Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 784 (2003) 243–253
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Mass spectrometric detection of nodularin and desmethylnodularin in mussels and flounders

Krister Karlsson[a], Vesa Sipiä[b], Harri Kankaanpää[b], Jussi Meriluoto[a,*]

[a]*Department of Biochemistry and Pharmacy, Åbo Akademi University, P.O. Box 66, 20521 Turku, Finland*
[b]*Finnish Institute of Marine Research, P.O. Box 33, 00931 Helsinki, Finland*

Received 4 June 2002; received in revised form 7 October 2002; accepted 8 October 2002

Abstract

Samples of mussels and flounders from the Baltic Sea were analysed for nodularin content on two different LC–MS instruments (triple quadrupole and ion trap). The triple quadrupole instrument was well suited for the quantitative analysis. The limit of detection in the selected ion recording mode was 5 pg and in the multiple reactant monitoring mode 500 pg on column for extracts of *Nodularia spumigena*. The fragmentation patterns of nodularin-R and desmethylnodularin-R were recorded and shown to be similar to those of microcystins. LC–MS proved to be an excellent tool for the analyses of cyanobacterial hepatotoxins in complex matrices.

Keywords: Mussel; Flounder; Nodularin; Desmethylnodularin

1. Introduction

The cyanobacterium *Nodularia spumigena* occurs regularly in the Baltic Sea during the summer [1,2]. Poisonings caused by *N. spumigena* have been reported since 1878 [3]. The main toxin produced by *N. spumigena* is nodularin-R (Nod-R), full name: cyclo(D-*erythro*-β-methylAsp(iso)-L-Arg-Adda-D-Glu(iso)-2-(methylamino)-2-(*Z*)-dehydrobutyric acid) where Adda corresponds to the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid [4] see Fig. 1. Nod-R shows great similarities with microcystins that are hepatotoxins produced by freshwater cyanobacteria. The toxicity of Nod-R is due to the inhibition of protein phosphatase 1 and 2A [5]. In addition to the hepatotoxicity Nod-R has also been reported as a direct carcinogen in rat liver and not only as a tumor promoter as microcystins [6]. It is also suggested that Nod-R penetrates hepatocytes more easily than microcystin-LR [6]. These facts suggest that even lower

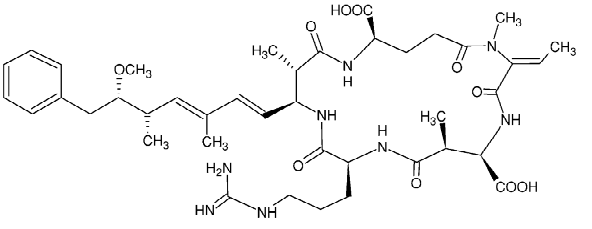

Fig. 1. The chemical structure of nodularin-R, desmethyl-nodularin-R is otherwise similar but lacks one methyl group in Asp at position 3.

*Corresponding author. Tel.: +358-2-215-4873; fax: +358-2-215-4745.
E-mail address: jussi.meriluoto@abo.fi (J. Meriluoto).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00802-4

exposures of Nod-R, than the World Health Organization (WHO) recommended tolerable daily intake (TDI) of 40 ng/kg body mass for microcystin-LR [7], can have serious effects on human health.

Microcystins and microcystin conjugates have previously been analysed in tissue with liquid chromatography–mass spectrometry (LC–MS) [8–10]. Watanabe et al. [10] and Kondo et al. [8] used frit-fast atom bombardment (FAB) LC–MS and Hormazabal et al. [9] used electrospray ionization (ESI) LC–MS, with single ion monitoring and full scan for detection of hepatotoxins. The multiple reactant monitoring (MRM) technique has been suggested for the analysis of cyanobacterial hepatotoxins in complex matrices [11]. It has recently been shown by ELISA (enzyme-linked immunosorbent assay) techniques that Nod-R indeed accumulates in mussels and flounders and other organisms in the Baltic Sea [12,13]. Other techniques besides LC–MS and ELISA that have been used for nodularin analyses in tissue are protein phosphatase inhibition (PPI) assay [12], magnetic resonance techniques [14] and detection of apoptosis induced by the toxins in hepatocytes [15]. Inhibition of PP was observed also for the negative standards which makes PPI an uncertain method for tissue analysis. The other detection techniques are not routine methods. Lemieux oxidation with MMPB (3-methoxy-2-methyl-4-phenylbutyric acid) analysis has been used successfully for microcystin analyses in crab larvae and fish [16,17]. Microcystins form covalent bonds with PP [18] in organisms and are thus only extractable to a certain degree that varied from 24% (salmon) [16] to less than 0.1% (mussels) [17]. Nod-R does not bind covalently to PP [19,20] and it should thus be more easy to extract Nod-R from tissue material. The enzyme glutathione-S-transferase catalyzes an addition reaction of microcystins and glutathione. This adduct formation has been suggested to be active in animals and plants as a part of the detoxification mechanism [21–23]. Probably is the same detoxification mechanism active for Nod-R [24].

The aim of the study was to set up quantitative LC–MS analyses of nodularins in tissue material. We also wanted to compare two different LC–MS instruments and different modes of detection on the triple quadrupole instrument for nodularin analyses. It was shown that LC–MS could be used for the quantitative analyses of nodularins in tissue.

