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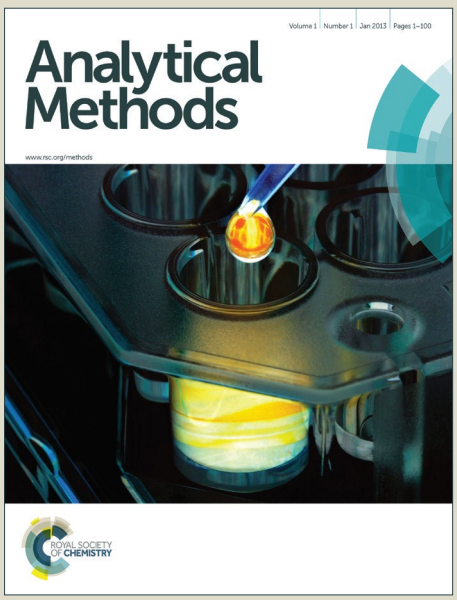

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Isotope-Labelling Derivatisation: A Broadly Applicable Approach to Quantitation of Algal Toxins by Isotope Dilution LC-MS/MS

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Abstract

Two methods were developed for the analysis of algal biotoxins in complex biological and environmental samples to demonstrate the concept of isotope-labelling derivatisation for quantitation. These methods are based on dansyl chloride derivatisation of samples and dansyl-d_6 chloride derivatisation of toxin standards. Derivatised sample and standard are then mixed to achieve isotope dilution calibration in liquid chromatography–tandem mass spectrometry analyses. Quantitation of the marine toxin domoic acid (DA) in mussel tissues and the freshwater toxins anatoxin-a (ATX) and homoanatoxin-a (hATX) in cyanobacteria is demonstrated. For DA, isotope-labelling was incorporated into existing dansylation methodology using inexpensive and commercially available reagents. For ATXs, a novel sample preparation procedure is presented that involves solid phase extraction on a mixed reverse phase/weak anion exchange column that facilitates simultaneous clean-up of the derivatised toxins and removal of excess dansylation reagent through covalent bonding. The challenge of achieving co-elution in LC between deuterated and non-deuterated dansylated toxins was addressed by modifying separation conditions from the usual reverse phase (RP) separation to hydrophilic interaction liquid chromatography in the case of DA and a shortened RP separation with high organic modifier content in the case of the ATXs. The new methods gave limits of detection between 10 and 60 µg/kg and allowed for precise, accurate and fast determination of toxins in spiked control samples and matrix reference materials. This work demonstrates that isotope-labelling derivatisation is broadly applicable to the field of algal toxin analysis where derivatisation is well established but isotopically-labelled standards are not available.

Keywords: Shellfish toxin, cyanobacterial toxin, isotope dilution, isotope-labelling derivatisation, mass spectrometry, anatoxin, domoic acid.

Introduction

Marine algal toxins pose a significant health risk to shellfish consumers and can have a negative impact on shellfish industries.[1,2] Freshwater toxins can contaminate drinking and recreational waters and pose a risk to humans and animals.[3] Therefore, reliable quantitation of such toxins is considered to be of high importance for minimising risks to public health and of negative economic impacts in Canada and abroad. Because of the need for low limits of detection and the ability to provide compound-specific quantitative data, liquid chromatography-mass spectrometry (LC-MS) has rapidly become one of the most widely used techniques for algal toxin analysis.[4-6] One limitation of this technique is the effect of sample matrix on electrospray ionisation (ESI) efficiency and the resulting problems in quantitative measurement of algal toxins in complex biological and environmental samples.[7,8] The available approaches for mitigating matrix effects include sample clean-up, sample dilution, matrix matched calibration, standard addition and stable isotope dilution, with the latter being the preferred approach. Isotope dilution uses an internal standard identical in structure to the analyte but enriched with an unnaturally high abundance of a heavy stable isotope. With this approach, any observed matrix effects are compensated for by measuring the ratio of the analyte response to that of the internal standard. The practical limitation to the isotope dilution approach, which is particularly true in the field of algal toxins, is the limited availability and prohibitive cost of obtaining isotopically labelled standards. Labelled standards are well accepted in mycotoxin research, where similar analytical challenges exist,[9] although there are also many mycotoxins for which there are no labelled standards. Of the hundreds of algal toxins that are measured by LC-MS for research and food safety regulatory purposes, only a few labelled toxins have been produced,[10-13] usually involving culturing toxin-producing algae in heavy media or challenging chemical synthesis. Still, no reliable commercial supply currently exists for the vast majority of algal toxins.

