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Liquid chromatography–electrospray ionization-mass spectrometry of cyanobacterial toxins

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ABSTRACT

Cyanobacterial neurotoxins such as anatoxin-a and saxitoxin, peptide hepatotoxins including microcystin variants and nodularin were studied by electrospray ionization-mass spectrometry following direct infusion of samples into the mass spectrometer. This technique was further exemplified when analysing the cyclic hepatotoxic peptides by means of on-line liquid chromatography–mass spectrometry. Peptide toxins were identified in extracts from toxic bloom material and cell cultures by comparison of spectral and chromatographic data to that of authentic materials. When authentic materials were not available, several peptides were tentatively identified by their molecular-weight information provided by this technique.

INTRODUCTION

Many freshwater cyanobacteria (blue-green algae) produce toxins (Fig. 1) which include: (a) the cyclic heptopeptides of microcystin and its analogues; (b) the cyclic pentapeptide nodularia toxin and (c) the neurotoxic alkaloids anatoxins and saxitoxin. Microcystins have the common structure cyclo(-D-Ala–L-X–D-MeAsp–L-Y–Adda–D-Glu–Mdha) where X and Y are variable amino acids (labelled X and Y, Fig. 1) [1], Nodularin contains four of the five amino acids found in microcystin and dehydrobutyrine [2]. Blue-green algae are found in eutrophic lakes and drinking water reservoirs and have been responsible for the deaths of domestic, farm and wild animals in many countries over several decades [3–5]. Consumption of drinking water contaminated by the toxins can seriously affect human health, and there is an interest in the tumor-promoting potential of the microcystins [6]. Complications arise when more than one species of cyanobacteria co-exist in the algal bloom, and in addition each species can produce more than one kind of toxin. Hence there is a need to detect, quantify and confirm the identity of these compounds for safety evaluation.

The most commonly used analytical method available for detecting the peptide hepatotoxins is high-performance liquid chromatography (HPLC) with ultraviolet detection. Those peaks collected which were shown to be toxic to mice were further characterised by mass spectrometry (MS), using fast atom bombardment ionization (FAB) [7]. Gas chromatography–mass spectrometry (GC–MS) seems to be a suitable technique for analysing anatoxins, but the toxins must be derivatized prior to

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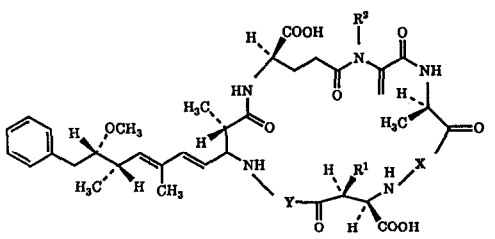
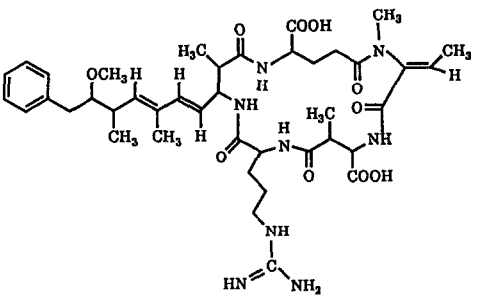
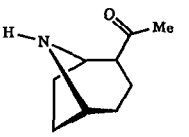


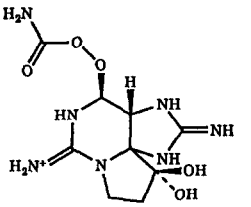
					M_r
MCYST-LR:	X = Leu;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	994
MCYST-FR:	X = Phe;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1028
MCYST-RR:	X = Arg;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1037
MCYST-RR: ([D-Asp^3])	X = Arg;	R^1 = H;	Y = Arg;	R^2 = CH3	1023
MCYST-RR: ([D-Asp^3,Dha^7])	X = Arg;	R^1 = H;	Y = Arg;	R^2 = H	1009
MCYST-YR:	X = Tyr;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1044
MCYSY-M(O)R:	X = Met(O);	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1028
MCYST-WR:	X = Trp;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1067



Nodularin



(+)-Anatoxin-a



Saxitoxin

Fig. 1. Structures of cyanobacterial toxins. MCYST = microcystin.

analysis [8–10]. Saxitoxin and its analogues are routinely analysed by fluorescence detection of their oxidised products [11].

It is feasible to study the peptide class of toxins by on-line LC–MS using FAB ionization [generally known as continuous flow (CF)-FAB] [12,13]. Since FAB requires matrices to assist ionization it is necessary either to develop a HPLC method with the matrix being incorporated into the mobile phase, or to introduce the matrix coaxially into the mass spectrometer ion source [14]. Care has to be taken to ensure a steady film of liquid being delivered to the FAB target. The amount of sample required by FAB to acquire a spectrum of the purified toxin is in the nanogram range.