2. Materials and methods

2.1. Chemicals

HPLC-grade methanol was purchased from Baker (Denver, The Netherlands) and HPLC S-grade acetonitrile from Rathburn (Walkerburn, UK). *n*-Butanol was of analytical or chromatographic grade and purchased from Fisher Scientific (Leicestershire, UK). Trifluoroacetic acid of protein sequencing grade was from Fluka (Buchs, Swizerland). Formic acid was of analytical grade and purchased from Riedel-de Haën (Seelze, Germany). Water was purified to 18.2 MΩ cm on a Milli-Q plus PF system (Millipore, Molsheim, France). The microcystin-LR was purified in the laboratory from *Microcystis* PCC 7820 (Institut Pasteur, Paris, France) [25]. The nodularins used as standards were extracts of *N. spumigena* AV1 (culture collection of Professor K. Sivonen, University of Helsinki, Finland). The solid-phase extraction (SPE) cartridges used at the Finnish Institute of Marine Research (FIMR) for set 1 were Bakerbond C_{18} (500 mg sorbent, Baker, Phillipsburg, NJ, USA). The SPE cartridges for set 2 used at the Department of Biochemistry and Pharmacy at the Åbo Akademi University (DBP) were Isolute C_{18} EC (100 mg sorbent, International Sorbent Technology, IST, Mid Glamorgan, UK).

2.2. HPLC conditions

The HPLC system for the diode array detection (DAD) consisted of a Degasys DG 2410 degasser from Uniflows (Tokyo, Japan), an FCV-10AL gradient mixer, an LC-10AT pump and an SIL-9A autosampler from Shimadzu (Kyoto, Japan). The column used was a Chromolith Performance RP-18e [26] (100 mm×4.6 mm I.D.) and the temperature was stabilized to 25 °C with a Merck–Hitachi L-7360 (Darmstadt, Germany) column heater. The mobile phase used was A: 0.05% TFA in water, B: 0.05% TFA in acetonitrile, and the flow-rate was 2

ml/min. The gradient was run from 25% B to 70% B in 5 min, held at 70% B for 0.5 min and then returned to 25% B, the injection interval was 10 min. Detection was performed with a Merck–Hitachi L-7450A photodiode-array detector at 200–300 nm. The absorbance data was analysed with Hitachi D-7000 HPLC system manager (HSM) software, version 3.1.1. The chromatograms recorded at 238 nm were used for automated integration of the peaks for quantitative analyses.

The same HPLC conditions and similar apparatus were used for both MS instruments. The HPLC apparatus was an Agilent 1100 series instrument (Agilent Technologies, Germany) consisting of vacuum degasser, binary pump, autosampler and thermostatted column compartment kept at 40 °C. The column was a Purospher STAR RP-18 endcapped (3 μm) 30 mm×4 mm I.D. (Merck). A Phenomenex (Torrance, CA, USA) C_{18} guard column was used. The mobile phase used was A: 0.5% formic acid in water, B: acetonitrile and the flow-rate was 0.5 ml/min. The gradient run was from 25% B to 70% B in 10 min, then to 90% B in 2 min. Acetonitrile was held at 90% for 1 min until the initial conditions were set. The injection interval was 18 min and 10 μl samples were injected for the analyses.

2.3. Mass spectrometers

The triple quadrupole mass spectrometer used was a Quattro Micro (Micromass, Manchester, UK) with ESI. Ions formed were detected in positive mode. The ionisation parameters were: capillary voltage 4.10 kV; cone voltage 75 V; source temperature 150 °C; desolvation temperature 350 °C; cone gas flow 100 l/h; desolvation gas flow 650 l/h. Fragmentation was achieved with argon collision gas pressure of $4 \cdot 10^{-3}$ mbar and collision energy of 60 eV, for the multiple reactant monitoring. The data acquisition was performed with Masslynx V3.5 software (Micromass) and the files were exported to Microsoft PowerPoint software in order to make publication quality chromatograms.

The ion trap instrument used was an Agilent 1100 series LC/MSD SL (Agilent technologies, Germany) mass spectrometer. ESI was used and ions were detected in the positive mode. Smart parameter setting (SPS) was used for m/z 825 with a 70% compound stability which gave a capillary voltage of 3.70 kV and trap drive voltage of 65 V. The nebulizer pressure was set to 15 p.s.i., the drying gas to 5 l/min and the dry temperature to 325 °C for the toxin infusion experiments (1 p.s.i.=6894.76 Pa). The nebulizer pressure was 40 p.s.i. and the dry gas flow 12 l/min for the ion trap LC–MS runs. The software used on the Agilent system was as follows: Chemstation for LC 3D for controlling the LC, LC/MSD Trap Control 4.1; MSD Trap Control version 5.0 for controlling the mass spectrometer and Bruker Daltonik Data Analysis version 2.1 to handle the data collected. The spectra were exported to PowerPoint software for the publication.

2.4. Mass detection

For the triple quadrupole instrument m/z values of 825.5 and 811.5 were chosen for Nod-R and dmNod-R, respectively, for the selected ion recording (SIR). Transitions from m/z 825.5 and 811.5 to m/z 135.1 were used for the multiple reaction monitoring. Microcystins were monitored in separate, non-spiked parallel samples of set 2 by choosing a selection of SIR channels corresponding to certain microcystins and also a full scan from m/z 900–1100. The channels and the corresponding microcystins were; 967.5 (didesmethylmicrocystin-LR), 981.5 (desmethylmicrocystin-LR), 995.5 (microcystin-LR), 1017.5 (didesmethylmicrocystin-YR), 1031.5 (desmethylmicrocystin-YR), 1045.5 (microcystin-YR), 505.7 (didesmethylmicrocystin-RR), 512.7 (desmethylmicrocystin-RR), 519.7 (microcystin-RR), 135.1 (fragment of Adda). The fragment of Adda was monitored as a general indication of microcystins and the corresponding protonated molecule would be identified in the scan mode.

In the ion trap instrument only m/z of 825.5 was collected for the SIR analysis. The fragmentation patterns of Nod-R and dmNod-R were collected as a product scan of the m/z 826.5 and 812.5 with an isolation window of 3 u, the scan range was from 200 to 900. The fragmentation mass spectrum was averaged over a period of 4 min for Nod-R and 8 min for dmNod-R and the fragmentation amplitude was 0.70 for both toxins.