An alternative approach to the synthesis of an authentic labelled standard for each analyte is the introduction of a site of isotope-labelling through chemical derivatisation. This approach is widely used in the fields of quantitative proteomics and metabolomics for achieving reliable relative quantitation by LC-MS.[14-17] Typically, different isotopomers of the same chemical derivatising reagent are used to label different test samples, which are then combined and analysed in a single LC-MS run. This has the effect of normalising matrix effects between samples in ESI analysis as well as any sample preparation steps carried out after derivatisation. A similar approach has been proposed for quantitation of target mycotoxins, flavanones and amino acids in complex food matrices by LC-MS when no labelled standard is available.[18-22] With this approach, different isotopomers of the derivatising reagent are used to differentially label the analyte in the sample and in the standard. The labelled standard is then spiked into the sample and used to perform isotope dilution quantitation.

Algal biotoxins are ideal candidates for the differential isotope-labelling derivatisation approach for a number of reasons. First, there is a long history of development of robust derivatisation chemistry for most classes of toxins, either to introduce chromophores for UV or fluorescence detection or to enhance separation in LC or capillary electrophoresis.[23-26] Also, a reliable supply of calibration solution certified reference materials (CRMs) has been developed for a wide range of algal toxins by the National Research Council Canada for development and calibration of analytical methods in research and regulatory testing. Combined, the availability of established derivatisation methods and CRMs for a large number of algal toxins and the absence of isotopically-labelled standards make differential isotope-labelling derivatisation a highly feasible approach to consider for quantitative analysis of algal toxins using LC-MS.

The goal of this communication is to demonstrate the concept of isotope labelling derivatisation for the quantitation of algal biotoxins in complex matrices. Our recent work showed that derivatisation with dansyl chloride (DNS-Cl) was useful in detection of trace levels of domoic acid (DA) in shellfish.[26] Here, we extend this work to the freshwater toxins anatoxin-a (ATX) and homoanatoxin-a (hATX) using a novel mixed reverse phase/weak anion exchange solid phase clean-up. This involves simultaneous quenching of the dansylation reaction through covalent bonding of excess reagent to the solid phase, and reverse phase clean-up of the derivatised toxin. Dansylation has the desirable effect of improving the retention of these polar toxins in RP-LC and the sensitivity of their detection by ESI-MS. We present two different methods developed for isotope-labelling derivatisation of DA in mussels and ATXs in cyanobacteria. This approach is broadly applicable to other classes of algal toxins for which derivatisation chemistry exists but isotopically-labelled standards are unavailable.

1. Experimental

HPLC grade acetonitrile, methanol and hexanes-200 were obtained from Caledon (Georgetown, ON, Canada). Formic acid (ACS grade, 98%) and ammonium formate (98.5% certified) were obtained from Fisher Scientific (Ottawa, ON, Canada) and disodium tetraborate (98%) was obtained from BDH Chemicals (Poole, England). Dansyl chloride (DNS-Cl) (95%) was obtained from Sigma Aldrich (Oakville, ON, Canada) and dansyl-d_6 chloride (98%) was obtained from Toronto Research Chemicals (North York, ON, Canada). Toxin reference materials were provided by the National Research Council Canada (Halifax, NS, Canada) and included certified calibration solutions for ATX at 30.0 ± 1.1 μM (CRM-ATX) and domoic acid at 327.1 ± 6.8 μM (CRM-DA-f), an in-house calibration solution for hATX at 20.2 ± 0.7 μM, a mussel tissue matrix CRM containing DA at 49 ± 3 mg/kg (CRM-ASP-Mus-d), a toxin-free

mussel tissue (CRM-Zero-Mus), and a pilot scale freeze-dried cyanobacterial reference material similar to that reported recently.[27] Control cyanobacteria consisted of freeze-dried *Microcystis aeruginosa* cultured in-house, which has been confirmed by LC-MS/MS not to contain ATX or hATX.

