PAPERS

OPTIMISING SPME-HPLC TO DETERMINE THE CYANOTOXIN NODULARIN IN WATER: A PRELIMINARY INVESTIGATION

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ABSTRACT

Nodularin is a cyclic pentapeptide produced by the cyanobacterium *Nodularia spumigena*. This cyanotoxin is responsible for the contamination of water bodies, and potentially can lead to human illness. Current analytical techniques that quantify nodularin often require several sample preparation steps that are time consuming and use organic solvents. Our objective was to develop a simple, relatively fast and solventless method for extracting and quantifying nodularin using solid-phase microextraction (SPME) coupled with high performance liquid chromatography (HPLC). Two concentrations of nodularin (1 ppm and 10 ppb) were used to evaluate extraction capabilities of Carbowax/Templated (CW/TPR, 50 μm) coated SPME fibres. Extraction efficiency of the nodularin solutions was optimised by adjusting the salt concentration to 30% (w/w), decreasing pH to 2 and exposing the SPME fibre to the nodularin solutions for either 60 or 120 minutes, depending on nodularin concentration. Under optimised conditions, the SPME-HPLC methodology produced linear, reproducible calibration curves using fresh or seawater samples spiked with nodularin. Results suggest that SPME-HPLC could be useful in screening nodularin-contaminated water samples, with the added advantage over other traditional extraction methods of being relatively quick, simple and solventless which avoids the dangers of occupational exposure and disposal of organic solvents.

Key words: nodularin; cyanobacteria; Solid Phase Microextraction (SPME).

INTRODUCTION

In an estuarine environment, *Nodularia spumigena* is often the dominant blue green alga causing toxic algal blooms. Reports of animal deaths from *Nodularia spumigena* blooms have been recorded from Australia since 1878 (Francis 1878; Codd et al. 1994) with the causative toxic agent being a cyclic pentapeptide named nodularin. Nodularin is regarded as a comparatively hydrophobic compound (Annila et al. 1996) that binds non-covalently to molecules (Bagu et al. 1997). It also has an unusual side chain termed ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (Carmichael 1989), which is considered important in toxicity (Carmichael 1992) as it strongly binds to the catalytic units of eukaryotic protein phosphatases 1 and 2A (PP) (Yoshizawa et al. 1990).

The high toxicity of nodularin even at comparatively low concentrations has focused development of a variety of techniques for detection and quantification. Biological methods include bacterial, invertebrate and mouse bioassays, biochemical assays (e.g., Protein Phosphatase Inhibition Assay), immunological detection (e.g., ELISA) and mammalian cells (e.g., rat cell cytotoxicity tests) (Harada et al. 1999) and their suitability, advantages and disadvantages have been reviewed (Lam et al. 2000). Analytical techniques such as HPLC/MS (High Performance Liquid Chromatography Mass Spectrometry), GC/MS (Gas Chromatography Mass Spectrometry), TLC (Thin Layer Chromatography), NMR (Nuclear Magnetic Resonance) and MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time of Flight), have also been developed and can achieve high sensitivity, but the cost and availability of instrumentation do not necessarily make these methods commonplace. HPLC with photodiode array detection is one analytical technique that is more typically available in government and private institutions and has been suggested as the best means for determining nodularin (Nicholson and Burch 2001).

As the nodularin levels in water and other more complex matrices are generally low, sample pre-treatment is often required to concentrate and separate nodularin from other compounds.

Typical methods for sample preparation use liquid/liquid or liquid/solid extraction with organic solvents, followed by clean-up and concentration steps (Siegelman et al. 1984; Krishnamurthy et al. 1986; Lawton and Edwards 2001). Many of these sample preparation techniques are regarded as time consuming, labour intensive and costly (Wardencki et al. 2007). Of more concern is the use of toxic solvents, their management, disposal and potential to cause environmental and occupational damage (Stewart et al. 2009). Development

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of solventless sample preparation techniques has great potential in terms of environmental and occupational benefits as well as reduced analytical costs.

