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# Development of a sensitive and selective liquid chromatography–mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction

Michael J. Boundy[a,*], Andrew I. Selwood[a], D. Tim Harwood[a], Paul S. McNabb[a], Andrew D. Turner[b]

[a] Cawthron Institute – 98 Halifax St, Nelson 7010, New Zealand
[b] Centre for Environment, Fisheries and Aquaculture Science (Cefas) – Barrack Rd, Weymouth, Dorset DT4 8UB, United Kingdom

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ABSTRACT

Routine regulatory monitoring of paralytic shellfish toxins (PST) commonly employs oxidative derivitisation and complex liquid chromatography fluorescence detection methods (LC-FL). The pre-column oxidation LC-FL method is currently implemented in New Zealand and the United Kingdom. When using this method positive samples are fractionated and two different oxidations are required to confirm the identity and quantity of each PST analogue present. There is a need for alternative methods that are simpler, provide faster turnaround times and have improved detection limits. Hydrophilic interaction liquid chromatography (HILIC) HPLC–MS/MS analysis of PST has been used for research purposes, but high detection limits and substantial sample matrix issues have prevented it from becoming a viable alternative for routine monitoring purposes. We have developed a HILIC UPLC–MS/MS method for paralytic shellfish toxins with an optimised desalting clean-up procedure on inexpensive carbon solid phase extraction cartridges for reduction of matrix interferences. This represents a major technical breakthrough and allows sensitive, selective and rapid analysis of paralytic shellfish toxins from a variety of sample types, including many commercially produced bivalve molluscan shellfish species. Additionally, this analytical approach avoids the need for complex calculations to determine sample toxicity, as unlike other methods each PST analogue is able to be quantified as a single resolved peak. This article presents the method development and optimisation information. A thorough single laboratory validation study has subsequently been performed and this data will be presented elsewhere.



## 1. Introduction

Paralytic shellfish toxins (PST) represent a diverse class of potent neurotoxins produced from dinoflagellates of the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium* that naturally accumulate in filter feeding shellfish [1–4]. More than 50 related PST analogues have been described to date [4], making the toxin group technically challenging to quantify by instrumental techniques. They are responsible for paralytic shellfish poisoning (PSP) syndrome [5], which poses a serious hazard to public health and threatens shellfish aquaculture in many parts of the world. The analysis of this toxin class demands high quality quantitative information and routine monitoring programmes to ensure production of safe seafood for global consumers and prevention of costly recalls. The current regulatory action limit for PST in bivalve molluscs is 0.8 mg STX·2HCl eq/kg [6].

PST are a class of guanadinium derivatives, with saxitoxin being regarded as the parent compound. Saxitoxin itself has two amine functional groups, whereas other analogues have a negatively charged C11-hydroxysulfate and/or N-sulfocarbamoyl functional groups. PST analogues can be categorised into three generic groups based on their net charge state (Fig. 1). Analogues that have both C11-hydroxysulfate and N-sulfocarbamoyl have an overall charge state of 0 (C toxins). Analogues that have either C11-hydroxysulfate or N-sulfocarbamoyl have a charge state of +1, and are commonly known as gonyautoxins (GTXs), but also include several other analogues such as the M toxins M1, M3, and M5 [3]. Analogues that do

* Corresponding author. Present address: Cawthron Institute, 98 Halifax St East, Nelson 7010, New Zealand. Tel.: +64 3 548 2319x325; fax: +64 3 546 9464.
*E-mail address:* michael.boundy@cawthron.org.nz (M.J. Boundy).

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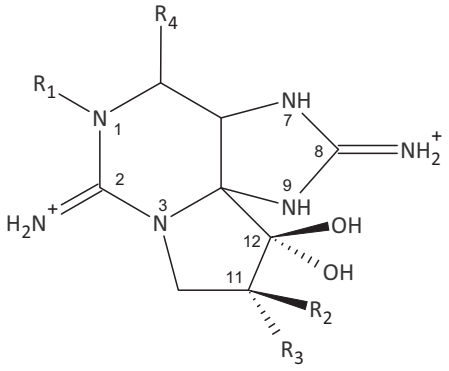


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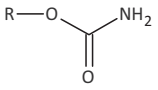
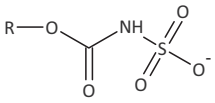



OCONHSO$_3^-$:




