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LC–ESI-Q-TOF-MS for faster and accurate determination of microcystins and nodularins in serum

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ABSTRACT

Microcystins (MC) and nodularins (Nod) are cyclic peptide hepatotoxins and tumour promoters produced by cyanobacteria. This study deals with liquid chromatography–mass spectrometry (LC–MS) analyses of 9 major cyanobacterial peptide toxins, starting with a comparison of six small particle size reversed-phase HPLC columns, from which one, Phenomenex Synergi Hydro-RP, was chosen for further chromatography with accurate mass MS studies in a complex biological fluid, serum. The instrumentation used for the serum sample analysis included a Bruker micrO-TOF-Q-MS coupled to an Agilent 1200RR LC system. Total analysis run time per sample was 8.5 min. The Q-TOF-MS instrument was operated on auto MS–MS mode to obtain fragment ions (such as the characteristic fragment m/z 135 from Adda amino acid residue) for toxin identification purposes. Detected mass errors in serum samples were in the range of from 0.3 mDa to 9.1 mDa. The narrow mass window (±20 mDa) for mass chromatograms used in quantitation gave benefits by background noise reduction. We conclude that a LC–ESI-Q-TOF-MS instrumentation is a powerful tool for identification and quantitation of cyanobacterial peptide toxins in a biological matrix.

1. Introduction

The cyanobacterial peptide toxins, microcystins (MCs) are produced by several genera of ubiquitous freshwater cyanobacteria, e.g. *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* [1,2]. The closely related nodularins (Nods) are produced by *Nodularia spumigena* in brackish waters [3]. Toxic cyanobacterial blooms are of concern to animal and human health when contaminated water bodies are used for drinking water, fishing, and irrigating crop plants. Microcystins and nodularins are hepatotoxins and tumour promoters [4,5] and Nod has been shown to be a direct carcinogen [6]. These toxins are known to specifically bind to protein phosphatases 1 and 2A [7] and in this way affect the cell signalling pathways. Microcystins and nodularins are also known to accumulate into tissues. The World Health Organization has acknowledged microcystins in drinking water as a potential health hazard and set a provisional guideline of 1 μg L^{-1} of MC-LR in drinking water [8].

Microcystins and nodularins are cyclic hepta- and pentapeptides, respectively, containing both common and uncommon amino acids. The characteristic unusual β-amino acid, Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid) [3] is required for the toxicity of these peptides, and the conjugated diene in Adda, the main chromophore absorbing at 238 nm, is also the source of absorbance spectra which is the basis for detection of microcystins and nodularins in HPLC–UV analysis. There are more than 90 structural variants of microcystins (partly reported in [9,10]) and more than 10 variants of nodularins identified this far (partly reported in [11,12]). The structure of microcystins is cyclo(-D-Ala-L-X-D-erythro-β-methyl-Asp(iso-linkage)-L-Z-Adda-D-Glu(iso-linkage)-N-methyldehydro-Ala) where the L-amino acid residues in positions 2 (X) and 4 (Z) are the most commonly varying amino acids in the structure and give the name to the variant. For example MC-LR (the most commonly occurring variant of microcystins) has leucine (L) in position 2 and arginine (R) in position 4 (Table 1). A very hydrophilic variant, MC-RR, has two arginines in these positions and, MC-LF, a more hydrophobic microcystin, has leucine and phenylalanine in these positions (Table 1). The demethylated variants, such as dmMC-LR and dmMC-RR, have one methyl group less (e.g. D-Asp instead of D-methyl-Asp in position 3). Another common demethylation site is position 7 where dehydroalanine is found instead of N-methyldehydroalanine [9]. The great number of closely related compounds makes the separation, identification and quantitation of the toxins more challenging. The overall structure is hydrophilic due to the carboxylic acid groups in positions 3 and 6 and the commonly occurring arginine residues in the structure, but the Adda residue (in position 5) makes the structure more hydrophobic. The nodularin structure is cyclo(-D-erythro-β-methyl-Asp(iso-linkage)-L-Z-Adda-D-Glu(iso-linkage)-2-methylamino-2(Z)-dehydrobutyric

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Table 1
Microcystins and nodularin used in this study.

	Position 1	Position 2	Position 3	Position 4	Position 5	Position 6	Position 7
Hepatotoxin variant							
dmMC-RR	D-Ala	L-Arg	D-Asp	L-Arg	Adda	D-Glu	Mdha
MC-RR	D-Ala	L-Arg	D-methyl-Asp	L-Arg	Adda	D-Glu	Mdha
MC-YR	D-Ala	L-Tyr	D-methyl-Asp	L-Arg	Adda	D-Glu	Mdha
MC-LR	D-Ala	L-Leu	D-methyl-Asp	L-Arg	Adda	D-Glu	Mdha
dmMC-LR	D-Ala	L-Leu	D-Asp	L-Arg	Adda	D-Glu	Mdha
MC-LY	D-Ala	L-Leu	D-methyl-Asp	L-Tyr	Adda	D-Glu	Mdha
MC-LW	D-Ala	L-Leu	D-methyl-Asp	L-Trp	Adda	D-Glu	Mdha
MC-LF	D-Ala	L-Leu	D-methyl-Asp	L-Phe	Adda	D-Glu	Mdha
Nod-R	D-methyl-Asp	L-Arg	Adda	D-Glu	Mdhb		

The microcystin and nodularin variants included in this study in both Extract Mixes (1 and 2). The grey background indicates residues that are variable or include modifications as compared to the common structure.

acid), where Z in position 2 is most often arginine (R) as in Nod-R [3].