DA was extracted from mussel tissue using a validated dispersive extraction procedure with 50% methanol as the solvent followed by a strong anion exchange solid phase extraction (SPE) clean-up (LC-SAX, 60 mg, Supelco).[28] Isotopically-labelled derivatisation was incorporated into our previously reported protocol for analysis of DA by dansylation-LC-MS/MS,[11] as shown in Fig. 1A. The DA calibration solution CRM was reacted with dansyl-d_6 chloride to form d_6-dansylated DA (DNS-d_6-DA), which was then spiked into the SPE-cleaned and dansylated mussel tissue extracts.

ATX and hATX were extracted from cyanobacteria using a procedure similar to that reported recently.[27] Briefly, 0.5 g samples of freeze-dried algae were extracted dispersively with 50 mL 0.1% AcOH in 50% acetonitrile. Acetonitrile was used as solvent since methanol was not compatible with the dansylation reaction, which was carried out directly on extracts. The isotope-labelling derivatisation protocol is summarised in Fig. 1B. Toxin standards were reacted with dansyl-d_6 chloride to form d_6-dansylated ATX (DNS-d_6-ATX) and hATX (DNS-d_6-hATX) and then spiked into dansylated algal extracts. The mixture was then loaded dropwise onto a mixed mode weak anion exchange SPE (60 mg StrataX-AW, Supelco) equilibrated with 30% acetonitrile. The column was washed with 6 mL of 30% acetonitrile and then eluted with 3 mL of 100% acetonitrile. This eluate was then evaporated under a gentle stream of nitrogen and re-constituted in 50% acetonitrile prior to injection (1 µL) into LC-MS.

