic acid.

2.4. LC-MS Analysis

LC-MS analysis was performed using an Agilent 1260 series LC system (Santa Clara, CA, USA) coupled to an AB-SCIEX 5500 QTRAP mass spectrometer with a TurboSpray ionization source (Concord, ON, Canada) operated in positive ionization mode with an ionization voltage (IS) of 5500 V, a curtain gas (CUR) of 20 psi (nitrogen), a source temperature (TEM) of 600 ^{o}C and auxiliary gases (GS1 and GS2) of 50 psi (nitrogen).

During method development, the dansylation reaction was monitored using a hydrophilic interaction liquid chromatography (HILIC) - mass spectrometry method similar to that reported previously for determination of DA in shellfish tissue [9]. Separations were carried out using an LC column (250 mm x 2 mm I.D.) packed with 5 µm TSK-gel Amide-80 (Tosoh, Grove City, OH) held at 30 ^{o}C with a mobile phase of water (A) and 95% acetonitrile (B), each containing 2 mM ammonium formate and 50 mM formic acid. Gradient elution was carried out at 200 µL/min and consisted of a 5 min isocratic period at 90% B followed by a linear gradient to 30% B over 5 min and a 5 min isocratic period at 30% B before a 10 min column re-equilibration. Mass spectral data was acquired in full scan mode over a range from *m/z* 150 to 650 with a declustering potential of 50 V. Spectral intensity at *m/z* values corresponding to $[M + H]^+$ and $[M + Na]^+$ of DA (*m/z* 312 and 334) and DNS-DA (*m/z* 545 and 567) were used to evaluate the degree of completion of the dansylation reaction.

For quantitative analysis, reverse phase LC separations were performed using an LC column (100 x 2.0 mm I.D.) packed with 2.5 µm Luna C18 (Phenomenex, Torrance, CA) held at 70 °C. Aqueous (A) and organic (B) mobile phases consisted of 0.2% (*v/v*) formic acid in water

and acetonitrile, respectively. Isocratic elution was carried out at 30% B for 15 min at a flow rate of 0.5 mL/min. All sample injection volumes used were 1 µL. The SRM transitions monitored were *m/z* 545 > 170 at collision energy (CE) = 50 V used for quantitative analysis, and two confirmatory transitions of *m/z* 545 > 453 at CE = 25 V and *m/z* 545 > 261 at CE = 30 V, all with a declustering potential of 60 V, a dwell time of 100 msec and collision gas setting of 8.

2.5. Alternative Procedure for Ultra-trace Analysis

In order to investigate the lowest limits of detection and quantitation achievable using this combination of techniques, an alternative set of cleanup, derivatization and analysis parameters was developed. This alternative procedure compensated for all sample dilution steps throughout the cleanup procedure and used a larger, 5 µL injection volume to achieve the highest possible signal-to-noise values for ultra-trace analysis. Changes to the method described above included a decrease in the volume to which the SAX eluate was reconstituted from 2 mL to 0.2 mL and a decrease in the total volume of the dansylation reaction from 2.5 mL to 0.3 mL. This was done by using 0.1 mL of more concentrated 150 mM borate buffer and eliminating the additional dilution with acetonitrile.

3. Results and Discussion

3.1. Dansylation Derivatization Reaction

The dansylation of amino acids (Fig. 1) proceeds under basic conditions as it is the neutral amine that reacts by nucleophilic attack on DNS-Cl [24]. Compared with proteogenic amino

acids, pH control is especially important for dansylation of DA because of its three carboxylic acid functional groups. The previously published SAX SPE sample cleanup [6] is highly selective and results in an extract that was suitable for dansylation. Elution of DA from the SAX SPE is accomplished using acid, which had to be removed from the sample prior to dansylation. Previously, this elution was done using 20 mM aqueous formic acid (FA) [6], but reducing 5 mL of aqueous sample to dryness to remove excess FA and pre-concentrate the sample was not practical. In order to elute DA from the SAX cartridge using more volatile acetonitrile, the concentration of FA needed to be increased to 1 M, which made complete drying of the sample prior to re-constitution particularly important. To compensate for the higher acidity of DA and the possibility of residual FA remaining in the sample, the concentration of the stock borate buffer was increased from 25 mM used in initial trials with standards to 100 mM, which results in a pH of 9 to 9.5 in the final reaction mixture. Since DA has been found to have poor stability in strongly acidic (HCl) solutions [5], it is recommended that acidic SAX eluates not be stored for extended periods of time. However, no evidence of DA degradation was observed in this study when eluates were evaporated to dryness following cleanup.