The use of HPLC for analysing microcystins in drinking and natural waters as well as in cyanobacterial material has been well established [13]. Other common cyanobacterial toxins, the cytotoxic cylindrospermopsin and the neurotoxic anatoxin-a can also be analysed by HPLC–UV and by LC–MS [14,15]. These compounds, however, are much more hydrophilic than the microcystins, and therefore both the sample preparation and the chromatographic conditions need to be conducted separately [16].

Modern HPLC columns are designed for high-speed and high-throughput analysis and have particle size below 3 μm. The recent developments in both LC and column techniques as well as in the detection instruments (MS ion sources) have made it possible to reduce significantly the analysis time per sample [17].

Mass spectrometry has been utilised in structural [18–20] and quantitative studies [21] of microcystins. The TOF-MS instrumentation is an established tool in the quantitative studies of small molecules (veterinary drug residues, antibiotics, etc.) in biological matrices utilising the narrow, ±20 mDa mass window for compound detection [22,23]. With the help of high accuracy mass, analytes have been detected in milk [22], urine [24], water samples and dietary supplements [25], and hair [26]. Plasma has been studied with TOF-MS instrumentation [27,28], but in these studies the quantitation is not based on accurate mass. For microcystin analysis, a TOF instrument has been used mainly for structural analysis [29–31] and for quantitative purposes [25,32]. The complementing information provided by the tandem MS fragmentation of the quadrupole has seldom been utilised. In one tandem MS study with TOF-MS analyser the daughter ions, not the parent accurate mass, were used for quantitation of glycopyrrolate in plasma samples [33].

In this study our first aim was to compare two sub-2 μm particle and four 2.5 μm particle size RP-HPLC columns in the chromatographic separation of microcystins and nodularins. Based on the chromatographic performance, the most suitable column was chosen for the second part of the study, in which microcystin and nodularin analyses were performed with a challenging biological matrix, serum. Here we utilised an accurate mass MS instrumentation with the additional certainty in analysis obtained from quadrupole (CID) fragmentation products.

2. Experimental

2.1. Toxins

The microcystins used in this study are described in Table 1. They originated from cultures of *Microcystis* PCC7820 (deposited at Institut Pasteur, Paris) and *Microcystis wesenbergii* NIES-107 (deposited at National Institute of Environmental Studies, Tsukuba, Japan) grown in our Finnish laboratory, and purified by reversed-phase chromatography as described by Meriluoto and Codd [34]. The identities of the toxins were confirmed by typical ESI-MS–MS product ion spectra [12,18,35] acquired with an ion trap (HCT Ultra, Bruker Daltonics, Bremen, Germany). PCC7820 has been previously shown to produce MC-LR, -LY, -LW and -LF [36] and NIES-107 produces mainly 3-demethyl-MC-RR, MC-RR, MC-YR, 3-demethyl-MC-LR and MC-LR (partially reported in [37]). Nod-R originated from Baltic Sea bloom consisting mainly of *N. spumigena* collected with plankton net in July 2003, purified as described by Karlsson et al. [38]. The toxin purity assessment was based on RP-HPLC analyses with UV-absorbance detection at 210 nm, 238 nm, 254 nm and 280 nm. For column comparison study the toxin extracts were then dissolved into 35% methanol, mixed together to result in an extract mixture (called Extract Mix 1) containing ca 0.1 $\mu g\,mL^{-1}$ of Nod-R, MC-RR and MC-LR and lower concentrations of the other toxins. The same extracts, but in different dilutions were used in the serum study (called Extract Mix 2; see Table 4). The concentrations specified (in vial) in Table 4 are the highest ones used in serum spiking studies. Dilutions down to 1:50 of the highest concentrations were made and analysed. The concentrations used for spiking studies therefore ranged between 0.24 $ng\,mL^{-1}$ and 12 $ng\,mL^{-1}$ in serum (MC-LY) to 3.6–180 $ng\,mL^{-1}$ in serum (MC-LR).

2.2. Column comparison study

These experiments were performed at the NRC Halifax Laboratory. Acetonitrile was distilled-in-glass grade (Caledon Labs, Georgetown, ON, Canada) and water was purified to 18.2 Ω cm with MilliQ purification system (Millipore Ltd., Bedford, MA, USA). Formic acid (FA) and ammonium formate (AF) were both AR grade and purchased form Fischer Scientific (Fair Lawn, NJ, USA). The instrumentation consisted of an Agilent 1200 Rapid Resolution (RR) LC coupled to an Applied Biosystems 4000QTRAP MS (Applied Biosystems, Sciex, Canada). The LC system included a binary high-pressure gradient pump, in-line degasser, refrigerated autosampler, and a temperature controlled column oven set at 40 °C. The injection volume was 5 μL.

Six 50 mm × 2 mm reversed-phase columns were tested (Table 2): Waters Acquity Bridged Ethyl Hybrid (BEH) C-18 50 mm × 2.1 mm, 1.7 μm column, Agilent Zorbax Rapid Resolution (RR) Stable Bond (SB) C-18 50 mm × 2.1 mm, 1.8 μm, and four Phenomenex Synergi 50 mm × 2.0 mm, 2.5 μm columns: Hydro-RP, Fusion-RP, MAX-RP and Polar-RP.