Recently Fenn and co-workers [15,16] have successfully interfaced electrospray ionization with mass spectrometry (ESI–MS), a development which has proved invaluable in the analysis of thermally labile polar molecules, especially for peptides and proteins. This report demonstrates that on-line ESI-MS is a simple, specific, reliable and rapid technique to monitor precisely and simultaneously various algal toxins in extracts from laboratory cultures or algal blooms present in eutrophic waters.

EXPERIMENTAL

Chemicals

Anatoxin-a was obtained from Biometric Systems (Eden Prairie, MN, USA). Saxitoxin was purchased from Calbiochem Novabiochem (Nottingham, UK). Microcystin-LR and microcystin-desmethyl-3,7-RR were purified from lyophilized cells of *Microcystis aeruginosa* PCC 7813 and Nodularin was purified from a laboratory culture of *Nodularia spumigena* as described by Lawton [17]. Microcystin-RR was a gift from Professor H.W. Siegalman (Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA).

HPLC grade solvents and glacial acetic acid were purchased from Romil Chemicals (Shepshed, UK) and Fisons PLC (Loughborough, UK), respectively.

Algal material

Microcystis aeruginosa PCC 7813 was grown in batch culture on BG 11 media as described [18]. Cells were harvested by continuous centrifugation and freeze dried. Toxic bloom material containing *Microcystis aeruginosa* was collected from Hart-

beespoort Dam in South Africa in August 1987 and Water Lane fish farm, Wessex, UK in November 1990. Toxic bloom material containing *Nodularia spumigena* was collected from the River Humber in 1990.

Preparation of samples from algal cells

Lyophilized cells (0.5 g/50 ml) were extracted twice in 5% (w/v) acetic acid for 30 min with continuous agitation. The extracts were centrifuged at 3000 *g* for 10 min. Both supernatants were pooled and filtered through a GF/C filter to remove particulate material. The filtrate was applied to a preconditioned environmental C_{18} Sep-Pak (Millipore) at a rate of 2 ml/min. The cartridge was washed with 20 ml 25% methanol and the peptides were eluted with 100% methanol. Solvent was removed *in vacuo* at 40°C and residues were retained for analysis. For the PCC 7813 0.5 g freeze dried material, 1/2000 μl was used for analysis, whereas for the *Nodularia*, South African and fish farm studies, each injection contained approximately 100, 135 and 400 ng equivalent amount of nodularin or microcystin-LR toxins, respectively (assessed by their extinction coefficients from the UV spectra).

Detection of cyanobacterial toxins by ESI-MS

Authentic toxin standards were dissolved in a mixture of methanol–water (50:50, v/v) containing 1% acetic acid. Each of these solutions was infused at 1 μl/min with a Harvard Apparatus infusion pump (model No. 22, Harvard Apparatus, Cambridge, MA, USA) into a Finnigan TSQ 700 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) fitted with an electrospray ion source (Analytica, Branford, CT, USA). A potential difference of 3500–3600 V was applied between the grounded needle and the metalized ends of the glass capillary tube that passes ions into the analyser. Nitrogen heated to 60°C was used as the drying gas. Data analysis was controlled by DEC 2100 data system. The deconvolution program is based on the Finnigan ICIS software version 5 [19]. Full scan spectra were acquired in the positive ion peak centroid or profile modes over the mass range of *m/z* 400–1200 at 6 s/scan.

Detection of cyanobacterial peptides by LC-ESI-MS

The HPLC system used was an ABI model 140A dual syringe pump (Applied Biosystems, San Jose, CA, USA) fitted with a Rheodyne 7125 injection valve equipped with a 5-μl sample loop. Separation was achieved using a 150 x 1 mm I.D. microbore column packed with Spherisorb 5-μm ODS material (Phase Separations Ltd., Clwyd, UK). The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% acetic acid. A gradient elution was employed: 0–10 min 40% B; 10–20 min, 40–55% B; 20–35 min, 55–65% B and then isocratic for 5 min at 65% B. The flow-rate was maintained at 45 μl/min. The effluent was directed to a Valco tee splitter. One outlet was connected directly with a fused silica capillary (1 m × 100 μm I.D., J & W Scientific) to the UV detector (Applied Biosystems, Model 785A) fitted with a micro flow cell (2.4 μl volume, 6 mm pathlength). The eluent was monitored by UV detection at 240 nm. The other outlet was linked to the stainless steel electrospray needle via a fused silica capillary 1.5 m × 50 μm I.D. The split ratio was maintained at 11:1 (UV:MS), so that approximately 3.5 μl/min of the effluent entered the ESI ion source. Full scan mass spectra were obtained in the peak centroid mode over the mass range of *m/z* 400–1200 at 6 s/scan. The individual samples were dissolved in an appropriate volume of methanol and an aliquots of 1 μl were analysed by LC–MS.