Also important was the amount and type of organic solvent used in the reaction. Acetonitrile was chosen over methanol as DNS-Cl reacts rapidly with methanol to produce an undesirable side-product. Both the DNS-Cl stock solution and the reaction mixture itself should contain at least 50% acetonitrile since DNS-Cl precipitates in higher percentage aqueous samples. Temperature and reaction time were less important variables in the dansylation derivatization. It was found that the reaction for dansylation of DA was extremely rapid and that even a 5 min

reaction time at ambient temperature gave complete conversion to the desired product as monitored by HILIC-MS (Fig. 2.). Also, longer reaction times investigated (up to 90 min) did not show any signs of undesirable side-products or loss of DNS-DA. It was important however to remove excess DNS-Cl reagent and dansyl hydroxide, the hydrolysis product of DNS-Cl, in order to make samples more suitable for injection into the LC-MS. This was effectively and easily carried out using a small-scale liquid-liquid extraction with hexane.

Throughout method development, progress of the dansylation reaction was monitored using a HILIC-MS method similar to one reported previously for the analysis of un-derivatized DA in shellfish tissue [9]. This method, chromatograms of which are shown in Fig. 2, was suitable for selective detection of both DA and DNS-DA and was used to establish a suitable molar excess of DNS-Cl for dansylation of standards (20:1) and extracts (2000:1). This excess ensured that the dansylation reaction (Fig. 1) proceeded to completion for tissue extracts, as well as for concentrated standard stock solutions used in preparation of calibration curves (up to 250 µM).

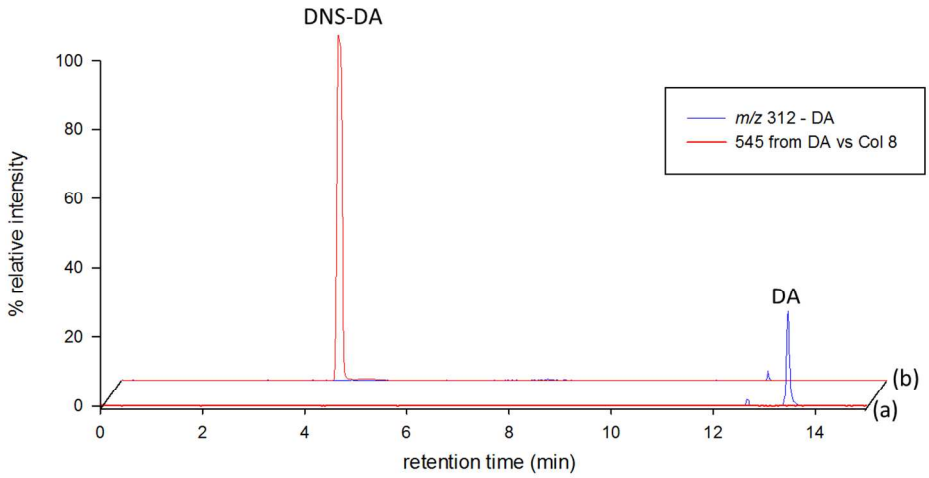

Fig. 2 - HILIC-MS monitoring of domoic acid dansylation. Trace (a) shows DA detection in an control sample without dansylation. Trace (b) shows the absence of DA and detection of DNS-

DA, respectively, after dansylation using a 5 min reaction time.