The solvents were A: water, B: 95% acetonitrile, both with 50 mM formic acid and 2 mM ammonium formate, pH 2.3. The tested gradients included a long gradient from 10% to 80% B over 30 min, 80% B held for 5 min, and the column then equilibrated for 15 min at 10% B, flow 0.2 $mL\,min^{-1}$; an intermediate gradient from 30% to 75% B over 5 min, 75% B held for 6 min, the column equilibrated at 30% B for 14.5 min, flow 0.2 $mL\,min^{-1}$; or a fast gradient from 35% to 65% B

Table 2
Column specifications for the reversed-phase HPLC columns tested in this study.

Column	Particle size (μm)	Pore size (Å)	Surface area ($m^2\ g^{-1}$)	Carbon load (%)	Endcapped	Length (mm)	I.D. (mm)	Phase description
Waters Acquity BEH C-18	1.7	130	185	18	Yes	50	2.1	Trifunctional C-18, proprietary end-capping
Phenomenex Hydro-RP	2.5	80	400	19	Yes	50	2.0	C-18, polar end-capping
Phenomenex Fusion-RP	2.5	80	400	12	Yes	50	2.0	Polar-embedded C-18
Phenomenex Max-RP	2.5	80	400	17	Yes	50	2.0	C-12
Phenomenex Polar-RP	2.5	80	400	11	Yes	50	2.0	Ether-linked phenyl, polar end-capping
Agilent Zorbax RR SB	1.8	80	180	10	No	50	2.1	Sterically protected di-isobutyl n-octadecylsilane

over 1.1 min, 65% B held for 2 min, column then equilibrated 35% B for 6 min, flow 0.5 mL min^{-1}. The flow rates and gradients were not separately optimised for each column to correspond the different dimensions and chemistries.

The single ion recordings used in the analysis were *m/z* 512.8 (dmMC-RR, $[M+2H]^{++}$), 519.8 (MC-RR, $[M+2H]^{++}$), 825.5 (Nod-R, $[M+H]^{+}$), 981.5 (dmMC-LR, $[M+H]^{+}$), 986.5 (MC-LF, $[M+H]^{+}$), 995.6 (MC-LR, $[M+H]^{+}$), 1002.6 (MC-LY, $[M+H]^{+}$), 1003.6 (MC-LF, $[M+NH_3]^{+}$), 1009.6 (methylMC-LR, $[M+H]^{+}$), 1019.6 (MC-LY, $[M+NH_3]^{+}$), 1025.6 (MC-LW, $[M+H]^{+}$), 1042.6 (MC-LW, $[M+NH_3]^{+}$) and 1045.6 (MC-YR, $[M+H]^{+}$). The parameters for the initial SIR analyses were set as follows: declustering potential 75 V (during final optimisation lowered to 40 V for the doubly charged MC-RR and dmMC-RR), source temperature 275 °C, dwell time 25 ms, curtain gas 20 psi, turbo gas 1 and 2 at 50 psi. Data analysis was performed with the ACD/Labs MS Manager 10.0.

2.3. Serum analysis study

These experiments were performed at the Åbo Akademi University, Turku, laboratory.

The mobile phase consisted of water purified to 18.2 MΩ cm with a MilliQ Synthesis purification system (Molsheim, France), and acetonitrile, formic acid and ammonium formate of LC–MS grade were from Fluka, Sigma–Aldrich, Steinheim, Germany. Two different aqueous mobile phases were tested: in the first, called FA, eluent A (water) was acidified only with formic acid 26.5 mM (1 mL L^{-1}); in the second one, called AF + FA, the eluent A contained both, ammonium formate 15.9 mM (1 g L^{-1}) and formic acid 79.5 mM (3 mL L^{-1}; pH 2.5). The eluent B, 100% acetonitrile was in both cases (in FA and in AF + FA mobile phases) only acidified with 26.5 mM formic acid (1 mL L^{-1}).

Only one column, Phenomenex Synergi Hydro-RP, was used for serum analysis study with an Agilent 1200RR LC system coupled to a Bruker micrO-TOF-Q instrument (Bruker Daltonics, Bremen, Germany). Injection volume was 5 μL. Both of the mobile phases were tested (FA and AF + FA) with the same gradient, from 28% B to 38% B in 3.5 min, up to 75% B in 0.2 min for 0.9 min and in 0.6 min to 90% for a washing period, which was held for 1 min. The flow rate was 0.5 mL min^{-1}, except for equilibration at 1.0 mL min^{-1} (flow directed to waste) for 2.3 min. The total gradient plus equilibration time was 8.5 min. The source parameters were: for nebuliser gas 1.6 bar, dry gas 12.0 L min^{-1}, dry temperature 220 °C. The scan range was from *m/z* 50 to 1500, spectra time in MS mode 0.5 s. The instrument was operated on auto MS–MS mode with a preference mass list. The collision energy for fragmentation was set with a table for the two different charge states: 1: *m/z* 250 (20 eV), *m/z* 500 (30 eV), *m/z* 1000 (50 eV), *m/z* 1500 (75 eV); charge state 2: 1: *m/z* 250 (12 eV), *m/z* 500 (25 eV), *m/z* 1000 (30 eV), with collision energy sweeping from 80% to 120%. Each run (TOF-MS-analyser) was calibrated internally with a sodium formate cluster calibrant. Data analysis was done with Bruker DataAnalysis version 4.0 and QuantAnalysis version 2.0.

The serum sample preparation was performed as described by Neffling et al. [39]. In short, the procedure included protein precipitation of 1 mL fetal bovine serum sample with acetonitrile acidified with trifluoroacetic acid (TFA) and solid phase extraction with Waters Oasis HLB (30 mg packing) SPE cartridges. Spiking of the sample with different dilutions of the Extract Mix 2 was performed either before or after the sample pre-treatment in triplicates in order to have comparable samples for recovery assessment.