RESULTS

Detection of cyanobacterial toxins by ESI-MS

The mass spectrum obtained by direct infusion of anatoxin-a in methanol containing 1% acetic acid gave a strong protonated molecule at *m/z* 166 $[M + H]^+$ and a natriated adduct at *m/z* 188 $[M + Na]^+$ (Fig. 2a). A weak signal at *m/z* 181 suggested the presence of a methylene analogue of anatoxin. This component has been characterised by Skulberg *et al.* [20], but further studies are required to confirm its presence.

The electrospray mass spectrum of saxitoxin is presented in Fig. 2b, approximately 2 picomol sample was consumed to give this full scan spectrum. The protonated molecule at *m/z* 300 and the methylated adduct at *m/z* 314 dominate the spectrum. The fragment ion at *m/z* 282 could be attrib-

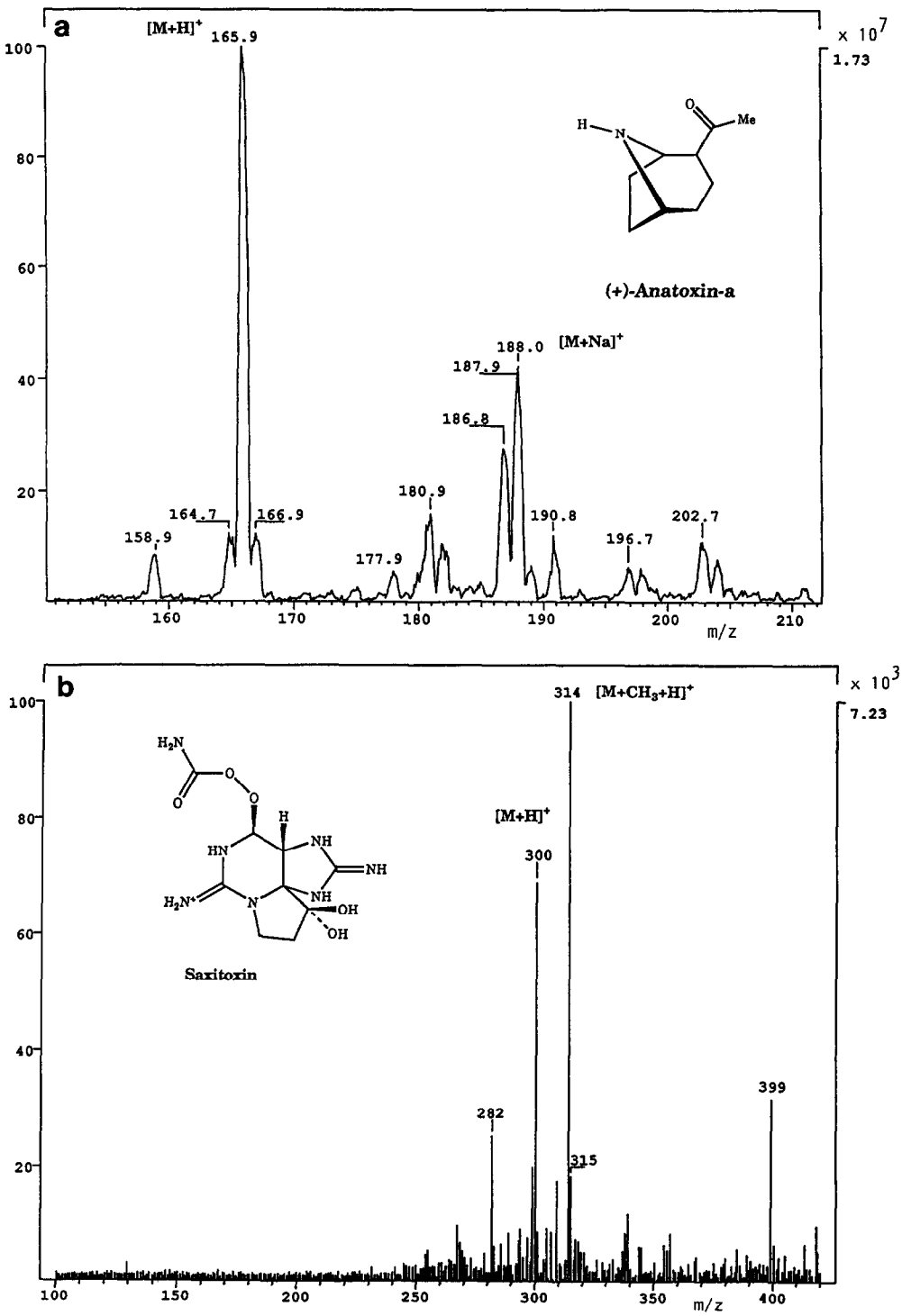

a
[M+H]+
165.9
x 10^7
1.73
(+)-Anatoxin-a
188.0
[M+Na]+
187.9
186.8
180.9
164.7
166.9
190.8
202.7
158.9
177.9
196.7
m/z
b
x 10^3
7.23
314
[M+CH3+H]+
[M+H]+
300
Saxitoxin
399
282
315
m/z

Fig. 2.

uted to either loss of water from m/z 300 or the loss of methanol from m/z 314. A similar observation has been reported by Quilliam *et al.* [21] during LC–ionspray-MS analysis.