2.5. Sample preparation

Blue mussel (*Mytilus edulis*) and flounder (*Platichthys flesus*) samples were collected in the Gulf of Finland by scuba diving (see Tables 2 and 3 for details of the sampling site and date). As negative controls were flounders from the North Atlantic Ocean and mussels from the Vigo Bay, Spain used. The flounder livers were separated, weighed and homogenized prior to freeze–drying. The freeze–dried samples were stored at −20 °C until further treatment. The mussel samples were pooled (100 individuals) and treated similar to the fish samples. Set 1 was extracted at FIMR as follows: 1 g of freeze–dried material was extracted with 30 ml of water–methanol–*n*-butanol (75:20:5, v/v) under shaking in a ultrasonic bath (50–60 °C) for 8 h. The samples were then centrifuged at 2500 *g* for 15 min. The supernatant was collected and reduced to ca. 1 ml with a rotary evaporator. The remains of the samples were transferred to Eppendorf tubes and centrifuged at 12 000 *g* for 15 min prior to SPE purification. The SPE cartridges were preconditioned with methanol and washed with water. The cartridges were washed with water and 10% methanol after the adsorption of the targeted compounds in extracts. The toxins were eluted with 100% methanol. The eluate was evaporated to dryness and stored at −20 °C until LC–MS analyses. The dry samples were sent to the DBP. At DBP the samples were reconstituted in 200 μl of 75% methanol and 100 μl was taken for the quadrupole LC–MS analyses. The rest was evaporated to dryness and stored at −20 °C. The samples were later reconstituted again with 100 μl of 75% methanol for the ion trap LC–MS analyses.

Set 2 was handled at DBP as follows: the mussel and flounder samples were treated similar to set 1 but sent to DBP as freeze–dried powder. A 0.175-g amount of the freeze–dried material was weighed and then extracted two times with 3 ml of 75% methanol in an ultrasonic bath for 15 min. The extracts were pooled and centrifuged at 10 000 *g* for 10 min. The volume was reduced by blowing argon gas at 50 °C, to a maximum of 1.5 ml prior to SPE purification. SPE purification was performed on IST cartridges in a similar way to the treatment of set 1. The eluate was blown to dryness with argon and reconstituted with 200 μl of 75% methanol for LC–MS analyses.

2.6. ELISA analyses

ELISA analyses were performed on the samples at FIMR and the samples were extracted with water–methanol–*n*-butanol (75:20:5, v/v), see details above. No SPE purification was performed for the ELISA tests but the samples were diluted to suitable concentrations. The ELISA was performed using an EnviroLogix, Microcystins plate kit (EnviroLogix, Portland, Maine, USA) according to the manufacturer's instructions. This kit has good crossreactivity between microcystins and nodularin. The concentration at 50% inhibition (50% Bo) is 0.50 ppb for microcystin-LR and 0.73 ppb for nodularin as reported by the technical specifications of the kit. The plates were read at 450 nm on a Benchmark Microplate Reader (Bio-Rad, CA, USA).

2.7. Toxin identification and calibration

The strain AV1 of *N. spumigena,* cultured at FIMR, was used as a source for Nod-R and dmNod-R standards. Collected cells were extracted with 75% methanol for 30 min by bath sonication. The toxin content in the extract was quantified with HPLC–DAD analysis. The HPLC–DAD system was calibrated for microcystin-LR by methods described in Ref. [25]. The molar absorption coefficient ϵ for nodularin-R was estimated to be the same as for microcystin-LR (39 800 l mol^{-1} cm^{-1}). The samples from the HPLC–DAD runs were further diluted and used as standards for the LC–MS calibrations.

Full scan (m/z 100–1400) MS analysis with the ion trap instrument was performed on the extract; m/z 825.5 corresponded to Nod-R and m/z of 811.5 to dmNod-R.

3. Results

3.1. Infusion experiments

In order to establish the optimal parameter settings for Nod-R, direct infusion analysis of the *N. spumigena* extract was performed on the ESI triple

quadrupole system. The infusion experiment clearly shows also a desmethylated variant (m/z 811.5) of Nod-R to be present in the cyanobacteria extract. The optimal signal response was obtained with the cone voltage of 75 eV and capillary voltage of 4.10 kV. Higher cone voltage produced more in-source CID (collision induced dissociation) that could be detected as a higher signal for the m/z of 135.1, which corresponds to a fragment of Adda. Daughter scan of m/z 825.5 and 811.5 also produced the m/z of 135.1, a collision energy of 60 eV gave the maximum signal for MRM runs. These parameters were used for the SIR, respectively, MRM quantitative runs on the triple quadrupole system.

Multiple MS analysis was performed on the ESI iontrap system in order to establish the fragmentation pattern of Nod-R and dmNod-R. The fragmentation patterns of Nod-R and dmNod-R from the infusion experiments are shown in Fig. 2. The experiment was performed as a daughter scan of the protonated molecule $[M+H]^+$ of both toxins. Similar cleavage patterns that have been reported for microcystins [27] could be observed in nodularins as well. Loss of ammonium, water or CO_2 from the protonated molecule was observed in both nodularins. The loss of the typical fragment m/z 135.1 could be seen as an m/z of 691.5 for Nod-R and 677.5 for dmNod-R. An m/z of 599.5 could be found for both nodularins and the ion consists of the common part of Arg–Adda–Glu, which is also found in most microcystins. The m/z 389.2 found for both Nod-R and dmNod-R corresponds to the fragment $C_{11}H_{15}O$+Glu+Mdhb+H [27]. This fragment proves the desmethylation site to be located in Asp at position 3, [D-Asp^3]Nod-R. See Table 1 for details of the fragments.