Also evident from Fig. 2 is the favorable impact of dansylation on ionization efficiency in the ESI source, resulting in improved sensitivity of LC-MS analysis compared to DA. In ESI, highly polar species often have relatively poor ionization efficiency when compared with ionizable species with significant non-polar character (e.g., surfactants). Introducing non-polar functionality in the form of a dansyl group into the polar DA structure has the desirable effect of increasing ionization efficiency and sensitivity by approximately 5-fold compared to direct analysis of DA. This increase in sensitivity, also observed in reverse phase (RP) LC, can at least partially be attributed to the higher % organic composition of the eluate for DNS-DA compared with DA in both HILIC and RPLC. These factors also have the desirable effect of reducing detection limits of the analysis, as described in subsequent sections of this work.

The stability of a derivative is an important consideration with automated analysis of a large number of samples, many of which may remain in an auto-sampler for several hours before analysis. A short-term stability study was carried out in order to verify the suitability of dansylation for use with automated analysis. This consisted of storing extracts and standards at + 4 ^{o}C, + 23 ^{o}C and + 38 ^{o}C and carrying out repeated analyses over a period of 7 days. The + 4 ^{o}C and + 23 ^{o}C samples showed no sign of degradation or isomerization over this period while the + 38 ^{o}C sample showed a small but not statistically significant decrease in concentration of around 5%.

3.2. Tandem Mass Spectrometry Detection of DNS-DA

Since tissue extracts contain abundant co-extractives that could react with dansyl chloride to form potentially interfering species, the selectivity of full scan MS used to monitor reaction progress in Fig. 2 was not considered adequate for trace analysis of DNS-DA in mussel tissue. Instead, a study of the tandem mass spectrometry reactivity of DNS-DA was carried out in order to develop a detection method that would exploit the superior selectivity of selected reaction monitoring (SRM). The full scan MS spectrum (Fig. 3A) of DNS-DA using positive ESI shows an abundant $[M + H]^+$ signal at *m/z* 545 with minor in-source fragmentation and only minor salt adducts corresponding to $[M + Na]^+$ and $[M + K]^+$ ions. The product ion spectrum of the $[M + H]^+$ precursor (Fig. 3B) at an intermediate collision energy is complex and includes several ions formed from multiple eliminations of H_2O and CO and/or HCOOH as reported previously for un-derivatized DA [9, 10], as well as cleavage at various positions around the dansylation site. Of these additional fragmentations, cleavage at the sulphonate bond in the DNS moiety to form the dimethylaminonaphthalenium ion as shown in Fig. 3B is the most abundant. This fragment ion is common to all dansylated amines and was the most intense fragment ion detected during a survey of the MS/MS reactivity of 32 dansylated amine containing metabolites [27]. The absence of product ions at *m/z* 500 for DNS-DA corresponding to elimination of dimethylammonia (-45 Da) is difficult to rationalize considering the previously proposed dimethylamino protonation site of dansylated amines [27]. Further study of the gas phase ion reactivity of protonated DNS-DA during collision induced dissociation would be required to establish whether protonation occurs at the highly resonance stabilized sulfone oxygen or on the

DA amino group. Both these possible protonation sites can be used to rationalize the observed reactivity of DNS-DA.