When the peptide toxins are analysed by ESI-MS, compounds with molecular weights less than 1000 are more likely to give strong singly charged protonated molecules $[M + H]^+$. This can be illustrated by nodularin which has a molecular weight of m/z 824 (figure 2c), whereas for toxins with molecular weights over 1000, their corresponding doubly charged ions $[M + 2H]^{2+}$ predominate. In the case of microcystin-LR, the singly charged ($[M + H]^+$ at m/z 995), doubly charged ($[M + 2H]^{2+}$ at m/z 498) ions as well as the natriated ($[M + H + Na]^{2+}$ at m/z 509) and kaliated ($[M + H + K]^{2+}$ at m/z 517) adducts of the doubly charged ions were all observed in the spectrum (Fig. 2d). A deconvoluted spectrum around the doubly charged cluster ions at m/z 500 gave m/z 995 ($[M + H]^+$), m/z 1017 ($[M + Na]^+$) and m/z 1033 ($[M + K]^+$) (Fig. 2e). Approximately 100 femtomoles of the sample were consumed to obtain a full scan mass spectrum, with signal-to-noise level in the order of 20:1 or better. The amount consumed to produce a full scan spectrum could easily be reduced.

Detection of cyanobacterial peptides by LC-ESI-MS

For the LC–MS system, a split ratio of 11:1 was applied and 3.5 μl/min of the LC eluent was directed into the mass spectrometer. The rest of the flow was diverted to the UV detector fitted with a 2.4-μl flow cell. Since a capillary flow cell (90 nl) suitable to monitor a low LC flow-rate was not available, the peaks on the chromatograms tended to be rather broad. There was a 20-s delay in receiving the signal at the mass spectrometer compared to the UV detector. The five standards available for this study, mcyst-LR, mcyst-YR, mcyst-desmethyl-3,7-RR, mcyst-RR and nodularin, were separated by reversed phase LC with mass spectrometric and UV detection (Fig. 3a and b). Although the compounds did not have baseline separation on the UV chromatogram, the reconstructed ion chromatogram

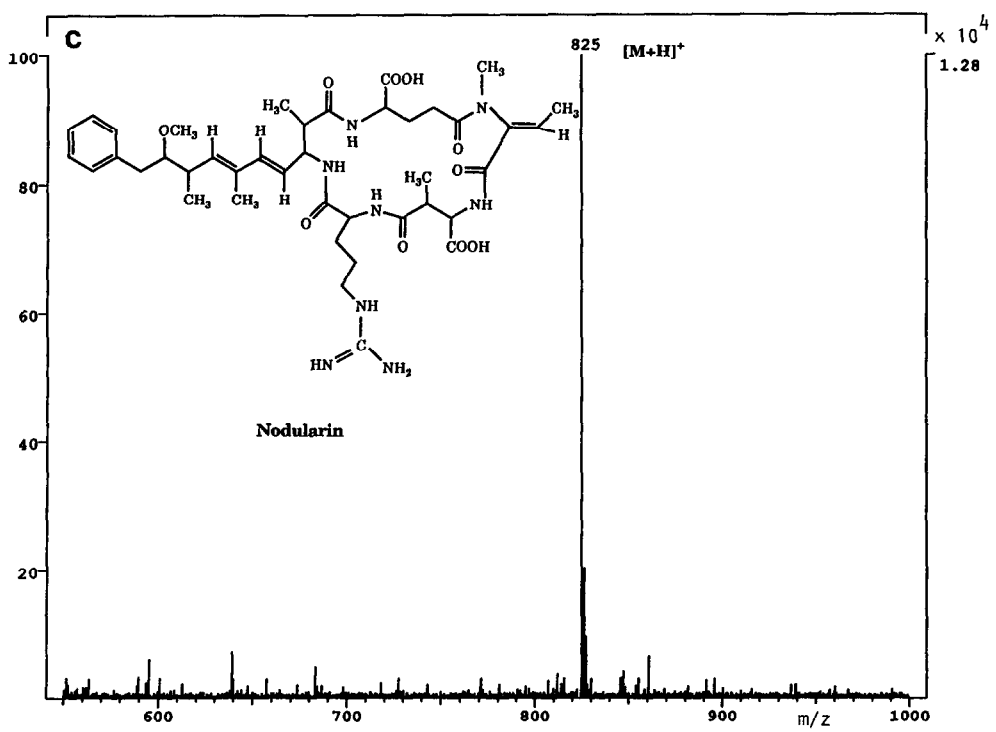


Fig. 2.