One such technique is solid-phase microextraction (SPME), which has shown promise in many scientific disciplines (Pawliszyn 1997; Peñalver et al. 1999; Prosen and Zupan-Kralj 1999; Alpendurada 2000; Lord and Pawliszyn 2000; Theodoridis et al. 2000; Ulrich 2000). It is regarded as fast, economical, sensitive and solventless (Wardencki et al. 2007). Originally devised by Arthur and Pawliszyn (1990), its application in algal toxin detection is now beginning to emerge (Poon et al. 2001; Namera et al. 2002; Ghassempour et al. 2005; Rodríguez et al. 2006; Rellán and Gago-Martínez 2007; Rellán et al. 2007).

SPME extracts an analyte from a complex matrix (e.g., air, soil, water, blood, urine) by using a polymer coating that is bonded to a support. Uptake into the SPME coating continues until an equilibrium exists between the coating and the sample matrix (e.g., water). After absorption into the SPME coating, the analyte can be directly desorbed into a conventional GC or HPLC for separation and quantification. The effectiveness of the SPME polymer fibre to concentrate an analyte is often dependent on variables such as fibre type, salt concentration, pH and extraction time.

MATERIALS AND METHODS

Nodularin was purchased from Calbiochem (La Jolla, Ca, USA). HPLC grade acetonitrile and methanol, analytical grade hydrochloric acid (HCl), sodium chloride (NaCl) and trifluoroacetic acid (TFA) were obtained from Riedel-de Haën (Seelze, Germany). Water was purified using a Milli-Q purification system (Millipore, Bedford, Ma, USA). Nodularin stock solutions were prepared in 50% methanol and stored at –20°C.

The SPME syringe holder, Carbowax/templated resin (CW/TPR, 50µm) coated fibres and a SPME–HPLC interface were purchased from Supelco (Canada). Carbowax/templated resin coated fibres were chosen because of their suitability in previous cyanotoxin research (Poon et al. 2001) and their use in other SPME-HPLC applications (Wardencki et al. 2007).

For the SPME optimising experiments, working concentrations of nodularin were spiked into Milli-Q water at either 10 ppb or 1 ppm. The low concentration was chosen based on what might be expected in contaminated waters, while the high concentration was selected to ensure a likely response. Linearity and precision experiments used nodularin concentrations of 10, 20, 50, 80, 100, 500 and 1000 ppb in Milli-Q water or seawater. Nodularin samples were dispensed into 10-mL glass vials and kept under constant agitation using a magnetic stirrer. Temperature for all samples was kept constant at 22°C. A SPME syringe holder containing the SPME fibre was suspended directly above the sample vial and secured using a retort stand and clamp. The needle of the syringe holder was lowered into the sample and by depressing the plunger of the syringe holder, the SPME fibre was exposed to the nodularin sample. Extraction efficiency was optimised by varying time, salt and pH concentrations in the sample.

After an extraction period, the SPME fibre was removed and briefly rinsed with Milli-Q water to remove excess salt that may interfere with the sensitivity of the HPLC column. The SPME fibre was then retracted back into the syringe holder before being inserted into the top of the SPME-HPLC interface. The syringe was clamped down and held tightly using a clamp – ferrule arrangement. The plunger was again pushed down to expose the SPME fibre into the desorption chamber. Extracted nodularin was desorbed from the SPME polymer coating by engaging the desorption rotodyne which effectively passed the mobile phase from the HPLC over the SPME fibre and into the HPLC separation column.

The HPLC system consisted of a Water 600S controller, 626 pump and 717 autosampler. Separation of analytes was through a Saulle 40 x 2 mm column, packed with 3 µm Gromsil 120 octyl-6. Detection of nodularin was achieved using a Waters 996 DAD (Diode Array Detector) multi-scanning UV (Ultra Violet) detector, scanning wavelengths between 210 and 280 nm, with optimal wavelength detection set at 238 nm. Analytes were eluted with a modified gradient of 20% acetonitrile in Milli-Q water, ramped up to 90% acetonitrile with 0.1% TFA over 8 minutes with a total run time of 15 minutes. The retention time of nodularin was approximately 6 minutes.