3.2. Quantitative LC–MS analyses

Quantitative calibrations of the instruments were performed immediately before the analyses of the real samples. Quantitative standards were embedded in the sample series in order to monitor the fluctuations of the signal response. It was found that the signal response could vary significantly especially in the triple quadrupole instrument when the cone in the

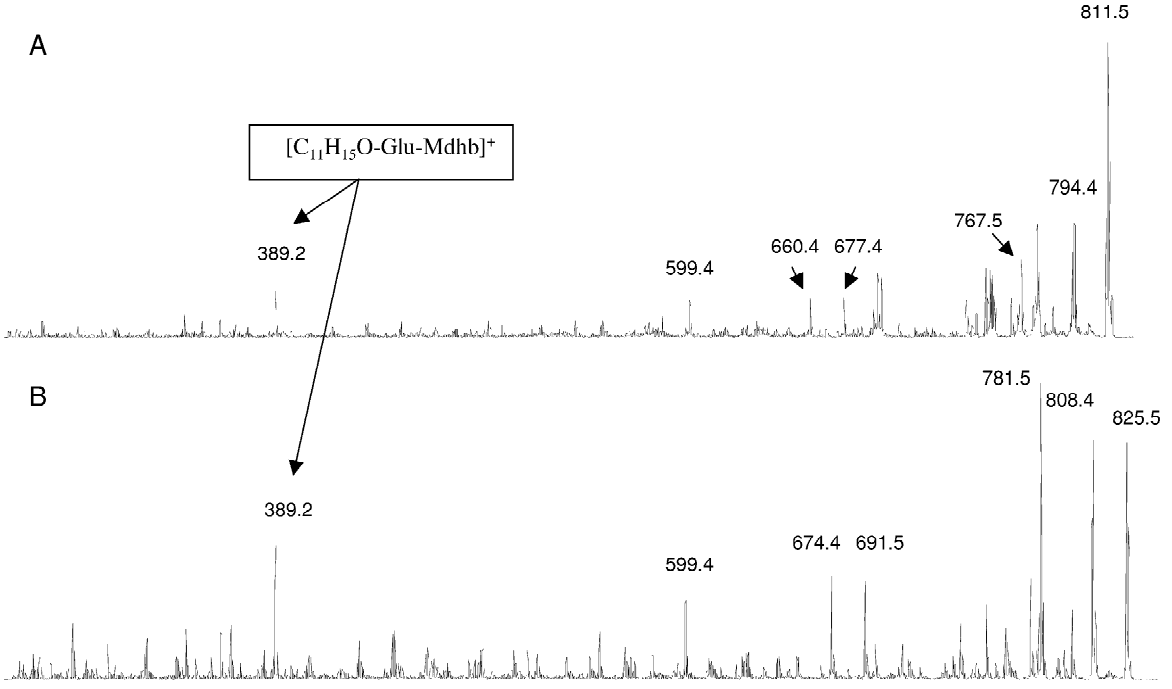

Fig. 2. The mass spectra of desmethylnodularin-R (A) and nodularin-R (B) as daughter scans of the protonated molecule $[M+H]^+$ on the ion trap instrument.

Table 1
The fragment ions of Nod-R and dmNod-R in MS–MS experiment on the Agilent ion trap ESI-MS system

Fragment ion	*m*/*z* dmNod	*m*/*z* Nod-R
$[M+H]^+$	811.5	825.5
$[M+H\text{-}NH_3]^+$	794.4	808.5
$[M+H\text{-}H_2O]^+$	793.5	807.5
$[M+2\ H\text{-}CO_2]^+$	767.5	781.5
$[M+2H\text{-}135]^+$	677.4	691.5
$[M+2H\text{-}135\text{-}NH_3]^+$	660.4	674.4
$[Arg\text{-}Adda\text{-}Glu+H]^+$	599.4	599.4
$[C_{11}H_{15}O+Glu+Mdhb+H]^+$	389.2	389.2

interface got dirty. See Fig. 3 for typical calibrations curves. A linear response was found for Nod-R reaching from 50 pg to 50 ng on column, the same molar response was used for both toxins. A total of 13 samples that were treated and pre-analysed by ELISA at FIMR were analysed on both LC–MS systems (set 1). These samples were analysed both in the SIR and the MRM modes on the triple quadrupole instrument, except where mentioned. See Table 2 for the results on the quantification of Nod-R and dmNod-R. The dmNod-R peak in the samples was sometimes broad and even double peaks were observed (see Fig. 4 for chromatograms), this is probably due to two different desmethylation sites. The standards contained only [D-Asp^3]Nod-R. The amounts of Nod-R and dmNod-R are given as ng in g tissue (dry weight, dw) and not corrected according to the recovery. A total of nine samples were extracted at DBP and analysed on the triple quadrupole instrument (set 2). The ELISA results are from analyses previously carried out at FIMR. See Table 3 for the results on the quantitation of Nod-R and dmNod-R. Here the concentration was too low for MRM analysis. Set 2 was spiked with microcystin-LR prior to the extraction in order to calculate the recovery. The recovery for microcystin-LR was at the highest 60% and at the lowest 20%, the mean value was 31%. No microcystins could be identified from set 2 in the additional microcystin analyses.