3. Results

3.1. Column comparison study, chromatographic performance

Column performance was assessed by resolution, selectivity and peak characteristics (see Table 3). Chromatograms of the well performing columns Acquity BEH C-18 and Hydro-RP columns are presented in Fig. 1.

The 5 min gradient gave somewhat better selectivity and resolution than the longer, 30 min gradient (Table 3), although the steepness of the gradient was 9% solvent B increase/min for the 5 min gradient, compared to 2.3% solvent B increase/min in the longer, 30 min gradient. Both of these gradients were run with the same 0.2 mL min^{-1} flow rate.

Two columns were chosen for testing the fast gradient: the Waters Acquity BEH C-18 column, which was able to resolve dmMC-LR/MC-LR, and the Phenomenex Synergi Hydro-RP because of its good performance in the longer gradients.

The peak characteristics were compared by peak height, width and shape, as well as signal to noise ratio (S/N; data not shown). In both, the 30 min and 5 min gradient runs, the Acquity BEH C-18 and the Zorbax RR columns gave the best peak characteristics. In the fast gradient (Fig. 1) the Acquity BEH C-18 performed better in the peak width comparison, but the Hydro-RP was better in the S/N and peak height assessment. Due to the excellent performance, and lower backpressure, the Hydro-RP column was chosen for the serum sample analysis study.

3.2. Serum analysis study

The recovery, or extraction efficiency, of the arginine-containing hepatotoxins (dmMC-RR, MC-RR, MC-YR, Nod-R, MC-LR and dmMC-LR) from serum matrix was from 78% to 94%, on average 87%. The chromatographic separation in the high-resolution MS studies were performed with the Hydro-RP column, and the FA mobile phase was compared to the AF + FA mobile phase with an optimised gradient with flow rate of 0.5 mL min^{-1} (Fig. 2). According to instrument ion source manufacturer (Bruker) recommendations, the flow rate was limited to 0.5 mL min^{-1}. The main difference between the two tested mobile phases FA and AF + FA was the separation of the

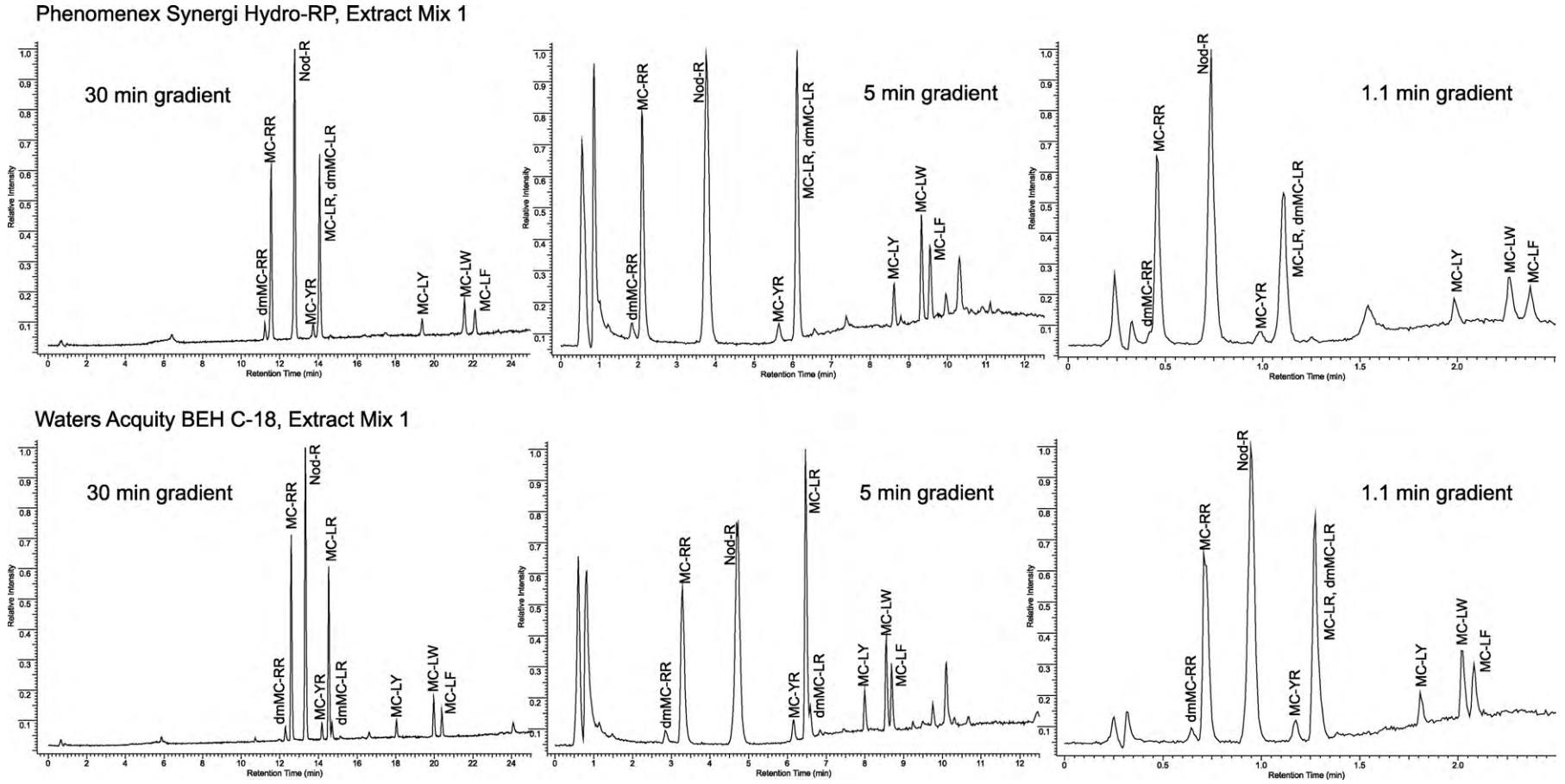

Fig. 1. Chromatograms of Extract Mix 1 produced by Phenomenex Synergi Hydro-RP and Waters Acquity BEH C-18 columns with the three tested gradients (see Sections 2.1 and 2.2 for sample and analytical details).