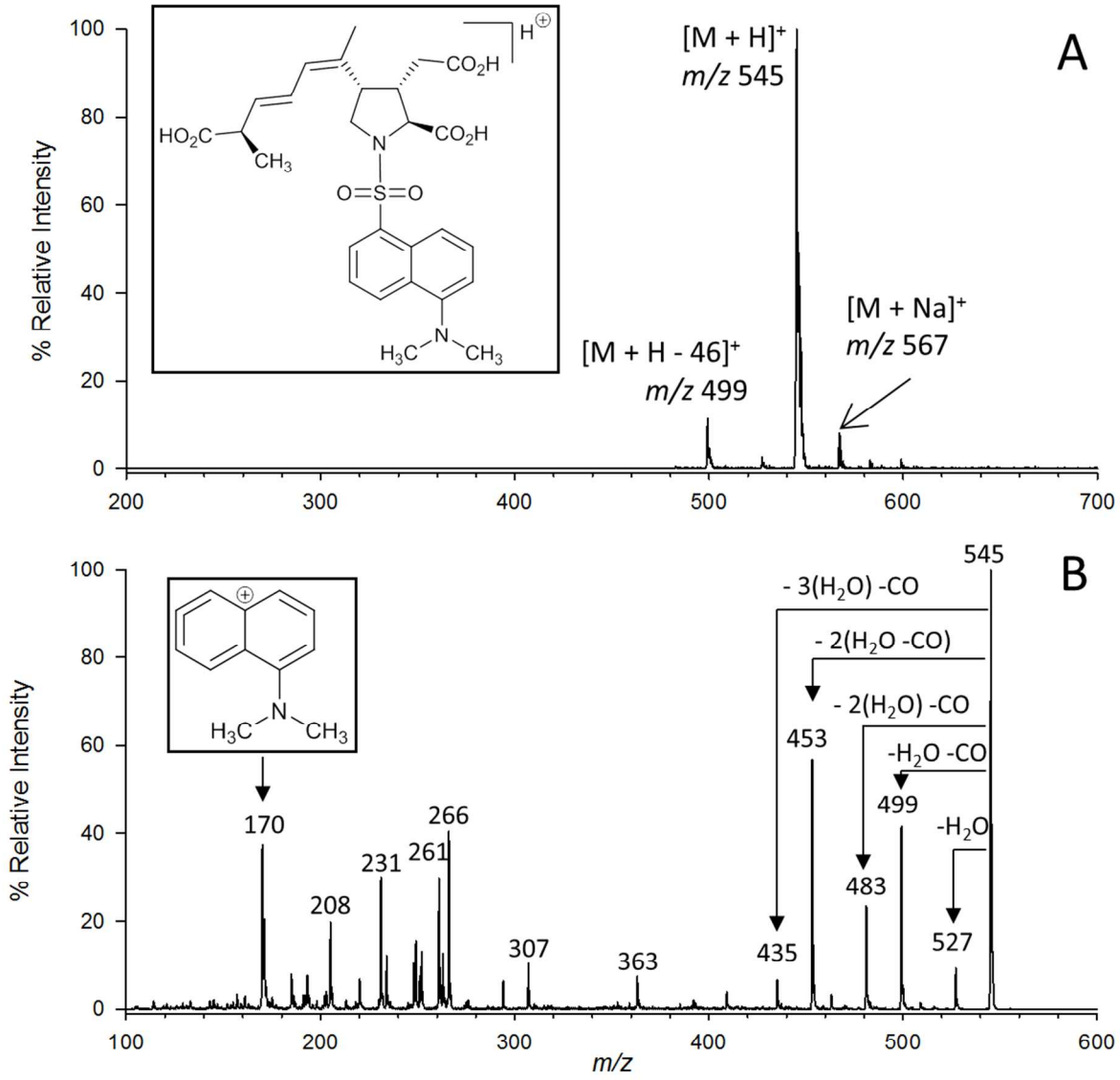

Fig. 3. Mass spectral analysis of DNS-DA. Full scan mass spectrum (A) and MS/MS spectrum (B) of *m/z* 545 precursor $[M+H]^+$ ion of DNS-DA.

In order to optimize the conditions for DNS-DA detection in SRM, product ion spectra were acquired at collision energies ranging from 10 V to 60 V at 5 V intervals using optimized source conditions (Fig. 4). From this energy resolved MS/MS data, the three most abundant product ions of the $[M + H]^+$ precursor were chosen to construct the most sensitive SRM detection method possible. The 545 → 170 (precursor *m/z* → product *m/z*) transition at CE = 50 V was the most sensitive SRM transition and was used for all quantitative analysis. Two additional qualitative transitions, 545 → 499 at CV = 25 V and 545 → 453 at CE = 30 V, were also monitored to insure the unequivocal identification of DNS-DA considering of the ubiquity of the $[M + H]^+$ → *m/z* 170 transition for dansylated compounds [27].