| Group (Charge state) | Analogue | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
| C toxins (0) | C1 | H | H | $OSO_3^-$ | $OCONHSO_3^-$ |
| | C2 | H | $OSO_3^-$ | H | $OCONHSO_3^-$ |
| | C3 | OH | H | $OSO_3^-$ | $OCONHSO_3^-$ |
| | C4 | OH | $OSO_3^-$ | H | $OCONHSO_3^-$ |
| GTXs (+1) | dcGTX2 | H | H | $OSO_3^-$ | OH |
| | dcGTX2 | H | $OSO_3^-$ | H | OH |
| | dcGTX1 | OH | H | $OSO_3^-$ | OH |
| | dcGTX4 | OH | $OSO_3^-$ | H | OH |
| | GTX2 | H | H | $OSO_3^-$ | $OCONH_2$ |
| | GTX3 | H | $OSO_3^-$ | H | $OCONH_2$ |
| | GTX1 | OH | H | $OSO_3^-$ | $OCONH_2$ |
| | GTX4 | OH | $OSO_3^-$ | H | $OCONH_2$ |
| | GTX5 (B1) | H | H | H | $OCONHSO_3^-$ |
| | GTX6 (B2) | OH | H | H | $OCONHSO_3^-$ |
| | M1α | H | H | OH | $OCONHSO_3^-$ |
| | M1β | H | OH | H | $OCONHSO_3^-$ |
| | M3 | H | OH | OH | $OCONHSO_3^-$ |
| STXs (+2) | doSTX | H | H | H | H |
| | dcSTX | H | H | H | OH |
| | dcNEO | OH | H | H | OH |
| | STX | H | H | H | $OCONH_2$ |
| | NEO | OH | H | H | $OCONH_2$ |
| | M2α | H | H | OH | $OCONH_2$ |
| | M2β | H | OH | H | $OCONH_2$ |
| | M4 | H | OH | OH | $OCONH_2$ |

**Fig. 1.** Structures of principal toxins associated with paralytic shellfish poisoning (PSP) syndrome, grouped by charge state.

not have C11-hyxdroxysulfate or N-sulfocarbamoyl (doSTX, dcSTX, STX, dcNEO, NEO, and other analogues such as M2, M4) have a charge state of +2 (STXs) [2].

The reference method for PSP is a mouse bioassay (AOAC 959.08) [7]. This method has recently been replaced in many testing laboratories with instrumental methods due to both ethical concerns and technical issues such as poor sensitivity, reproducibility and interferences [8–12]. There are currently three alternative AOAC accepted official methods for analysis of PST: pre-column oxidation LC-FL (AOAC 2005.06) [13], post-column oxidation LC-FL (AOAC 2011.02) [14], and the receptor binding assay (RBA; AOAC 2011.27) [15]. There are associated benefits and drawbacks with each approach.

Pre-column oxidation LC-FL [13] uses a simple liquid chromatography fluorescence detection system, with samples extracted and fractionated over a carboxylic acid solid phase extraction cartridge that exploits the differences in charge states of the various PST groups. In recent years this methodology has been refined and incorporated into both the UK and New Zealand official control monitoring programmes for the analysis of a range of different shellfish species [16–20]. Two oxidation conditions are used to produce fluorescent products of the PST various analogues present in the sample. This method can be fully automated, although sample analysis times are long due to the multiple injections required per sample. In addition, data interpretation and toxin quantitation is time consuming and complex due to common fluorescent oxidation products originating from different PST.

Post-column oxidation LC-FL [14] uses a complex ion pairing liquid chromatography system with an in-line post-column oxidation reaction chamber located between the analytical column and the fluorescence detector. The instrumentation configuration is much more complex than the pre-column oxidation LC-FL, although it can also be automated. Two separate chromatography conditions are required in order to analyse the full suite of regulated PST, resulting in long total analysis time per sample. Sample preparation and quantitation is simple as no pre-column fractionation or oxidation is required, and each PST is able to be determined as a single chromatographic peak. However, chromatographic column life is problematic and this is primarily due to the use of ion pairing reagents that accumulate on the column [21].

The RBA [15] has also demonstrated suitability for routine shellfish testing purposes. The assay quantifies the composite PST toxicity in shellfish samples based on the ability of sample extracts to compete with $^3$H saxitoxin for binding to voltage-gated sodium channels in a rat brain membrane preparation. Quantification of binding can be carried out using either a microplate or traditional scintillation counter. This methodology has many good features although there is concern over the continued availability of the radiolabelled tracer and it has limitations in the research setting as no toxin profile information is obtained for samples.

There has also been significant effort directed towards the development of methods for PST based on liquid chromatography–mass spectrometry [2,22–26]. However, high detection limits and substantial sample matrix issues due to the presence of salts and co-extractives have prevented it from becoming a viable alternative for routine testing of shellfish samples [2,24]. This paper describes efforts to overcome these effects through effective sample preparation processes. Once a suitable sample preparation method has been developed electrospray ionisation mass spectrometry (ESI-MS) becomes a highly sensitive method for PST detection as the saxitoxin backbone is readily ionisable [22].

Hydrophilic interaction liquid chromatography (HILIC) is well suited for the separation and determination of polar compounds such as PST [2]. The high organic mobile phase content allows for efficient desolvation and ionisation, both key requirements for ESI-MS detection [27]. HILIC also has the advantage of not requiring the use of ion pairing reagents. HILIC coupled with ESI-MS detection has been implemented in a research setting for the detection of PST in both algal [2,26], and shellfish samples [23–25]. However, the reported sample preparation procedures for shellfish are prohibitively long for routine analysis, which in a regulatory setting are subject to strict turnaround time requirements.

In this article we describe the development of a sensitive and selective method for the analysis of PST from a variety commercially produced shellfish species. The sample extraction procedure allows fast sample preparation with reduced complexity, and the