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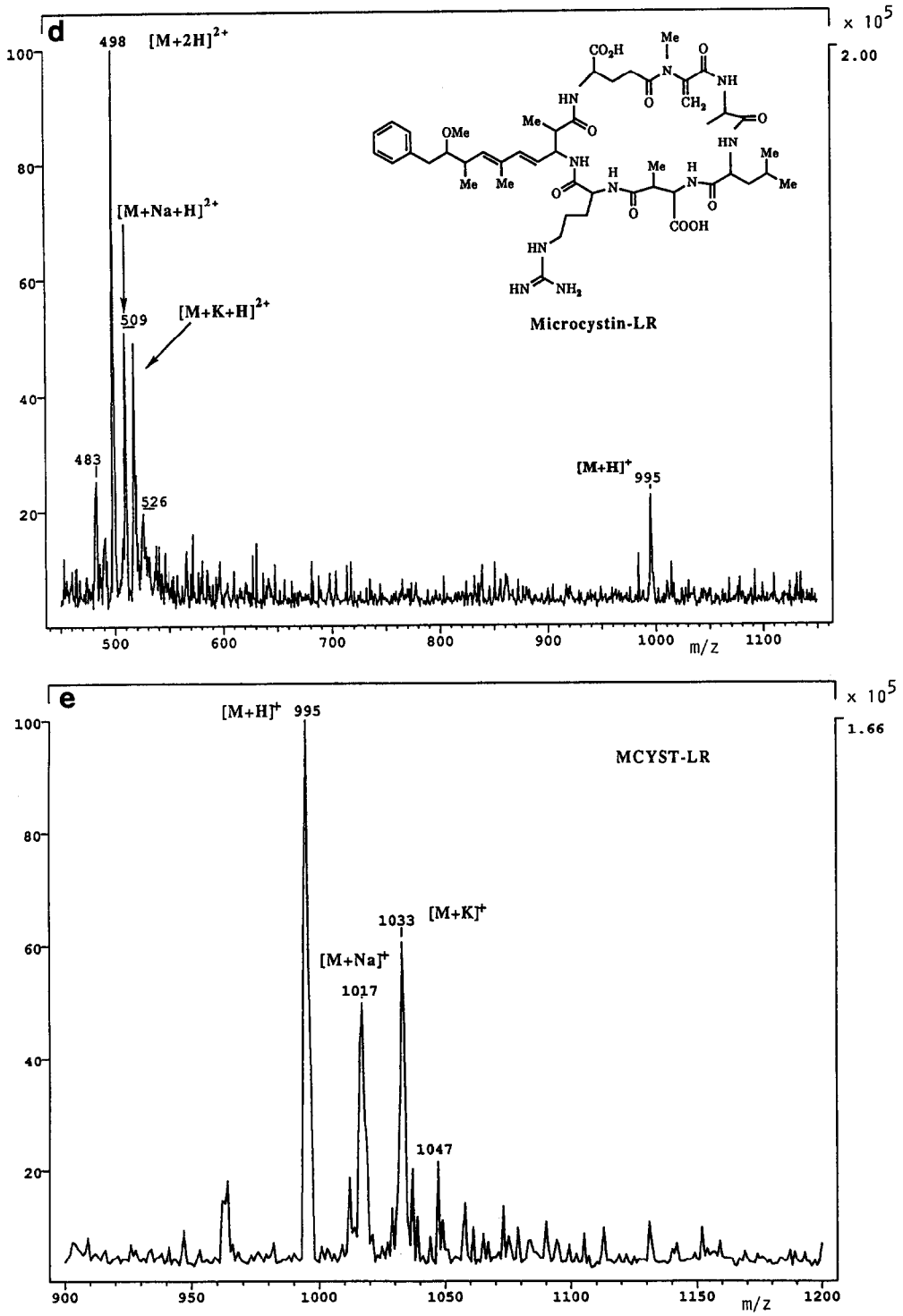

Fig. 2. Electrospray mass spectra obtained from freshwater cyanobacterial toxins, (a) (+)-anatoxin-a, (b) saxitoxin, (c) Nodularin and (d) microcystin-LR. (e) Deconvolution of the spectrum around the doubly charged cluster ions at m/z 500 gave m/z 995 ($[M + H]^+$), m/z 1017 ($[M + Na]^+$) and m/z 1033 ($[M + K]^+$).