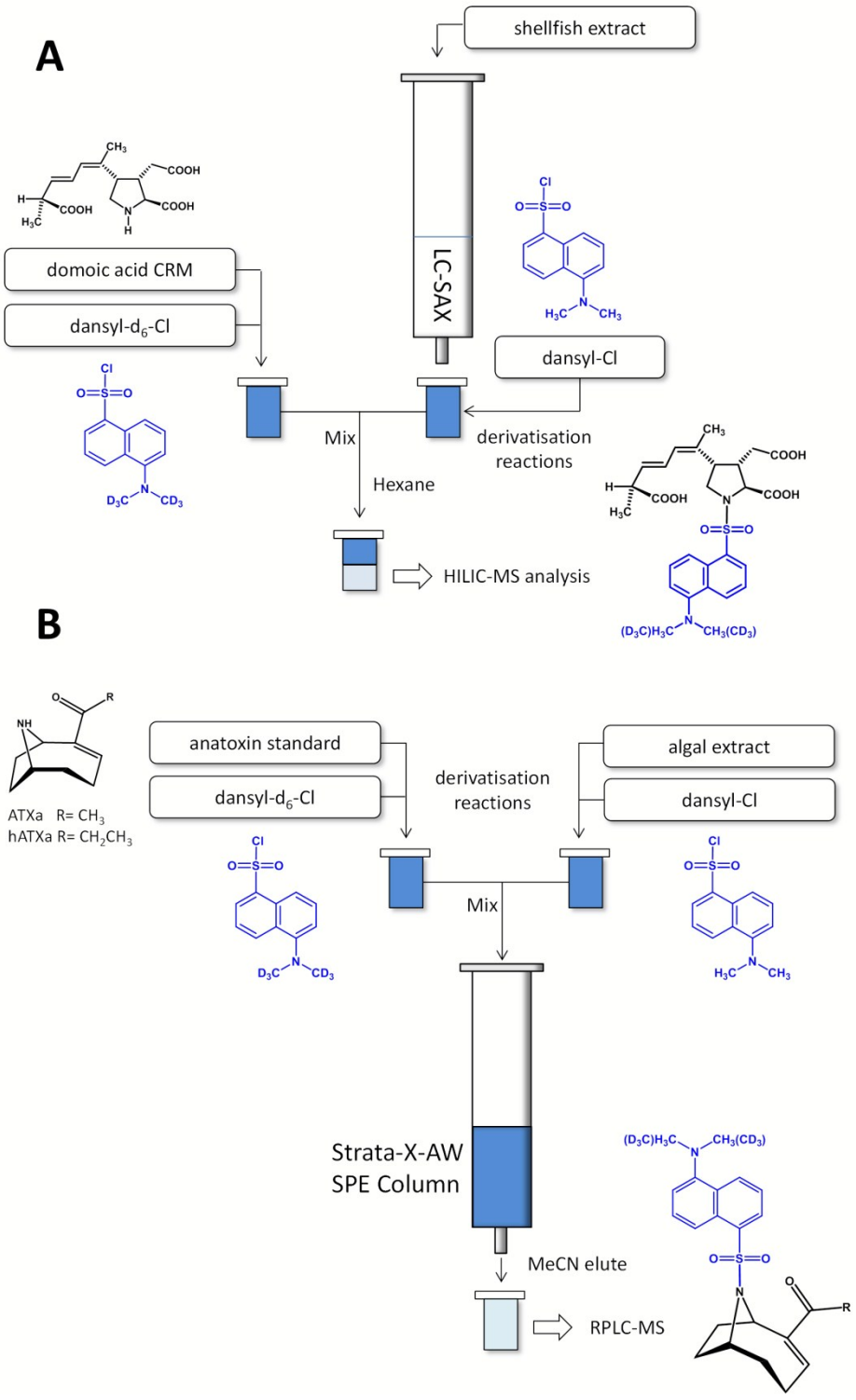

Fig. 1 Schematic representation of sample clean-up and differential derivatisation procedures used for (A) DA in mussel tissue extracts and (B) ATX-a and hATXa in algal samples. Dark blue colour symbolises excess dansyl chloride derivatising agent that was removed prior to analysis.

Liquid chromatography-mass spectrometry was carried out on an Agilent 1260 LC system (Palo Alto, CA, USA) coupled to a 5500 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a Turbospray source operated in positive ionisation mode. Chromatographic conditions used for quantitative analysis of DNS-DA consisted of a 2 × 250 mm, 5 μm TSK-Gel Amide-80 HILIC column (Tosoh Bioscience, Montgomeryville, PA) and isocratic elution using 2 mM ammonium formate and 50 mM formic acid in 9:1 acetonitrile:water at a flow rate of 0.2 mL/min. Conditions used for quantitative analysis of DNS-ATX and DNS-hATX consisted of a 2 × 50 mm 1.8 μm C_{18} Luna column (Phenomenex, Torrance, CA) and isocratic elution using the same buffer and flow rate as DA but at 70% acetonitrile. Detection was carried out in selected reaction monitoring (SRM) mode using previously reported instrumental parameters for DA and its dansylated derivative.[26] Analogous transitions were used for ATX and hATX and included *m/z* 399 >170 and *m/z* 399>355 for quantitation and confirmation of dansylated ATX and *m/z* 413 >170 and *m/z* 413 > 349 for quantitation and confirmation of dansylated hATX, respectively. Isotopically-labelled (d_6) dansylated derivatives were detected under the same conditions as their unlabelled isotopologues except for the precursor and product ion masses used in SRM, which were offset by + 6 Da in all cases.

2. Results and Discussion

In the ideal case for isotope dilution quantitation, the labelled and unlabelled isotopologues of an analyte exhibit identical physicochemical properties, which results in identical chromatographic and mass spectrometric behaviours. The ESI and MS/MS behaviour of each pair of d_0- and d_6- dansylated isotopologues of DA, ATX and hATX were equivalent, resulting in equimolar response from LC-MS/MS analysis in SRM mode. The fragmentation of the

dansylated DA derivative has been described previously and is dominated by a cleavage at the sulfonate bond in the DNS moiety to form the dimethylaminonaphthalenium product ion at *m/z* 170.[26] Analogous fragmentation for dansylated ATXs was observed here. These most sensitive SRM transitions, $[M + H]^+ > m/z$ 170 and $[M + H]^+ > m/z$ 176 for d_0- and d_6-dansylated toxins, respectively, were used for all quantitative analyses and were detected along with additional qualitative transitions for each analyte.