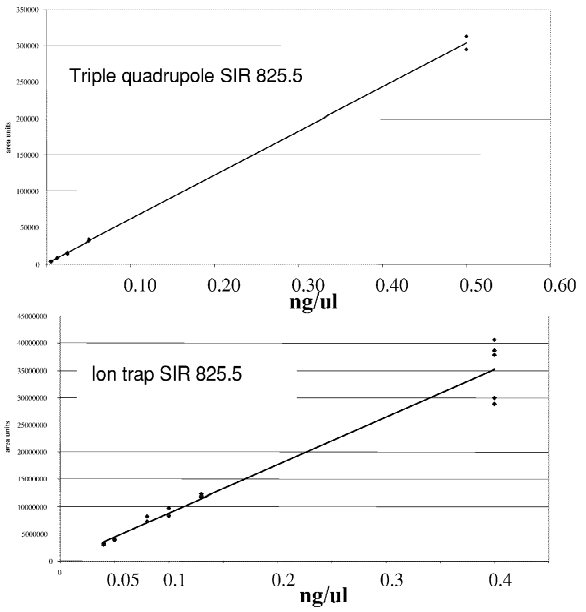

Fig. 3. Examples of the calibration curves that was achieved with injection of different concentrations of nodularin-R. The range of linear response was typically at least four decades, 5 pg to 50 ng on column. Each amount was injected at least three times and the individual responses can be seen on the graph.

It was found that the nodularins have longer retentions times in the flounder samples than in the standards and the mussel samples. This was verified by spiking of flounder sample in set 2, see Fig. 5 for the chromatograms. Nod-R had a 0.3 min longer retention time in the flounder samples as compared to the mussel samples. Although this change is small, it is significant, repeatable and it could be observed on both LC–MS systems. The concentrations of Nod-R and dmNod-R in the flounder samples were calculated in the same manner that the mussel samples were. Spiking the negative control flounder sample, gave retention times equal to the standards.

4. Discussion

This study has shown that low concentrations of Nod-R and dmNod-R in tissue can be analysed with LC–MS. ELISA and LC–MS complement each other and together they form a powerful method which can be used for routine tissue analyses.

4.1. LC–MS analyses, comparison and evaluation

The linearity of the calibration curve was better for the triple quadrupole instrument as compared to

Table 2
The sampling site and dates and toxin contents by both LC–MS instruments (triple quadrupole and ion trap ESI) and detection modes (SIR and MRM) and the ELISA results for set 1, see Sections 2.2 and 2.3 for conditions

	Sample	Sampling site and depth	Sampling date	LC–MS SIR (ng/g dw)		LC–MS MRM (ng/g dw)		LC–MS IT (ng/g dw), Nod-R	ELISA (ng/g dw), total toxins
				dmNod-R	Nod-R	dmNod-R	Nod-R		
1	Mussel	Sundholm, 3 m	08/07/1999	4	31	5	28	13	196
2	Mussel	Sundholm, 3 m	20/08/1999	12	84	11	70	32	2100
3	Mussel	Sundholm, 3 m	24/09/1999	12	105	13	89	32	360
4	Flounder liver	Sundholm	25/08/2000	nd	25*	nd	23*	5*	nd
5	Flounder liver	Sundholm	17/07/2001	4*	100*	4*	98*	25*	640
6	Mussel	Grunbacken, 4–5 m	25/07/2001	32	303	31	245	148	350
7	Flounder liver	Sundholm	31/07/2001	nd	6*	nd	6*	nd	250
8	Flounder liver	Furuskär	17/08/2001	nd	15*	nd	16*	nd	240
9	Flounder liver	Sundholm	28/08/2001	nd	28*	2*	25*	nd	nd
10	Mussel	Lågland, 1 m	15/07/2001	–	–	10	33	–	nd
11	Mussel	Lågland, 5 m	15/07/2001	7	27	7	41	–	nd
12	Mussel	Western Gulf of Finland, 5 m	16/08/2001	–	–	6	45	–	500
13	Mussel	Hanko, 10 m	26/08/2001	–	–	13	58	4	nd

The ELISA results from 1999 have been adapted from Ref. [13] and results from 2000 from Ref. [31].
* Later eluting Nod-R and dmNod-R.
''nd'': Not detected.
''–'': Not analysed.