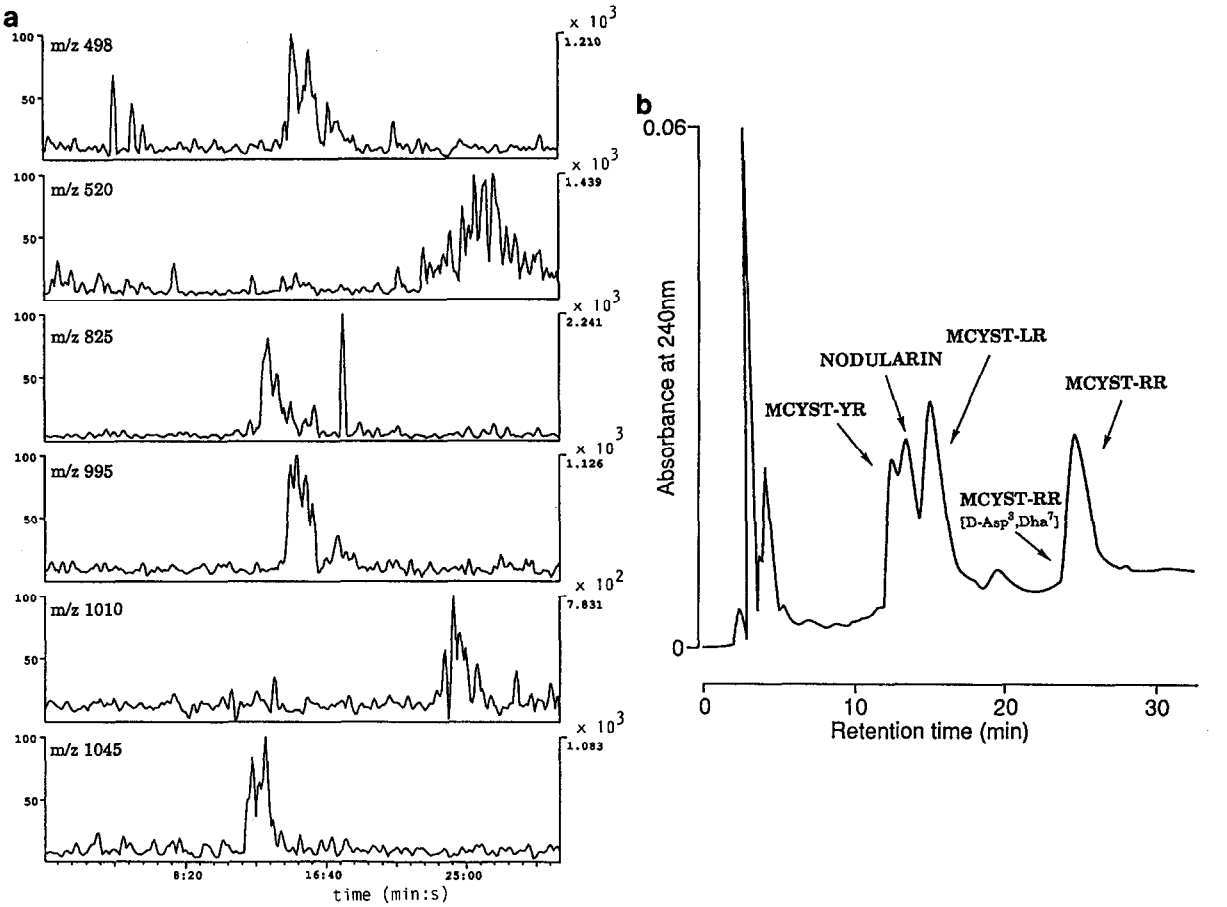

Fig. 3. Separation of toxic peptide standards by reversed-phase LC and displayed as (a) reconstructed ion chromatograms; (b) LC chromatogram with UV detection.

shows the compounds to be adequately resolved. They were easily recognised either as their singly or doubly charged protonated ions. Nodularin had a mass spectrum with a predominant peak at m/z 825 ($[M + H]^+$) (data not shown). The mass spectra of mcyst-LR, -YR and desmethyl-3,7-RR consisted of doubly and singly charged ions. Mcyst-RR only formed a doubly charged ion at m/z 520 $[M + 2H]^{2+}$ but deconvolution of this ion gave the protonated ion at m/z 1038.

Once the system was shown to be satisfactory with the known standards, extracts from cyanobacterial cultures and blooms were analysed.

The extract from the bloom material collected from Hartbeespoort Dam contained several microcystin variants as shown by the reconstructed ion chromatograms and UV chromatogram (Fig. 4a and b). At t_R 13.30 min mcyst-YR was observed which gave a protonated ion at m/z 1045.7 and a doubly charged ion at m/z 523. Mcyst-LR was detected at t_R 16.00 min with m/z 995, followed by mcyst-FR at t_R 20 min. The mass spectrum of mcyst-FR contained mainly a singly charged protonated ion at m/z 1029 and a weak signal (20% intensity) of its doubly charged ion at m/z 514. Mcyst-WR was detected at t_R 21.30 min (m/z 1069). Finally a broad peak of mcyst-RR was present as its doubly charged ion at m/z 520. All toxins mentioned above provide very good mass spectra as shown in Fig. 5.