Although less expensive and more accessible, the drawback to using deuterium rather than ^{13}C- or ^{15}N-labelling for isotope dilution is that it can alter chromatographic behaviour. This was the case when pairs of d_0- and d_6-isotopologues of DA, ATX and hATX were analysed using standard RP-LC separation conditions (Figs. 2A and 3A). Because the parent toxins are highly polar, the relatively non-polar dimethylaminonaphthalene moiety of the dansyl group (Fig. 1) represents the primary hydrophobic interaction of the dansylated toxin with the C_{18} stationary phase. This leads to significant isotope effects in LC and baseline separation using standard conditions. In fact, separation of isotopologues was observed under all RP conditions examined, which included various C_{18}, C_8 and polar RP stationary phases.

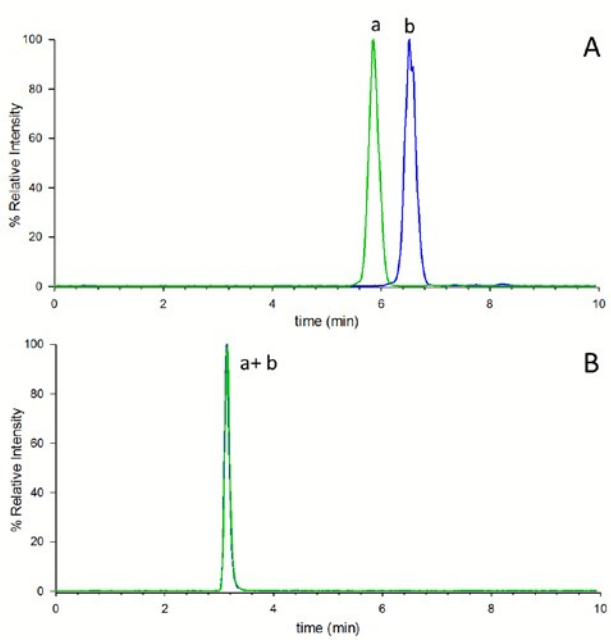

Fig. 2 LC-MS/MS analysis of differentially dansylated domoic acid in mussel tissue extract showing separation of DNS-d_6-DA (a) from DNS-DA (b) by RPLC (A) and their co-elution by HILIC (B).

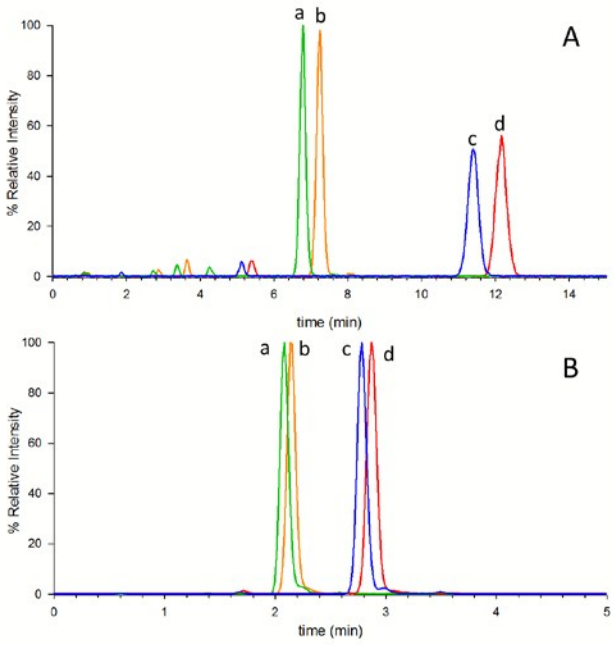

Fig. 3 LC-MS/MS analysis of differentially dansylated anatoxins in algal extract showing separation of DNS-d_6-ATXa (a) from DNS-ATXa (b) and DNS-d_6-hATXa (c) from DNS-hATXa (d) using RPLC with 50% acetonitrile mobile phase (A). Separation of differentially labelled derivatives is minimised using 70% acetonitrile (B).