To ensure that the HPLC set-up procedure was sensitive and responsive to nodularin, a standard curve of pure nodularin in 50% methanol was initially trialled by injecting 0, 0.2, 0.4, 1, 4, 10, 40 and 100 ppm directly into the HPLC system. The resulting standard curve showed the set-up to be sensitive and linear in response with a correlation coefficient (r^2) of 0.9974. Furthermore, the effects of salt and pH on HPLC column sensitivity were tested using 1 ppm nodularin in Milli-Q water. Salt effects were verified by acidifying 1 ppm nodularin to pH 2 and adjusting the NaCl concentrations (0, 5, 10, 20, 25, 30% w/w) before direct injection into the HPLC. Similarly, pH effects (pH 1.5, 2, 4, 6, 8) were investigated after adjusting the pH of the nodularin sample prior to direct injection into the HPLC. Results showed that only very high concentrations of salt (30% w/w) caused a significant decrease in column sensitivity to nodularin (data not shown) and column sensitivity was not affected by pH. As the SPME method removes most salt from the polymer fibre, these results suggest column sensitivity to nodularin should not be affected by pH and NaCl.

RESULTS AND DISCUSSION

Development of the SPME method required optimisation of extraction conditions suitable for nodularin. Three parameters were optimised: concentration of sodium chloride, pH value and extraction time profile. Initial optimisation tests were carried out on spiked Milli-Q samples with a nodularin concentration of 1 ppm.

Effects of salinity

In general, the effectiveness of the SPME fibre to extract nodularin (measured in terms of peak area) increased with sodium chloride concentration before maximising at 25-30% NaCl (Figure 1). The nodularin sample was initially acidified to pH 2.0 after previous studies had shown increased adsorption of nodularin in soils under acidic

conditions (Miller et al. 2001). Since the optimal time for the establishment of equilibrium between the SPME fibre and the matrix containing nodularin was unknown, a time of one hour was initially selected. The results suggested that sodium chloride increased the ionic strength in the sample solution, thereby reducing the solubility of nodularin and improving the partitioning of nodularin onto the polymer coating. This salting out effect has also been demonstrated for other algal toxins (Poon et al. 2001; Namera et al. 2002; Ghassempour et al. 2005; Rodríguez et al. 2006; Rellán and Gago-Martínez 2007; Rellán et al. 2007). As the maximum extraction efficiency did not increase beyond 30% NaCl, this salt concentration was used for all other experiments.

Effects of pH

Nodularin adsorption onto the SPME fibre was investigated at pH 1.5, 2.0, 4.0, 6.0 and 8.0. Salt concentration was kept at 30% NaCl and extraction time was again selected for one hour. Lowering pH in the sample increased the extraction of nodularin onto the SPME fibre, with a maximal extraction efficiency occurring between pH 2.0 and 4.0. The greatest change occurred between pH 4.0 and 6.0 (Figure 2). De Maagd et al. (1999) have shown that the structurally similar algal toxin, microcystin, increases its lipophilic nature at low pH, and nodularin also seems to behave in the same way. Further to this, the optimal pH value to extract microcystin using SPME is 2.0 (using the same extraction conditions to those reported here) (Poon et al. 2001). Maximum extraction of nodularin by SPME was between pH 2.0 and 4.0 and suggests subtle differences in the chemistry of nodularin and microcystin. Like microcystin, nodularin resembles a polar lipid, with an unusual hydrophobic ADDA side chain (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and a hydrophilic polar cyclic moiety (Spassova et al. 1995). Decreasing pH may affect the ionisation states of the functional groups on the cyclic moiety of nodularin, thus favouring partitioning onto the SPME fibre. As maximum extraction efficiency did not increase beyond pH 2.0, and for comparative purposes to other cyanotoxin SPME research (e.g., Poon et al. 2001) this pH value was used for all other experiments.

Effects of extraction time

The optimal time for SPME to extract nodularin from water samples was studied at nodularin concentrations of 1 ppm and 10 ppb. Initially, 1 ppm was extracted at 10, 20, 40, 60 and 120 min. The low concentration of 10 ppb nodularin was extracted at 40, 120, 240 and 720 min. During the investigation salinity was optimised at 30% and the pH adjusted to 2. Extraction of nodularin at 1 ppm increased sharply until equilibrium existed after 60 minutes (Figure 3.). Nodularin at 10 ppb also showed a sharp increase between 40 and 120 minutes, at which point the uptake slowed and increased marginally over time (Figure 4.). Although the LOD (Limit of Detection) could be slightly improved by extending the time beyond several hours, our objective was to develop a relatively simple and quick method for nodularin extraction. As such, a 2-hour extraction period was determined to be suitable for low concentrations of nodularin.