Table 3
Chromatographic performance data from the column comparison study.

Column	Resolution			Capacity factor		Selectivity[a]
	dmMC-LR/MC-LR	MC-YR/MC-LR	MC-LW/MC-LF	dmMC-RR	MC-LF	MC-LF/dmMC-RR
Long gradient: from 10% to 80% of solvent B over 30 min						
Waters Acquity BEH C-18	**1.3**	2.7	2.8	19.6	33.1	1.7
Phenomenex Hydro-RP	0.1	2.0	**2.9**	19.7	39.8	2.0
Phenomenex Fusion-RP	0.1	1.9	1.8	18.5	38.5	**2.1**
Phenomenex Max-RP	0.1	1.9	2.7	19.3	38.0	2.0
Agilent Zorbax RR	0.6	**3.1**	2.8	20.6	35.6	1.7
5 min gradient: from 30% to 75% of solvent B over 5 min						
Waters Acquity BEH C-18	**1.3**	2.7	1.4	3.7	13.5	3.6
Phenomenex Hydro-RP	0.3	2.9	**2.0**	2.4	16.6	7.0
Phenomenex Fusion-RP	0.0	2.8	1.5	2.3	16.1	**7.1**
Phenomenex Max-RP	0.1	2.9	1.7	2.4	16.0	6.7
Agilent Zorbax RR	0.6	**3.1**	1.4	4.2	14.8	3.5
Short gradient: from 35% to 65% of solvent B over 1.1 min						
Waters Acquity BEH C-18	**0.7**	2.1	1.5	1.6	7.5	4.6
Phenomenex Hydro-RP	0.0	**2.3**	**1.7**	0.8	9.0	**11.7**

The best value for each parameter in each gradient is in bold face.
[a] Selectivity of the column has been calculated as the ratio of capacity factors for the last eluting compound, MC-LF and the first eluting compound, dmMC-RR.

MC-LR/dmMC-LR pair. With AF + FA mobile phase the analytes were separated with *R*s value of 1.0, whereas with the FA mobile phase the *R*s value was only 0.5 (see Fig. 2 for chromatogram comparison). However, the signal intensity for the analytes (detected ions listed in Table 4) with the AF + FA mobile phase were about half of the signal intensity obtained with the FA mobile phase (see Fig. 2). No significant $[M+NH_4]^+$ ions were detected. Also the S/N values were lower, typically only about 25% of those obtained with the FA mobile phase: e.g. the concentration of 180 ng mL^{-1} in vial of the analyte dmMC-LR spiked in serum samples gave S/N values around 40 with FA mobile phase, and S/N around only 10 with the AF + FA mobile phase.

Following parameters were used to assess the performance of micrO-TOF-Q: limit of detection (Table 5), quantification range (Table 5) and spectral quality (MS and auto MS–MS modes) and mass errors in MS mode (Table 4). The accurate and detected masses for the nine different compounds in the different matrices (with FA mobile phase) are listed in Table 4. The spectra often also contained fragment ions resulting from loss of the Adda residue (134) (e.g. *m*/*z* 861.5 $[M-134+H]^+$ for MC-LR, $[M+H]^+$ *m*/*z* 995.5), and in some cases