Despite these isotope effects, it was possible to alter the separation conditions in such a way as to make the d_6-dansylated toxins useful as internal standards. Even after dansylation, DNS-DA is still somewhat polar allowing hydrophilic interaction liquid chromatography (HILIC) to be used in place of RP-LC. In this separation mode, the polar carboxylic acid groups of the domoic acid moiety represent the primary interactions with the polar stationary phase and isotope effects of the dansyl moiety are minimised. Under the conditions used, DNS-d_6-DA co-eluted with the unlabelled isotopologue (Fig. 2B) making it ideal for use as an internal standard. However, these conditions did not allow for the separation of DA from its epimer C5'-epi-domoic acid (epi-DA). Due to interconversion between DA and epi-DA, both routine measurements in shellfish and certified values in reference materials are typically given as the sum of the two toxins, which are usually integrated together even when separation is achieved.

Unlike DNS-DA, DNS-ATX did not show sufficient retention in HILIC to allow a similar approach to be used to achieve co-elution between d_0- and d_6-isotopologues. Instead, this was accomplished by reducing their retention in reverse phase by using a higher composition of organic modifier than had been used previously. This gave a minimum acceptable retention factor (k' ~ 3) while minimising the undesirable isotope effect (Fig. 3B). Depending on the sample matrix and observed matrix effects, improved quantitative results may be possible in the case of improved resolution (Fig. 3A) or improved co-elution (Fig. 3B). Future work to synthesise ^{13}C-labelled dansyl chloride, as recently reported,[14] could be carried out to eliminate these isotope effects and allow for more selective separation to be used in the future.

The different sample preparation methods used for the two toxin classes required isotope-labelled derivatisation to be incorporated into the methods using different approaches (Fig. 1). Because of the complexity of the mussel tissue extracts, a highly selective strong cation

exchange (SAX) SPE clean-up was used to clean up extract samples prior to dansylation of DA.[26,28] In this configuration, d_6-dansylation of the DA calibration solution CRM was carried out in parallel with d_0-dansylation of the SAX eluate (Fig. 1A). After reaction, an aliquot of the d_6-standard was spiked into the sample reaction mixture. Excess DNS-Cl was then removed from the reaction mixture using a μL-scale liquid-liquid extraction with hexane.

Extracts of ATXs in algae samples were dansylated directly prior to any sample clean-up. In this case the d_6-dansylated ATX/hATX standard was spiked into the d_0-dansylated sample reaction mixture immediately. Liquid-liquid extraction with hexane could not be used to remove excess DNS-Cl because DNS-ATX is relatively non-polar and is partitioned into the hexane layer. Instead, a novel approach was developed using a mixed reverse phase/weak anion exchange solid phase extraction cartridge to carry out simultaneous reagent removal and sample clean-up. Excess DNS-Cl reacted readily with the secondary amine functionality of the stationary phase binding it covalently as shown in Fig. 4. Simultaneously, DNS-ATX and DNS-hATX were retained by a reverse phase mechanism allowing for sample de-salting and clean-up before elution of the derivatised toxin. Recovery from SPE, as well as any matrix effects in the LC-MS analysis, were corrected for by the internal standard. The potential for interconversion or exchange between DNS-Cl and DNS-d_6-Cl during this simultaneous clean-up was ruled out by spiking a DNS-ATX standard with high levels of DNS-d_6-Cl before clean-up, which showed no detection of DNS-d_6-ATX. For all analytes, the progress of the dansylation reaction was monitored throughout development using additional SRM transitions for the un-derivatised toxin, as described previously.[26,27]