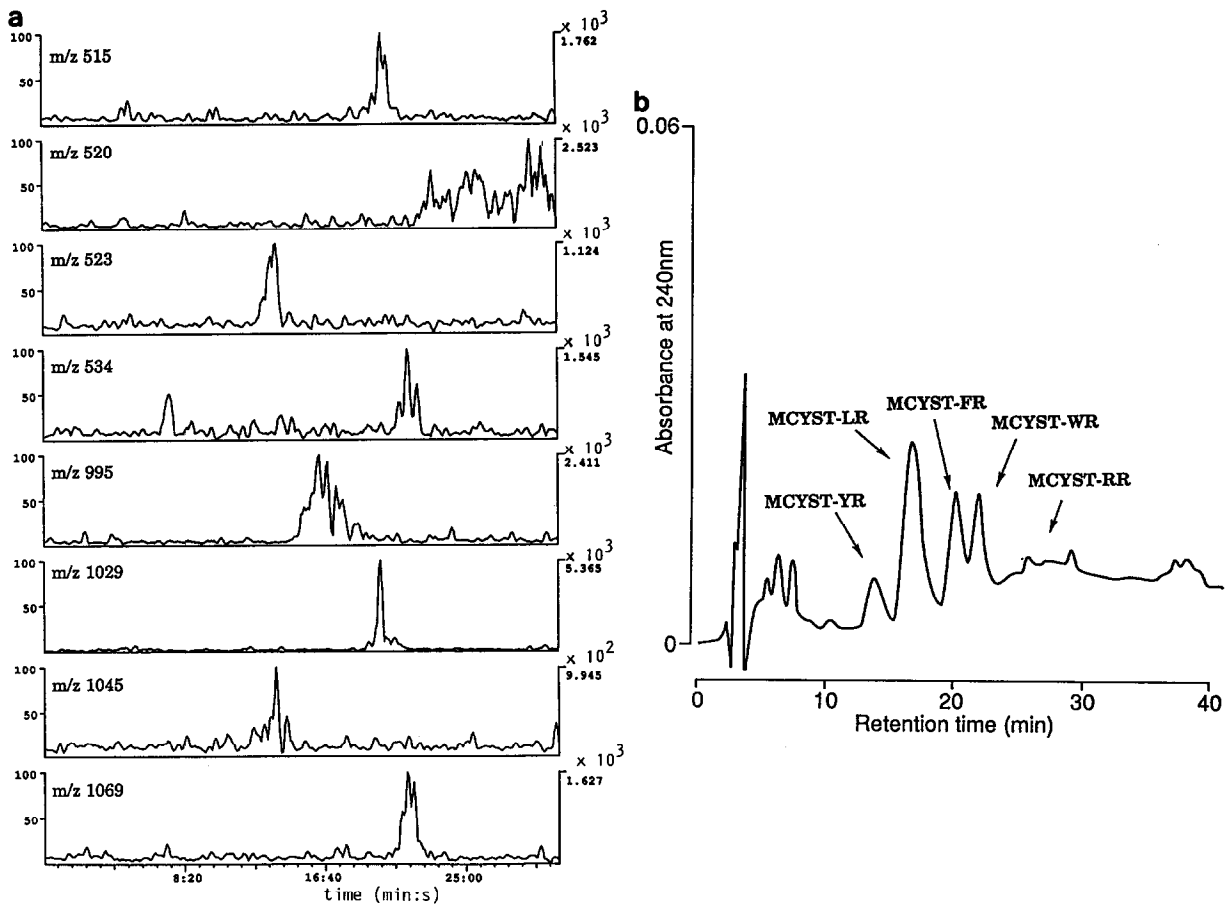

Fig. 4. Separation of microcystins in an extract from bloom material from Hartbeespoort Dam, South Africa. (a) Reconstructed ion chromatograms; (b) LC chromatogram with UV detection.

Analysis of the extract of the bloom containing predominantly *Nodularia spumigena* showed the presence of the pentapeptide, nodularin at t_R 12.70 min (Fig. 6a and b). Examination of the reconstructed ion chromatograms (Fig. 6a) demonstrated the presence of trace amounts of mcyst-LR (*m/z* 995 at t_R 16.00 min) and possibly mcyst-RR [D-Asp^3] at t_R 28 min which had a mass spectrum with a prominent ion at *m/z* 512 and a deconvoluted spectrum with the protonated ion at *m/z* 1024 (Fig. 6d).