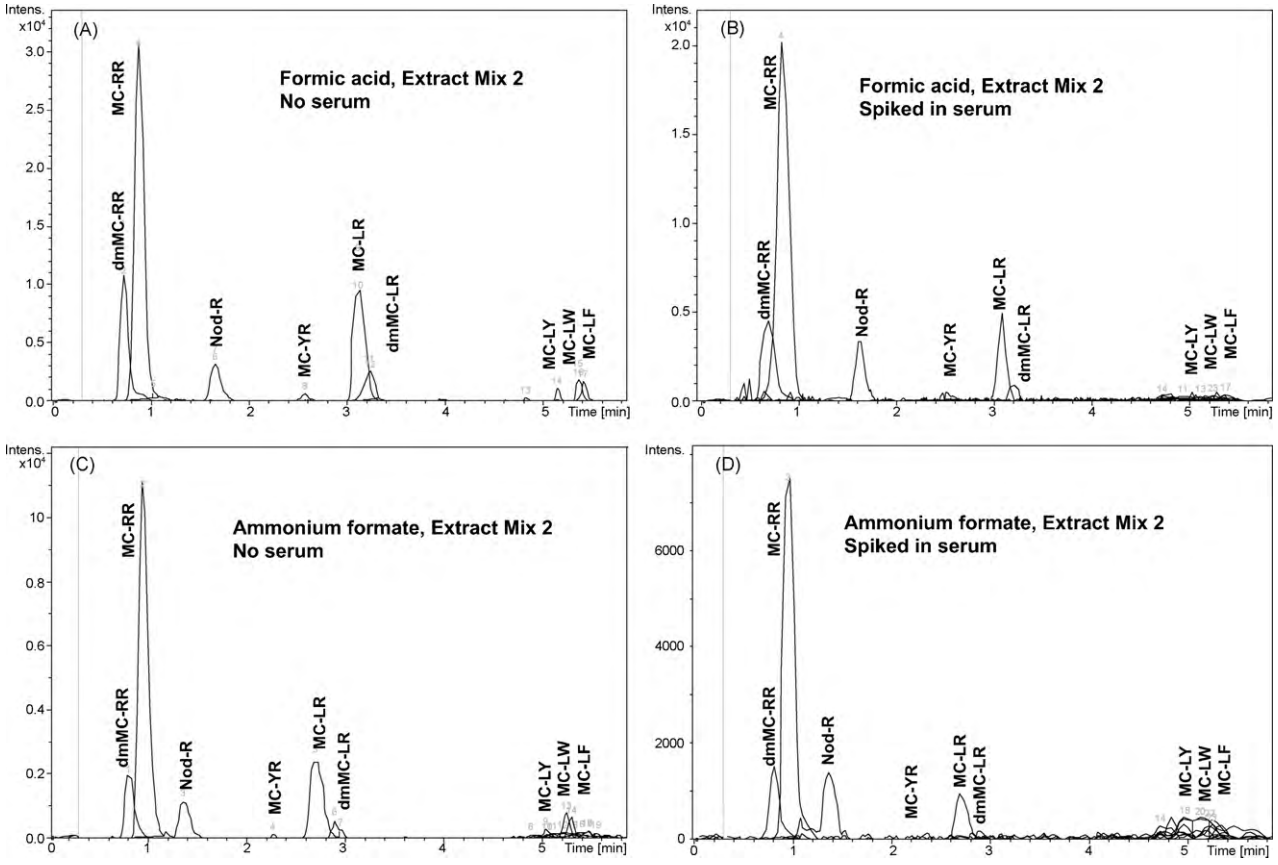

Fig. 2. Chromatograms of Extract Mix 2, with (B and D) and without (A and C) serum matrix, produced by the Phenomenex Hydro-RP column. Chromatograms of both of the two mobile phases (FA: A and B; AF + FA: C and D) tested are shown. See Section 2.3 for analytical details.

Table 4
Extract Mix 2 toxin detection in the serum study.

Hepatotoxin variant	Elemental composition, monoisotopic mass	Accurate masses for detected ions, included as quantitation traces	Toxin concentration in vial (ng mL^{-1})	Detected masses in culture extract	Mass error (mDA)	Detected masses in serum extract	Mass error (mDa)	Confirmatory fragments by auto MS–MS function
dmMC-RR	$C_{48}H_{74}N_{13}O_{12}$ 1023.5502	512.7824 $[M+2H]^{2+}$	250	512.7824	0.09	512.7821	0.28	135.1[a] 289.2[a] 213.1[a]
MC-RR	$C_{49}H_{76}N_{13}O_{12}$ 1037.5658	519.7902 $[M+2H]^{2+}$	610	519.7890	1.2	519.7893	0.86	135.1[a] 200.1[a] 298.7[a] 329.2[a]
Nod-R	$C_{41}H_{60}N_8O_{10}$ 824.4432	825.4505 $[M+H]^+$	200	825.4462	4.31	825.4469	3.58	135.1[a] 227.1[b] 389.2[b]
		691.3774 $[M-Adda+H]^+$		691.3766	0.73	691.3683	9.06	
MC-YR	$C_{52}H_{72}N_{10}O_{13}$ 1044.5280	1045.5353 $[M+H]^+$	85	1045.5290	6.35	1045.5270	8.34	135.1[a] 213.1[a] (higher concentration)
		911.4621 $[M-Adda+H]^+$		911.4586	3.56	911.4562	5.96	
MC-LR	$C_{49}H_{74}N_{10}O_{12}$ 994.5488	995.5560 $[M+H]^+$	900	995.5518	4.27	995.5519	4.13	135.1[a] 213.1[a] 375.2[c]
		509.2726 $[M+H+Na]^{2+}$		509.2692	3.46	509.2713	1.37	553.3[c] 599.3[c]
		861.4829 $[M-Adda+H]^+$		861.4796	3.27	861.4766	4.65	
dmMC-LR	$C_{48}H_{72}N_{10}O_{12}$ 980.5331	981.5404 $[M+H]^+$	180	981.5374	3.02	981.5342	6.15	135.1[a] 163.1[a] 213.1[a]
		502.2648 $[M+H+Na]^{2+}$		502.2620	2.81	502.2628	1.99	375.2[c] 599.3[c] (higher concentration)
		847.4672 $[M-Adda+H]^+$		847.4626	4.62	847.4585	8.77	
MC-LY	$C_{52}H_{71}N_7O_{13}$ 1001.5110	1002.5183 $[M+H]^+$	600	1002.5153	3.01	1002.5126	5.69	135.1[a] 213.1[a] 375.2[c]
		1024.5002 $[M+Na]^+$		1024.4976	2.62			
MC-LW	$C_{54}H_{72}N_8O_{12}$ 1024.5270	1025.5342 $[M+H]^+$	220	1025.5272	7.09 5.54	1025.5389	4.67	135.1[a] 213.1[a] 375.2[c] 397.2[a]
		1047.5162 $[M+Na]^+$		1047.5107		1047.5098	6.41	
MC-LF	$C_{52}H_{71}N_7O_{12}$ 985.5161	986.5233 $[M+H]^+$	140	986.5170	6.34	1008.5001	5.21	213.1[a] 375.2[c]
		1008.5053 $[M+Na]^+$		1008.5001	5.21			

Elemental compositions, concentrations and the detected masses in MS and MS–MS mode (in FA mobile phase) of the individual toxins in Extract Mix 2 used in the serum study.