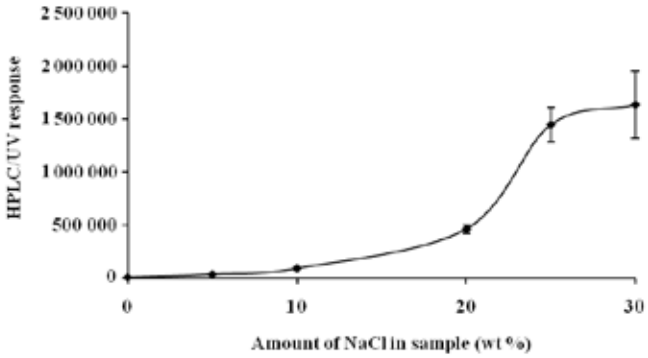

Figure 1. Effects of NaCl on SPME-HPLC extraction of Milli-Q water samples spiked with 1 ppm nodularin. The SPME sampling time was 1 hour. All samples were adjusted to pH 2. (n = 4; error bars represent ± SD)

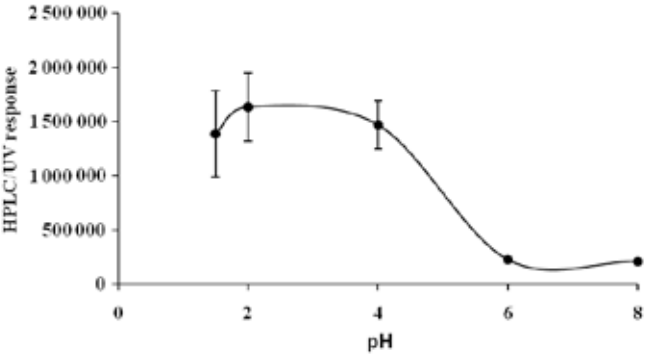

Figure 2. Effects of pH on SPME-HPLC extraction of Milli-Q water samples spiked with 1 ppm nodularin. The SPME sampling time was 1 hour. All samples contained 30% NaCl. (n = 4; error bars represent ± SD)

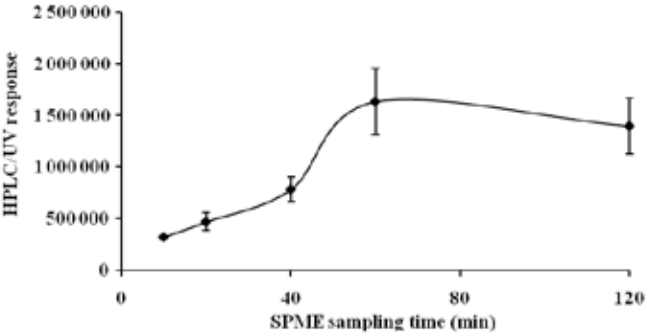

Figure 3. SPME absorption - time profile of nodularin at a spike level of 1 ppm in Milli-Q water. All samples contained 30% NaCl and were adjusted to pH 2. (n = 3; error bars represent ± SD)

Linearity and Precision

Linearity of the calibration curves was initially tested with spiked nodularin in Milli-Q water over two orders of magnitude with the following seven concentrations: 10, 20, 50, 80, 100, 500 and 1000 ppb. Nodularin concentrations were again tested in filtered seawater that could be considered more analogous to a natural water sample. Both calibration curves showed good linearity (Figure 5), with correlation coefficients (r^2) ranging from 0.9879 for nodularin in Milli-Q water and 0.9992 for spiked seawater samples. The nodularin