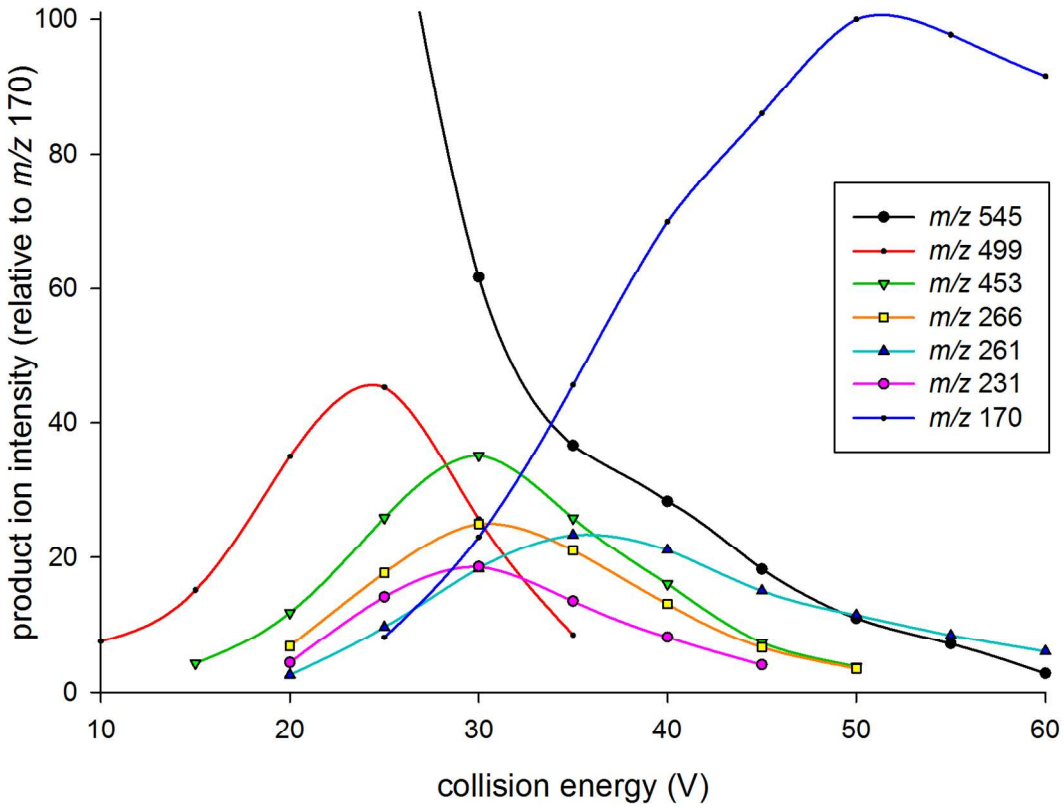

Fig. 4. Energy resolved MS/MS reactivity of DNS-DA showing the most abundant product ions of the $[M + H]^+$ precursor at their optimized collision energies chosen for SRM analysis.

3.3. Liquid Chromatography Separation

Since tandem mass spectrometry alone is not able to distinguish DNS-DA from its isomers, C5'-*epi*-domoic acid (*epi*-DA) and isodomoic acid A, D and E, an LC method capable of completely separating these species needed to be developed. Like DA, DNS-DA is quite acidic due to its three carboxyl groups and therefore an acidic mobile phase was required to minimize peak tailing due to interactions between ionized DNS-DA and residual silanol groups of the stationary phase. An acidic pH also leads to better retention of polar DNS-DA on the non-polar C18 stationary phase. Trifluoroacetic acid (TFA) is an effective ion pair agent in reverse phase LC separations of DA and was also found to be effective for the analysis of DNS-DA by LC with optical detection in preliminary experiments. However, TFA is not compatible with LC-MS as it causes significant ionization suppression in ESI. Formic acid at 0.2% (v/v) was found to be good an alternative to TFA providing both acceptable LC retention and ESI ionization efficiency in the current LC-ESI-MS/MS analysis. When the type and percentage of organic modifier in the mobile phase was examined, it was found that acetonitrile gave better retention than did methanol and good separation of DA from its isomers including *epi*-DA (Fig 5).

Resolution of DNS-DA and its isomers *epi*-DA and isodomoic acids A, D and E could be optimally achieved using isocratic elution on a C18 stationary phase using acidified aqueous acetonitrile mobile phase (Fig. 5). The identities of the DNS-DA isomers in Fig. 5 were assigned based on their well characterized relative abundance in the CRMs used in this study.