[a] Typical fragments identified from Yuan et al. (1999) [18].

[b] Typical fragments identified from Mazur-Marzek et al. (2006) [12].

[c] Typical fragments identified from Diehnelt et al. (2005) [19].

Table 5
Detection and quantitation of the toxins in Extract Mix 2. The linear quantitation ranges in Extract Mix 2 as well as limits of detection values for Extract Mix 2 in spiked 1 mL serum sample, quantified with ±20 mDa mass window. Linear quantitation ranges are given for Extract Mix 2, calculated by the Bruker QuantAnalysis program linear mode with the accuracy of ±20%. The *R*-value for the given range for the obtained quantitation slope was ≥0.994 (FA mobile phase) or ≥0.989 (AF + FA mobile phase).

Hepatotoxin variant	Linear quantitation range in Extract Mix 2, FA mobile phase (ng mL^{-1})	LOD in serum extract, FA mobile phase, concentration in serum (ng mL^{-1})	Comments on FA mobile phase	Linear quantitation range in Extract Mix 2, AF + FA mobile phase (ng mL^{-1})	LOD in serum extract, AF + FA mobile phase, concentration in serum (ng mL^{-1})	Comments on AF + FA mobile phase
dmMC-RR	5–2500	Below 1 (limit not reached)	Signal suppression	25–2500	2.5/	Some signal suppression
MC-RR	12.2–6100	Below 2.4 (limit not reached)	Minor signal suppression	30.5–6100	Below 2.4 (limit not reached)	Signal enhancement
Nod-R	20–2000	2	Signal enhancement at lower concentrations	40–2000	4	Severe signal enhancement
MC-YR	85–850	3.4	Signal enhancement at lower concentrations	85–850	17	Severe signal enhancement
MC-LR	45–9000	Below 3.6 (limit not reached)	Signal enhancement at lower concentrations	45–9000	9	Signal enhancement
dmMC-LR	36–1800	3.6	Signal enhancement	90–1800	18	Signal enhancement
MC-LY	12–600	–/(12)[a]	Severe signal suppression	60–600	–/– (signal not detected in spiked samples)	
MC-LW	44–2200	–/(22)[a]	Severe signal suppression	220–2200	–/(22)[a]	Signal enhancement
MC-LF	14–1400	–/(14)[a]	Severe signal suppression	70–1400	28	Signal suppression

[a] Samples with the limit of detection corresponding to a sample spiked after sample preparation, since no signal was detected in samples spiked before sample preparation.

Fig. 3. Three chromatograms for the same run using a ±0.02 Da (A), ±0.1 Da (B) or ±0.5 Da (C) mass windows. The sample is the Extract Mix 2 spiked into serum. Here the FA mobile phase is used. See Section 2.3 for analytical details.

sodium adduct ions (e.g. *m*/*z* 1008.5 $[M+Na]^+$, for MC-LF $[M+H]^+$ *m*/*z* 986.5). Doubly charged $[M+2H]^{2+}$ ions were observed with the arginine-containing dmMC-RR, MC-RR, dmMC-LR and MC-LR, and in the case of dmMC-LR and MC-LR, the $[M+H+Na]^{2+}$ ions were strong. In Table 4 some CID fragments that can be used for further confirmation have been listed. The fragments have been identified from Yuan et al. [18], Diehnelt et al. [19] and Mazur-Marzek et al. [12]. The MS–MS signal intensity was occasionally too low for good quality spectra. However, MS–MS provided a selection of fragment ions that can be used for confirmatory purposes, such as the *m*/*z* 135 fragment from the characteristic Adda amino acid residue (Table 4).

The limits of detection in serum samples (calculated to the original sample concentration and volume, 1 mL) with the FA mobile phase were from below 1 ng mL^{-1} for dmMC-RR (the lower limit was not reached), to 22 ng mL^{-1} for MC-LW (Table 5). In all cases the limits of detection were higher (if reached) with the AF + FA mobile phase. When using the FA mobile phase, the arginine-containing compounds suffered from signal enhancement and suppression effects only at the lower concentrations. The signal responses with higher concentrations were roughly equal to the responses obtained with the Extract Mix 2 without serum matrix (as can be seen in Fig. 2). The microcystins not containing arginine (MC-LY, MC-LW and MC-LF) suffered from severe signal suppression and were not reliably detected with the serum matrix (Fig. 2 and Table 5). The signal responses in the AF + FA mobile phase were more vulnerable to matrix effects, especially signal enhancement, perhaps because the signal response was lower from the beginning (see Fig. 2). The detection and quantitation was performed with ±20 mDa mass window (Fig. 3, chromatogram for Extract Mix 2 spiked in serum, with the FA mobile phase). The narrow mass win-

dow efficiently reduced the background noise (Fig. 3A–C) whereas the signal intensity did not suffer.

The linear quantitation ranges in Extract Mix 2 without serum, are given in Table 5. The signal response linearity was not in all cases axiomatic even though the limit of detection was low enough to have several data points per curve.

Some basic method validation data for three major toxins in serum matrix is given in Table 6.