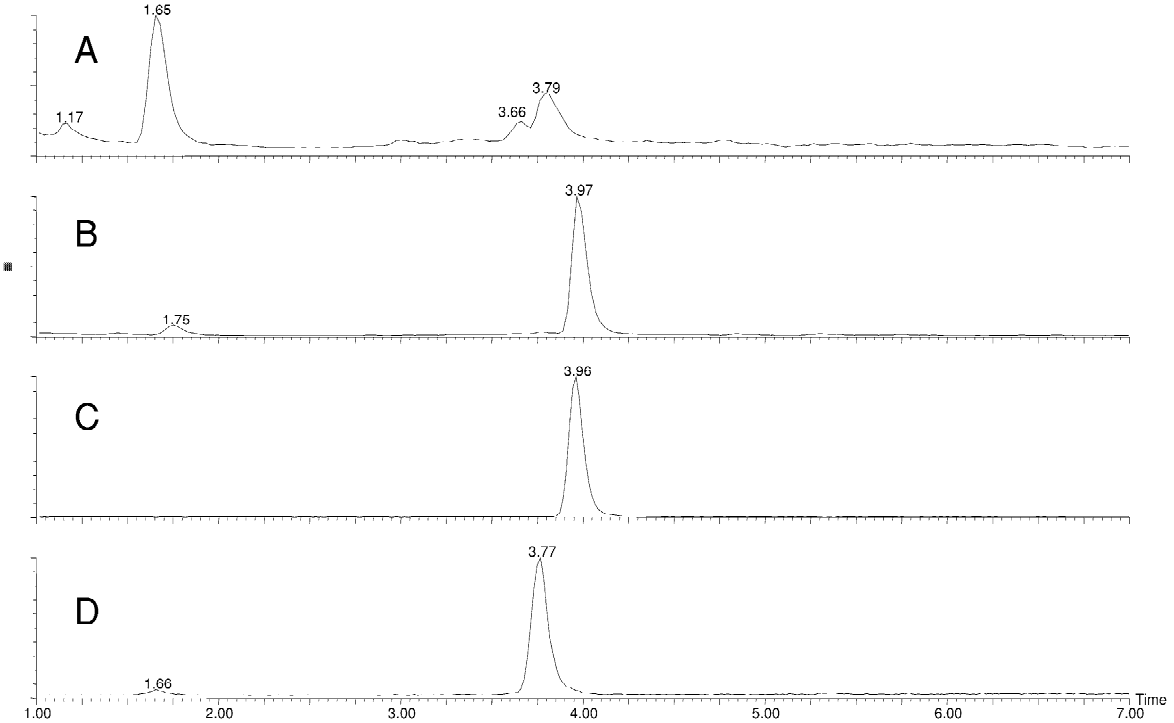

Fig. 4. Examples of mass chromatograms of the samples and standards see Section 2.2. for chromatography details and Section 2.3 for mass spectrometry details. SIR of 811.5 (dmNod-R) (A) and SIR 825.5 (Nod-R) (B) for sample 3 in set 1. SIR 825.5 (C) and SIR 811.5 (D) of the standard. The double peak in (A) suggests two different desmethylated nodularins, this have not been verified by collection of fragmentation pattern.

Table 3
The sampling site and dates and the toxin contents by LC–MS (SIR mode in triple quadrupole ESI) and ELISA results for set 2, see Sections 2.2 and 2.3 for conditions

	Sample	Sampling site and depth	Sampling date	LC–MS SIR (ng/g dw), Nod-R	ELISA (ng/g dw), total toxins
1′	Flounder liver	Sundholm	26/07/1999	256*	136
2′	Flounder liver	Rymättylä	15/08/1999	nd	nd
3′	Mussel	Sundholm, 3 m	10/09/1999	85	177
4′	Flounder liver	Sundholm	July 2000	294*	320
5′	Mussel	Eira, 27–28 m	06/07/2000	49	120
6′	Mussel	Eira, 27–28 m	27/07/2000	109	95
7′	Mussel	Eira, 27–28 m	15/09/2000	105	620
8′	Mussel	Helsinki, 7 m	14/10/2000	116	130
9′	Mussel	Helsinki, 13 m	14/10/2000	85	nd

The ELISA results from 1999 have been adapted from Ref. [13] and results from 2000 from Ref. [31].
* Later eluting Nod-R.
''nd'': Not detected.

the ion trap instrument. The sensitivity was equally high in both instruments. The samples were additionally dried and reconstituted for the ion trap analyses which gave lower toxin amounts compared to the triple quadrupole analyses.

The SIR mode was more sensitive than the MRM mode in the triple quadrupole instrument. This is probably due to insufficient fragmentation in the collision cell and a low level of noise in the SIR analyses. MRM gave better confirmation of peak identity than SIR but it was unnecessary for most runs. It is technically possible to monitor several SIR

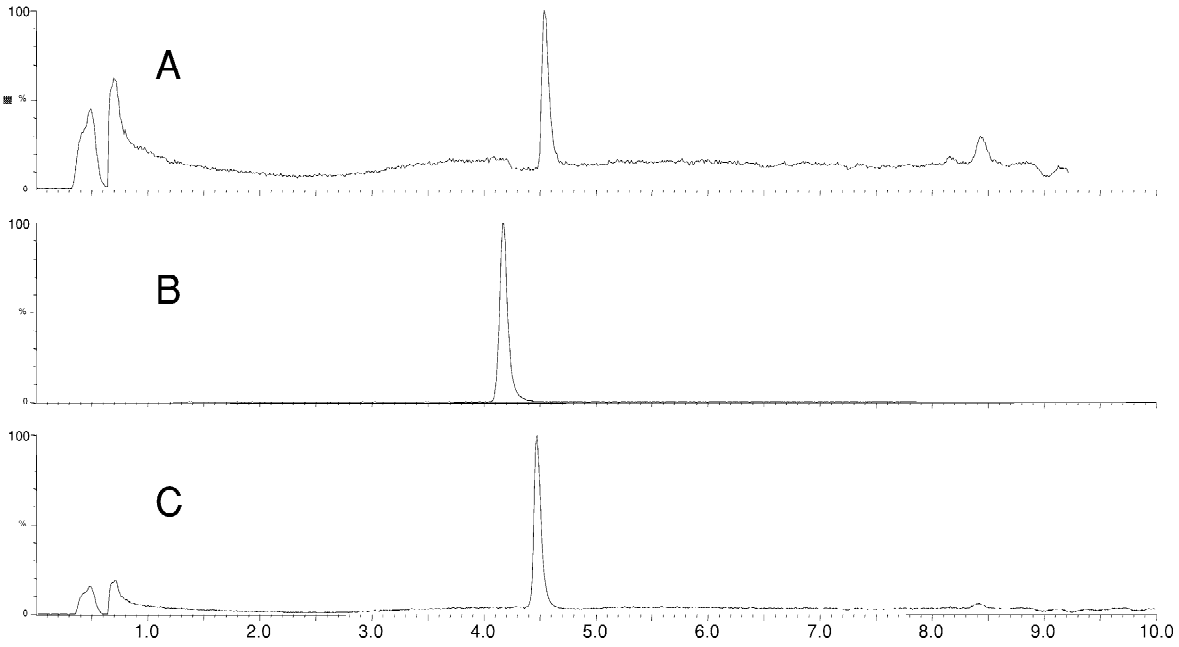

Fig. 5. The SIR chromatograms of m/z 825.5, flounder liver (sample 4) from set 2 (A), standard *N. spumigena* extract (B) and sample 4 spiked with the standard (C). The intensities were normalized to 100%.

channels simultaneous only in the triple quadrupole instrument. In the ion trap instrument can only one channel, in practice, be chosen for SIR monitoring at a certain time. Yuan et al. [28] reported excellent linearity in the ESI-MS response for microcystins containing only one arginine residue (compare nodularins) and recommended SIR analyses of the protonated molecule for trace analyses of microcystins. Zweigenbaum et al. [29] recommended ion trap mass spectrometry over triple quadrupole instrument for microcystins in biological samples. They scanned for unspecific microcystins over a certain range and collected MS–MS data automatically. We wanted a high sensitivity for two distinct toxins. For this kind of analyses and with these compounds the triple quadrupole instrument was superior to the ion trap instrument. The concentrations of toxins and possible conjugates are too low in tissue samples for scan analyses, for which the ion trap instrument certainly is better suited.