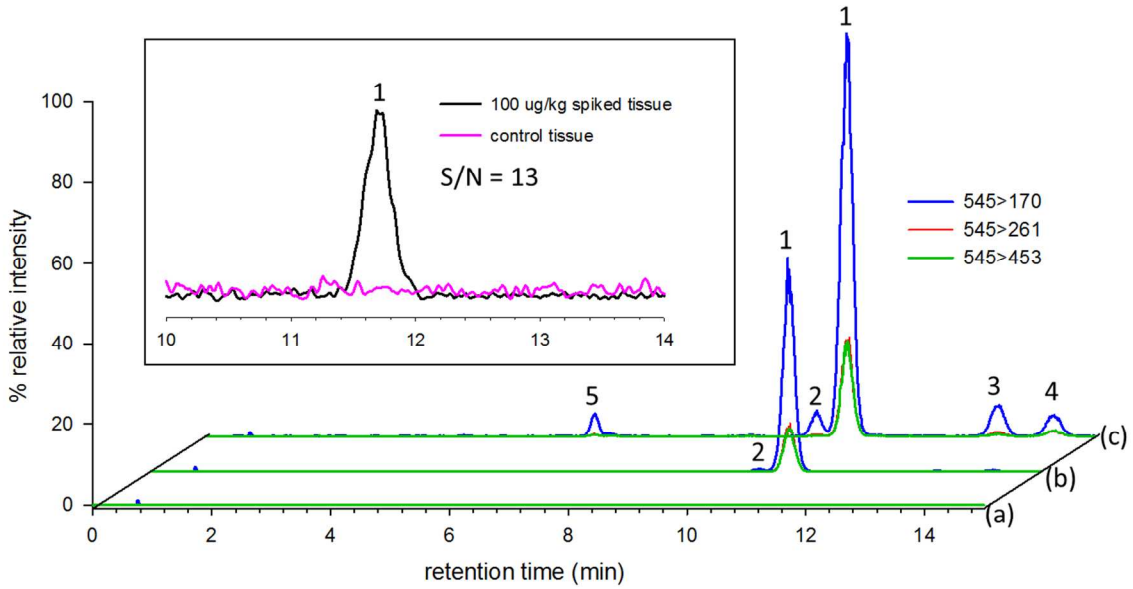

Fig. 5. Analysis of DA in mussel tissue by dansylation LC-ESI-MS/MS. Trace (a) shows control tissue extract, trace (b) shows a DNS-DA spiked control and trace (c) shows a dansylated DA-containing tissue matrix CRM. The inset shows the 545 → 170 transition for the analysis of DA spiked mussel tissue near the method limit of quantitation compared to control tissue. Labeled peaks represent 1 = DNS-DA, 2 = DNS-5'-*epi*-DA (epi), 3 = isodomoic acid-D, 4 = isodomoic acid-A and 5 = isodomoic acid-E.

3.4. Quantitative Method Verification

In order to evaluate the quantitative capabilities of dansylation derivatization in DA analysis, various mussel tissue samples were analyzed using the optimized method, which combines aqueous methanol extraction, SAX SPE cleanup, dansylation derivatization and LC-MS/MS methods reported herein. Because of the potential for interconversion between DA and 5'-*epi*-domoic acid (*epi*-DA), as well as the difficulty of separating the two isomers using some methods, certified values of the CRMs and quantitative results of DA analysis are typically given as the sum of the two analogues. The samples quantified included a mussel tissue CRM that contains DA at a level of 49 ± 3 mg/kg, and another toxin-free mussel tissue CRM analyzed as a

control, as well as after spiking with DA at two levels, 10 mg/kg and 1 mg/kg, both below the regulatory limit for DA of 20 mg/kg in shellfish tissue. These samples were quantified using a matrix matched calibration approach, which included a 5-point calibration curve spanning a concentration range from 0.5 nM to 5000 nM. Matrix matched standards were prepared using a dansylated extract of the DA-free mussel tissue CRM at an identical dilution factor to the analyzed test samples. These standards were prepared in triplicate, analyzed in duplicate and showed a good fit to a linear least squared regression ($R^2 > 0.9999$). The results of these quantifications are presented in Table 1 and show good agreement to certified values and good between-sample reproducibility ($\leq$ 5% RSD).