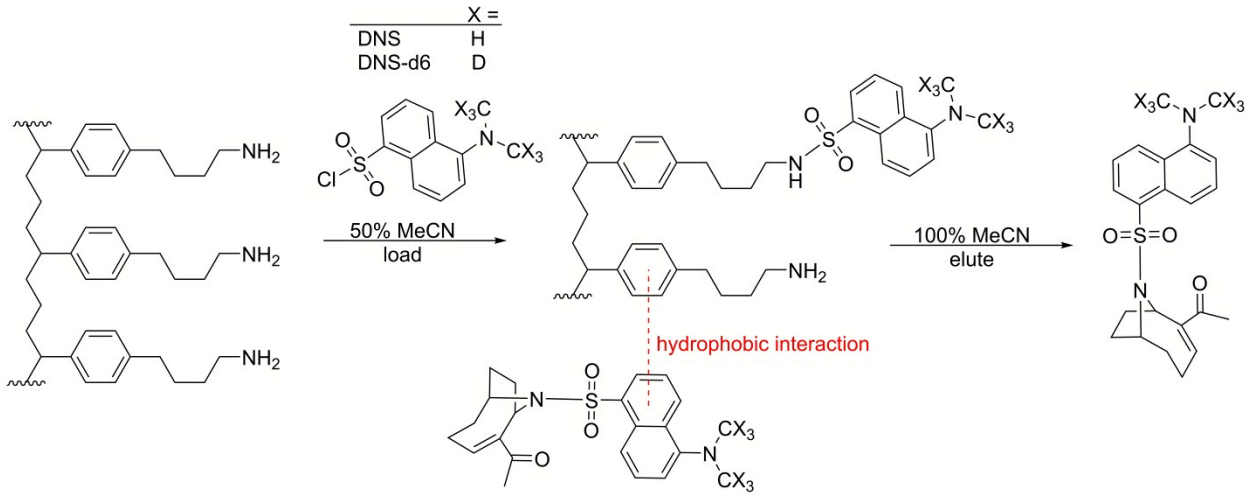

Fig. 4 Covalent bonding of excess dansyl chloride by mixed mode reverse phase/weak anion exchange SPE stationary phase followed by selective elution of dansylated anatoxins.

The range of ratios of analyte to internal standard concentration over which a linear calibration function can be observed is a critical parameter that must be established before isotope dilution can be used without careful matching of the levels of analyte and standard. This was verified by constructing matrix matched calibration curves consisting of a constant level of each d_6-dansylated toxin as an internal standard and a range of values for the corresponding d_0-toxin. This experiment showed linear response of d_0/d_6 peak area ratio for concentrations ranging from 5 nM to 1.25 µM for ATX (Fig. S1) and hATX (Fig. S2) and from 5 nM to 5.5 µM for DNS-DA (Fig. S3). The d_6-dansylated internal standard can therefore be used to correct for matrix effects across this broad range of analyte concentrations, reducing the need for extensive sample pre-screening.

In order to demonstrate the capabilities of differential isotope-labelling for the quantitation of algal toxins, results obtained using our isotope-labelling derivatisation methods were compared to certified values or those obtained using conventional techniques. The method

for DA analysis was evaluated by analysing a mussel tissue matrix sample certified for DA as well as a zero-level control mussel tissue spiked with DA calibration solution CRM at two lower levels equivalent to 1/2 and 1/20 the regulatory limit of 20 mg DA/kg tissue. The method for ATX/hATX analysis was evaluated by analysing a pilot scale freeze-dried algal matrix reference material similar to that described recently,[27] as well as by spiking control cyanobacteria samples with ATX and hATX calibration solutions at a level of 1 mg/kg each. The results of these analyses are presented in Table 1 and showed good agreement with certified values (DA and spike ATX) or those obtained using an established calibration method of standard addition (hATX and ATX in cyanobacterial reference material). Precision between replicate samples ranged from 1 to 12% relative standard deviation, typical values for LC-MS analysis of matrix samples. There appears to be a small overall negative bias in the quantitative results in Table 1. This is unlikely to be due to less efficient derivatization of toxins in the presence of matrix than in neat standards because throughout development, the progress of the reaction was monitored by detection of un-derivatized toxins in the LC-MS/MS method, as described previously [26].

Table 1: Quantitation of toxins in matrix reference materials and negative control samples spiked with standards using isotope-labelling derivatisation approaches.