concentrations were replicated three times and showed good reproducibility with average relative standard deviations (RSD) of 10.6% in seawater and 12.3% in freshwater samples. HPLC peak response was similar for each concentration in seawater and freshwater except 500 ppb nodularin, where a possible concentration error occurred. Nodularin standard curves constructed using SPME-HPLC can provide the basis to further measure unknown nodularin samples in aquatic environments to at least 10 ppb. Bloom conditions of *Nodularia spumigena* can liberate up to 54 µg/L of nodularin in Australian estuarine lakes (Davies in prep) and this method should enable rapid detection of such bloom levels. The lowest nodularin detection limit of 10 ppb by the current SPME-HPLC method limits its application in monitoring health level concentrations in drinking water (Fitzgerald et al. 1999) as Australian drinking water quality guidelines go below this limit (NHMRC 2004). For this, future SPME research is needed and should be directed towards improving sensitivity of the SPME method to detect nodularin to at least an order of magnitude lower, as recent immunoassay screening kits can now lay claim to such sensitivity (Lawton et al. 2010). Analytical instrumentation (i.e., HPLC-MS) can also achieve high sensitivity but often only after lengthy clean-up steps. Using the SPME method combined with other analytical instrumentation such as HPLC-MS may improve sensitivity for nodularin analysis and avoid solvent clean-up and concentration steps. Heating the matrix that nodularin is contained in may improve release of the toxin, as elevated temperature provides energy to overcome bonding with the matrix (Ghiasvand et al. 2006). Further study in SPME technology should investigate new SPME fibre coatings and the application of Thin-film microextraction (TFME) for sensitivity improvement (Risticevic et al. 2009). Ultimately, future studies should aim at applying SPME for the fast and sensitive determination of nodularin in more complex samples such as natural unfiltered water, wastewater and animal or plant tissue.

CONCLUSION

A relatively quick, simple and safe method for nodularin detection and quantification in water is presented. SPME presents the possibility of eliminating solvent use, often necessary for analytical determination of nodularin. This has benefits in reducing cost, time and dangers to the environment and personnel working in the field. The SPME-HPLC procedure was optimised and validated for nodularin and found to be linear and reproducible over a large concentration range. Although the SPME-HPLC method was shown to detect nodularin to 10 ppb other sensitive methods are available. With this in mind, further development is needed and current application of SPME-HPLC may be suited to the quick screening of potentially contaminated waters.

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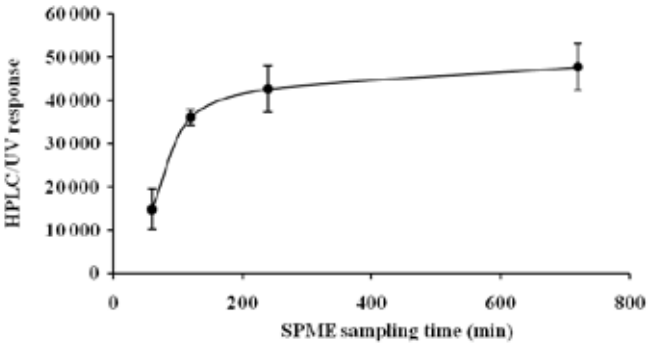

Figure 4. SPME absorption - time profile of nodularin at a spike level of 10 ppb in Milli-Q water. All samples contained 30% NaCl and were adjusted to pH 2. (n = 4; error bars represent ± SD)

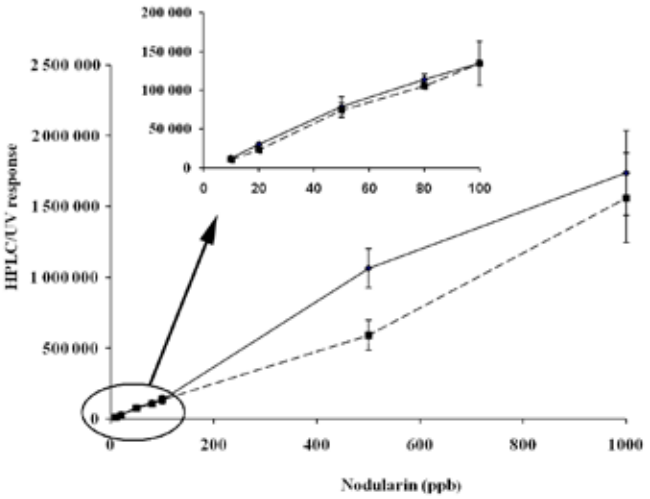

Figure 5. Nodularin (10-1000 ppb) standard curve after extraction using SPME-HPLC. Solid line represents nodularin in Milli-Q water. Dashed line represents nodularin in seawater. Samples contained 30% NaCl and were adjusted to pH 2, extraction time was 1 hour. (n = 3; error bars represent ± SD)

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