4. Discussion

4.1. Chromatographic performance

Rapid analyses of microcystins and nodularins in cyanobacterial samples have already been described for LC–MS [17] and for HPLC-DAD [40,41]. The longer 30 min gradient used in this study did not provide further benefits as measured by selectivity and resolution as compared to the 5 min gradient. Analysis of complex biological matrices require high selectivity and resolution, but with large numbers of samples high-throughput is beneficial for both reduced instrument time and reduced sample storage time. The low backpressure of the Synergi Hydro-RP column was considered an advantage, also because they can be used on regular LC instruments.

In microcystin analysis there are a few pairs of microcystins that are both commonly occurring and also sometimes difficult to separate in a conventional C-18 RP column with acidic mobile phases. These pairs are for example dmMC-LR and MC-LR; MC-YR and MC-LR; and MC-LW and MC-LF. Nodularins are usually not found in same (freshwater) samples as the microcystins, and therefore the separation of nodularin from microcystins is mainly of interest if nodularins are used as internal standards. Previously, an excellent resolution of the usually co-eluting pair MC-LR/dmMC-LR has been achieved with an amide embedded reversed-phase column [42]. This has not been achieved with the small particle size columns in fast gradients with acidic (TFA or FA modified mobile phases) [17,40,41]. Here, the separation was achieved with AF + FA mobile phase, but not with FA only mobile phase. However, due to the lower S/N values, with the AF + FA mobile phase, the FA based mobile phase is recommended in case the separation of the dmMC-LR/MC-LR is not considered crucial.

Cyanobacteria produces other toxins besides microcystins and nodularins, including cylindrospermopsin and anatoxin-a, hydrophilic toxins that require the use of almost 100% aqueous conditions for LC analysis. Conventional RP C-18 columns do not tolerate these conditions well, sometimes undergoing a phase collapse and loss of performance. For an analytical laboratory it would be preferable that one column could be used for different types of analytes, especially for samples of the same origin, with one set of chromatographic conditions [43].

4.2. High-resolution mass spectrometry

The differences in limits of detection and matrix effects (signal suppression/enhancements) between the different microcystin variants can be explained by ionisation efficiency. The presence of one or two arginines in the analyte structure makes the ionisation more efficient. Furthermore the serum sample complexity in the region where the more hydrophobic MC-LY, MC-LW and MC-LF elute make the matrix effects for those compounds more prominent. As noticed by Ortelli et al. [22], the limit of detection could not be based on S/N values given by the data analysis automatic peak detection, since the S/N values were not considered reliable estimates. The mass accuracy is of great importance when the quantitation is based on a narrow m/z window. In Table 5

Table 6
Method validation data for three major toxins in serum matrix for the optimised method using FA based mobile phase.

Parameter	MC-RR		Nod-R		MC-LR	
Retention time mean ± standard deviation (in min) (n = 108)	1.35 ± 0.02		2.13 ± 0.02		3.58 ± 0.02	
		Conc. ng mL^{-1} in serum samples		Conc. ng mL^{-1} in serum samples		Conc. ng mL^{-1} in serum samples
Extraction recovery mean ± standard deviation (higher conc.) (n = 3, duplicate injections)	77.5 ± 12.7%	122	80.9 ± 11.8%	40	80.8 ± 10.6%	180
Extraction recovery mean ± standard deviation (lower conc.) (n = 3, duplicate injections)	82.3 ± 6.6%	6.1	80.1 ± 13.6%	2	62.4 ± 9.9%	9
Matrix effect[a] mean ± standard deviation (higher conc.) (n = 3, duplicate injections)	92.6 ± 6.4	122	102.1 ± 4.6%	40	78.4 ± 4.9%	180
Matrix effect[a] mean ± standard deviation (lower conc.) (n = 3, duplicate injections)	99.8 ± 3.5%	6.1	161.9 ± 17.7%	2	108.8 ± 16.5%	9
Quantitation range[b] ng mL^{-1} in serum samples		7.7–122		6.0–40		10.8–180

[a] Signal response of a spiked sample in serum matrix divided by signal response of a standard in solvent matrix.
[b] LOQ (=LOD × 3) – highest tested concentration.

the upper limit of linearity in quantitation is the highest concentration of analyte used in the runs. The narrow range of analyte concentrations (considered as relevant for the sample type in question) is the most likely reason for why three orders of magnitude quantitation ranges were not seen, and two orders of magnitude (or better) only shown for the dmMC-RR, MC-RR, Nod-R and MC-LR.

The mass window to be used is determined by the mass error for the given compound. In our case the Bruker specifications of mass errors of equal to or less then 5 ppm were not always met, problems occurred especially with the later eluting compounds. The lowest errors (from 0.09 mDa) were seen with the doubly charged, early eluting species and the largest errors (up to 9 mDa) in the MS spectra with the microcystin and nodularin post-source fragment ions. In the current study the mass window used (±20 mDa) gave a clear advantage in noise reduction over using a wider detection mass window (Fig. 3). On the other hand, the Q-TOF-MS instrumentation allows the detection of confirmatory fragments from the analytes. This can be done using two different (MS only and MS–MS) runs for more reliable quantitation and separate confirmation, or, as in our case within a single run utilising a preference list in the auto MS–MS function for the presumed analytes.

New instrument and column technologies enable analyses of microcystins and nodularins in complex biological samples. The limits of detection for the arginine-containing microcystins are in the range of concentrations of microcystins reported in human serum samples detected by ELISA assay [44,45].

5. Conclusions

We suggest that conventional reversed-phase long columns and lengthy gradients could be replaced with fast, high performance columns and shorter run times for microcystin and nodularin analyses in various matrices, including complex biological matrices. The Q-TOF-MS instrumentation gave secure identification of the analytes when a combination of retention time, narrow mass window, as well as MS–MS fragmentation products were used.

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