Table 1: Quantitative verification of dansylation LC-MS/MS method for analysis of DA in mussel tissue.

Sample	Analyte (mg/kg)	Certified/accepted value (mg/kg ± μ)	Experimental value (mg/kg ± SD, n = 3)	% Recovery
CRM-ASP-Mus-d	DA + epi-DA	49 ± 2	44 ± 2	89
CRM-Zero-Mus spiked with CRM-DA-f	DA + epi-DA	10.2 ± 0.1	9.0 ± 0.4	88
CRM-Zero-Mus spiked with CRM-DA-f	DA + epi-DA	1.02 ± 0.01	1.09 ± 0.06	107
CRM-Zero-Mus spiked with CRM-DA-f [a]	DA + epi-DA	0.100 ± 0.001	0.09 ± 0.01	88
CRM-Zero-Mus spiked with CRM-DA-f [a,b]	DA + epi-DA	0.00300 ± 0.00003	0.0027 ± 0.0001	83

[a] 0.1 and 0.003 mg/kg samples was analyzed in order to accurately determine the method LOD/LOQ. As an additional measure of recovery near the LOD/LOQ, quantification was carried out using a one point matrix matched calibration at the equivalent DNS-DA concentration to the samples.

[b] 0.003 mg/kg sample used the alternative procedure described in Materials and Methods.

A third set of spiked samples was prepared close to the limit of quantitation estimated from the higher-level samples. From these 100 μg/kg spiked samples it was possible to accurately determine the limits of detection and quantification of the developed method for DA analysis in mussel tissue. These were calculated by extrapolating the signal obtained from the spiked sample (S/N = 13, shown in inset of Fig. 5) to values of S/N = 3, which gave an LOD of 23 μg/kg, and S/N = 10, which gave an LOQ of 80 μg/kg in mussel tissue. It should be noted that LOD/LOQ values are for a relatively small sample injection volume of 1 μL, which was chosen to avoid signal saturation of the sensitive MS detector used when analyzing the relatively high level tissue matrix CRM.

An alternative set of cleanup, derivatization and analysis parameters was also investigated in order to establish the practical lower limit to LOD/LOQ that can be achieved using the current methodology. This method compensated for all sample dilution throughout the procedure by pre-concentrating the solid phase extraction eluate, reducing the total volume of the dansylation reaction and using a 5 μL injection volume. Using this modified method, a sample spiked at 0.003 mg/kg was analyzed which gave an average S/N of 11, corresponding to a LOD of 1.1 μg/kg and a LOQ of 3.7 μg/kg. Recovery of this modified procedure around the LOQ was verified by using single point matrix matched calibration and gave a recovery of 83% and a between-sample RSD of 5%.

4.0. Conclusions

In this work we have shown how derivatization with DNS-Cl can be used for the sensitive analysis of the marine algal biotoxin DA in shellfish tissue. Mussel tissue extracts obtained after using a highly selective SAX SPE cleanup were suitable for direct dansylation with the reaction proceeding to completion in only 5 min. Compared with un-derivatized DA, DNS-DA showed a 5-fold increase in molar response using LC-MS/MS, as well as improved retention on a C18 stationary phase. A study of the MS/MS dissociation of DNS-DA allowed sensitive and selective SRM transitions to be established for quantification and confirmation of the derivatized toxin in a complex tissue extract sample matrix. The quantitative capabilities of the dansylation LC-MS/MS method were evaluated by analysis of a mussel tissue matrix CRM certified for DA as well as a control tissue matrix CRM spiked with DA CRM calibration solution. The results obtained from these analyses using a matrix matched calibration approach gave good quantitative agreement to certified values, good recoveries of spiked toxin and good reproducibility. The low limits of detection and quantification observed compare favorably to existing methodology and make the described method suitable for trace analysis of DA in shellfish and early detection of DA toxicity events. This method is also of utility in certified reference materials production as it serves as an alternative confirmatory method alongside existing methods. Future work will include extension of the dansylation approach to trace DA analysis in seawater and marine algae, as well as to other amine containing algal toxins.

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