Some structural information could be derived from the analyses of Nod-R and dmNod-R, including a tentative site of the desmethylation in dmNod-R. There are three possible desmethylation sites in nodularins, in Adda, in 2-(methylamino)-2-(*Z*)-dehydrobutyric acid (Mdhb) at position 7 or in MeAsp at position 3. Because the desmethylated nodularin produced the 135.1 and the 389.2 fragments it leaves only MeAsp as possible site for desmethylation

We were not able to show the existence of the Nod-R glutathione conjugate in this study, although it has been found in Baltic Sea flounder earlier [30]. We did not see a peak at m/z 1132, corresponds to Nod-R-glutathione, in the full scan analyses of the samples. SIR analysis of m/z 1132 gave no peaks with shorter retention times than Nod-R, which would be expected from the conjugate [21].

4.2. Comparison of LC–MS vs. ELISA analysis

The observed toxin amounts were significantly lower in the LC–MS experiments than the ELISA tests. ELISA can detect glutathione conjugates of microcystin [31]. It is possible that the samples contain nodularin conjugates and the higher toxin concentration in the ELISA analyses could be described as the amount of nodularin conjugates. However we have not been able to show the existence of nodularin conjugates by LC–MS due to the lack of standards, or other technical reasons such as instability of the conjugates in the source. We have not yet been able to synthesize the nodularin glutathione conjugate. There is also a possibility of toxin loss between the ELISA and LC–MS analyses due to degradation or adsorption to tissue debris. The samples were extracted the same way for both the LC–MS and the ELISA analyses in set 1 but the LC–MS samples were purified with SPE cartridges where you can have a loss of toxin. A different extraction protocol in set 2 was used for the LC–MS analyses compared to that of the ELISA analyses. These results are in slightly better agreement with the ELISA results. The use of ELISA alone is not optimal for tissue samples and it is recommended only for water or cyanobacterial samples by the manufacturers of the kit. The manufacturers report a sufficient crossreactivity and a reasonable limit of detection of nodularin compared to microcystin-LR. There is no reason to question the reactivity of the ELISA kit to desmethylated nodularin, although it has not been specifically tested. The ELISA assay is useful for screening the total toxin and toxin conjugate content and has been successfully used for tissue analyses with recoveries ranging from 40 to 60% [32].

4.3. Chromatography

Nod-R and dmNod-R had longer retention times in the flounder samples compared to those of the mussel samples and the standards. The peak identification as Nod-R was positive in both SIR and MRM mode in the triple quadrupole and in the ion trap. This phenomenon of longer retention time could be due to ion-pairing of molecules present in the extract with the carboxylic acid groups or the guanidino group in arginine, both present in nodularin. In theory, more lipophilic substances with a suitable structure could also be non-covalently associated to the Adda moiety and thus give a higher capacity factor to the analyte. The spiking of the negative flounder sample gave not rise to a longer retention time for Nod-R. HPLC–DAD analysis of flounder samples will be extremely difficult and the selectivity of LC–MS analysis is of paramount importance. HPLC–DAD analysis can be performed if the toxin

amount is sufficiently high for identification of the toxins based on the absorption spectrum. Adequate selectivity in HPLC–DAD calls for longer analysis time. In this study was the injection interval 18 min but this was optimized for microcystins as well as nodularins and could be reduced even more if only nodularins were to be analysed.

There were differences in the consistency of the freeze–dried samples and some samples clearly contained oily substances that could not be completely dried. The last mussel samples (5′–9′) in set 2 varied less than the rest of the samples in their consistency and the recovery of spiked microcystin-LR was stable for these samples. The baseline was more unstable in set 2 as compared to that of set 1. This can be due to lower concentrations but also to poorer extraction conditions.

4.4. Possible health effects

This study has shown that significant amounts of nodularins can be found in edible fish from the Baltic Sea. The highest amount of toxin found in flounder liver was 300 ng/g. With the weight of a flounder liver being roughly 3 g (dw) and with a 30% recovery and a TDI of 40 ng/kg can we conclude that it is possible to exceed the TDI by consumption of one fish including the liver tissue. Although fish is not eaten every day and the liver is usually not consumed, the toxins from the liver can contaminate the rest of the fish. Freitas de Magalhães et al. found microcystins in muscle from fish in the Jacarepaguá lagoon in Brazil in concentrations that exceeded the TDI when the daily consumption of fish was estimated [33]. Consumption of fish is clearly a possible route of nodularin and microcystin exposure with potential health effects. It is still uncertain how much of the toxins are in bound form and how the bound toxin affects health. Appropriate risk assessments regarding exposure to cyanobacterial hepatotoxins by consumption of fish should be undertaken for the management of the possible health risk.

Acknowledgements

We wish to thank the Academy of Finland for financial support (RC for Natural Sciences and Engineering, project 47664 of J.M. and K.K., and RC for Biosciences and Environment, project 48019 of V.S.). K.K. also acknowledges the support from the National Graduate School of Informational and Structural Biology and the Magnus Ehrnrooth Foundation.