Sample	Analyte	Reference value (mg/kg ± sd)	Internal standard experimental value (mg/kg ± sd, n = 3)
Mussel tissue matrix CRM-ASP-Mus	DA + epi-DA	49 ± 2[a]	46 ± 2
Negative control mussel tissue spiked with DA	DA + epi-DA	10.2 ± 0.1[b]	8.9 ± 0.1
		1.02 ± 0.01[b]	0.9 ± 0.2
Cyanobacterial RM	anatoxin-a	95 ± 9[c]	80 ± 5
	homoanatoxin-a	28 ± 4[c]	25 ± 3
Control *Microcystis sp.* spiked with ATXs	anatoxin-a	1.00 ± 0.02[b]	1.00 ± 0.04
	homoanatoxin-a	1.09 ± 0.02[b]	0.92 ± 0.03

[a] certified value

[b] spiked value. Certified reference material calibration solutions used for DA and ATX. hATX calibration solution quantitated by LC-MS and quantitative nuclear magnetic resonance spectroscopy.[29]

[c] as determined by HILIC-MS of un-derivatised toxin using standard addition calibration

The signal-to-noise values obtained from analysis of 1 mg/kg spiked matrix samples was used to estimate the limits of detection (LOD) for DA in mussel tissue as 0.06 mg/kg and that of ATX and hATX in freeze-dried algae as 0.02 mg/kg and 0.04 mg/kg, respectively. Limits of quantitation are estimated as three times higher than these LODs. These values are consistent with LOD/LOQ values reported recently for DA using dansylation and matrix matched calibration, with the possibility to extend these limits down to 0.001 mg/kg using a higher degree of pre-concentration.[26]

Conclusions

This communication demonstrates two examples of simple, cost effective ways in which isotope-labelling derivatisation can be used to perform quantitative analysis of algal toxins using isotope dilution when no labelled standard is available. Though more readily available than reagents labelled with ^{13}C or ^{15}N, deuterium-labelled DNS does introduce isotope effects into chromatographic separations. We demonstrate how these effects can be minimised in the case of d_0/d_6-dansylated toxins by manipulating the selectivity or retention characteristics of the LC separation. This approach was used to provide good quantitative results for three different algal toxins in mussel and cyanobacterial matrix samples.

This work, coupled with the abundance of robust derivatisation chemistry for algal toxins, the availability of a wide range of toxin CRMs and the scarcity of isotopically-labelled standards suggests that isotope-labelling derivatisation is broadly applicable in the field. Future work will be directed towards expanding the utility of this approach for algal toxin analysis by LC-MS. This will include expanding the approach to other toxin classes where matrix effects have a greater potential to hinder accurate quantitation by LC-MS. Also of interest will be to move away from the use of deuterated reagents towards the synthesis of ^{13}C and ^{14}N labelled derivatisation reagents in order to minimise the impact of labelling on chromatographic separations. The approach used here of derivatising a calibration solution with the labelled reagent along with each sample set is shown to be an effective method of quantitation. In the future, labeled reagent could be used more efficiently by preparing labelled, derivatised standards in bulk, which could then be used for direct spiking into derivatised samples.

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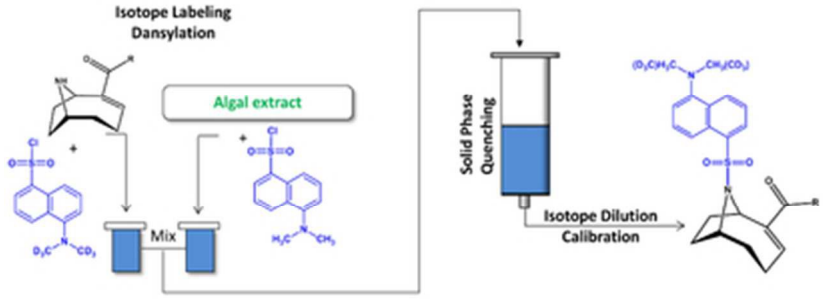

Derivatisation with a labelled reagent allows for isotope dilution calibration of algal toxins for which no labelled standard is available.
34x14mm (300 x 300 DPI)