References

[1] K. Kononen, J.-M. Leppänen, Deutsche Hydrographische Zeitschrift Suppl. 6 (1996) 33.
[2] K. Sivonen, K. Himberg, R. Luukkainen, S.I. Niemelä, G.K. Poon, G.A. Codd, Toxic. Assess. 4 (1989) 339.
[3] G. Francis, Nature (London) 18 (1878) 11.
[4] K.L. Rinehart, K.-i. Harada, M. Namikoshi, C. Chen, C.A. Harvis, M.H.G. Munro, J.W. Blunt, P.E. Mulligan, V.R. Beasley, A.M. Dahlem, W.W. Carmichael, J. Am. Chem. Soc. 110 (1988) 8557.
[5] S. Yoshizawa, R. Matsushima, M.F. Watanabe, K.-i. Harada, A. Ichihara, W.W. Carmichael, H. Fujiki, J. Cancer Res. Clin. Oncol. 116 (1990) 609.
[6] T. Ohta, E. Sueoka, N. Iida, A. Komori, M. Suganuma, R. Nishiwaki, M. Tatematsu, S.-J. Kim, W.W. Carmichael, H. Fujiki, Cancer Res. 54 (1994) 6402.
[7] I. Chorus, J. Bartram, E and FN Spon, Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management, London, 1999.
[8] F. Kondo, Y. Ikai, H. Oka, H. Matsumoto, S. Yamada, N. Ishikawa, K. Tsuji, K.-i. Harada, T. Shimada, M. Oshikata, M. Suzuki, Nat. Toxins 3 (1995) 41.
[9] V. Hormazabal, Ø. Østensvik, B. Underdal, O.M. Skulberg, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 185.
[10] M.F. Watanabe, H.-D. Park, F. Kondo, K.-i. Harada, H. Hayashi, T. Okino, Nat. Toxins 5 (1997) 31.
[11] C. Edwards, L.A. Lawton, K.A. Beattie, G.A. Codd, S. Pleasance, G.J. Dear, Rapid Commun. Mass Spectrom. 7 (1993) 714.
[12] V. Sipiä, H. Kankaanpää, K. Lahti, W.W. Carmichael, J. Meriluoto, Environ. Toxicol. 16 (2000) 121.
[13] V.O. Sipiä, H.T. Kankaanpää, J. Flinkman, K. Lahti, J.A.O. Meriluoto, Environ. Toxicol. 16 (2001) 330.
[14] R.A. Towner, S.A. Sturgeon, N. Khan, H. Hou, H.m. Swartz, Chem.-Biol. Interact. 139 (2002) 231.
[15] K.E. Fladmark, M.H. Serres, N.L. Larsen, T. Yasumoto, T. Aune, S.O. Døskeland, Toxicon 36 (1998) 1101.
[16] D.E. Williams, M. Craig, S.C. Dawe, M.L. Kent, C.F.B. Holmes, R.J. Andersen, Chem. Res. Toxicol. 10 (1997) 463.
[17] D.E. Williams, S.C. Dawe, M.L. Kent, R.J. Andersen, M. Craig, C.F.B. Holmes, Toxicon 35 (1997) 1617.
[18] R.W. MacKintosh, K.N. Dalby, D.G. Campbell, P.T.W. Cohen, P. Cohen, C. MacKintosh, FEBS Lett. 371 (1995) 236.
[19] J.R. Bagu, B.D. Sykes, M.M. Craig, C.F.B. Holmes, J. Biol. Chem. 272 (1997) 5087.

[20] M. Craig, H.A. Luu, T.L. McCready, D. Williams, R.J. Andersen, C.F.B. Holmes, Biochem. Cell Biol. 74 (1996) 569.
[21] F. Kondo, Y. Ikai, H. Oka, M. Okumura, N. Ishikawa, K.-i. Harada, K. Matsuura, H. Murata, M. Suzuki, Chem. Res. Toxicol. 5 (1992) 591.
[22] F. Kondo, H. Matsumoto, S. Yamada, N. Ishikawa, E. Ito, S. Nagata, Y. Ueno, M. Suzuki, K.-i. Harada, Chem. Res. Toxicol. 9 (1996) 1355.
[23] S. Pflugmacher, C. Wiegand, A. Oberemm, K.A. Beattie, E. Krause, G.A. Codd, C.E.W. Steinberg, Biochim. Biophys. Acta 1425 (1998) 527.
[24] V. Sipiä, Ph.D. Thesis, Åbo Akademi University, Turku, 2001.
[25] J. Meriluoto, L. Lawton, K.-i. Harada, in: O. Holst (Ed.), Bacterial Toxins: Methods and Protocols, Humana Press, Totowa, NJ, 2000, p. 65.
[26] L. Spoof, J. Meriluoto, J. Chromatogr. A 947 (2002) 237.
[27] M. Yuan, M. Namikoshi, A. Otsuki, K.L. Rinehart, K. Sivonen, M.F. Watanabe, J. Mass Spectrom. 34 (1999) 33.
[28] M. Yuan, M. Namikoshi, A. Otsuki, M.F. Watanabe, K.L. Rinehart, J. Am. Soc. Mass Spectrom. 10 (1999) 1138.
[29] J.A. Zweigenbaum, J.D. Henion, K.A. Beattie, G.A. Codd, G.K. Poon, J. Pharm. Biomed. Anal. 23 (2000) 724.
[30] V.O. Sipiä, H.T. Kankaanpää, S. Pflugmacher, J. Flinkman, A. Furey, K.J. James, Ecotoxicol. Environ. Safety, in press.
[31] J.S. Metcalf, K.A. Beattie, S. Pflugmacher, G.A. Codd, FEMS Microbiol. Lett. 189 (2000) 155.
[32] H.T. Kankaanpää, K.M. Vuorensola, V.O. Sipiä, J.A.O. Meriluoto, Chromatographia 55 (2002) 157.
[33] V. Freitas de Magalhães, R. Moraes Soares, S.M.F.O. Azevedo, Toxicon 39 (2001) 1077.