Several microcystin analogues were detected in the extract from the bloom material collected from Water Lane Fish Farm (Fig. 7a and 7b) including mcyst-LR and -YR at t_R 11.30 min and t_R 14.80 min, respectively. Several other peaks were detected which may be other microcystin variants. A weak spectrum with *m/z* 1029 was tentatively identified as mcyst-M(O)R (Fig. 7c). At low concentration, no *m/z* 515 doubly charged ion was obtained. However at a high concentration, the *m/z* 515 ion was present, but it was masked by the two abundant doubly charged ions at *m/z* 537 and 519 (data not shown). This component has the same molecular weight as mcyst-FR but differs in retention time. The relatively short retention time suggests the presence of a sulphoxide functional group in the molecule. Another component was detected at t_R 12.30 min which had a strong doubly charged ion at *m/z* 519 and a deconvoluted ion at *m/z* 1037. This compound could be mcyst-L-Harg (Fig. 7d and e) as

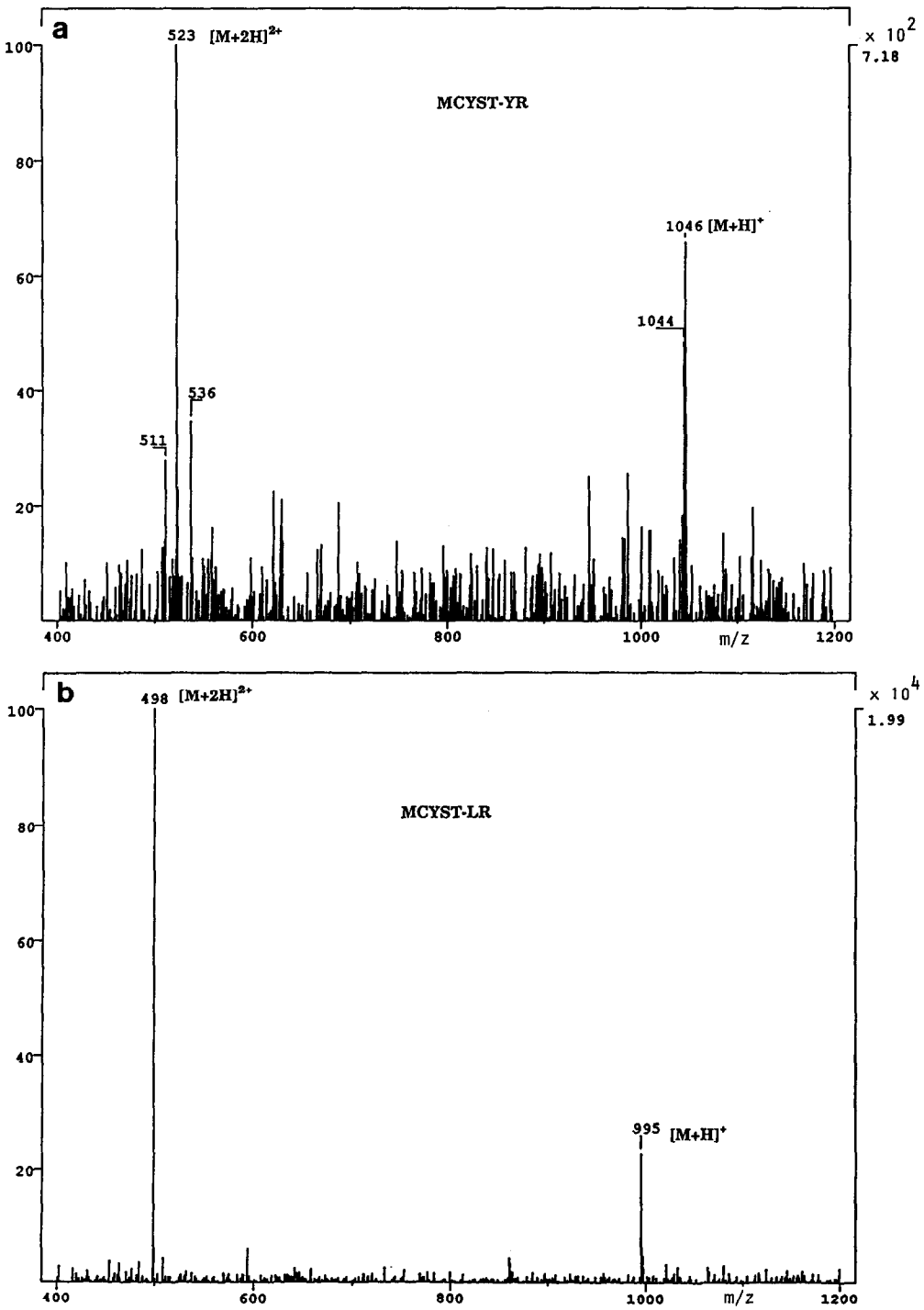

Fig. 5.

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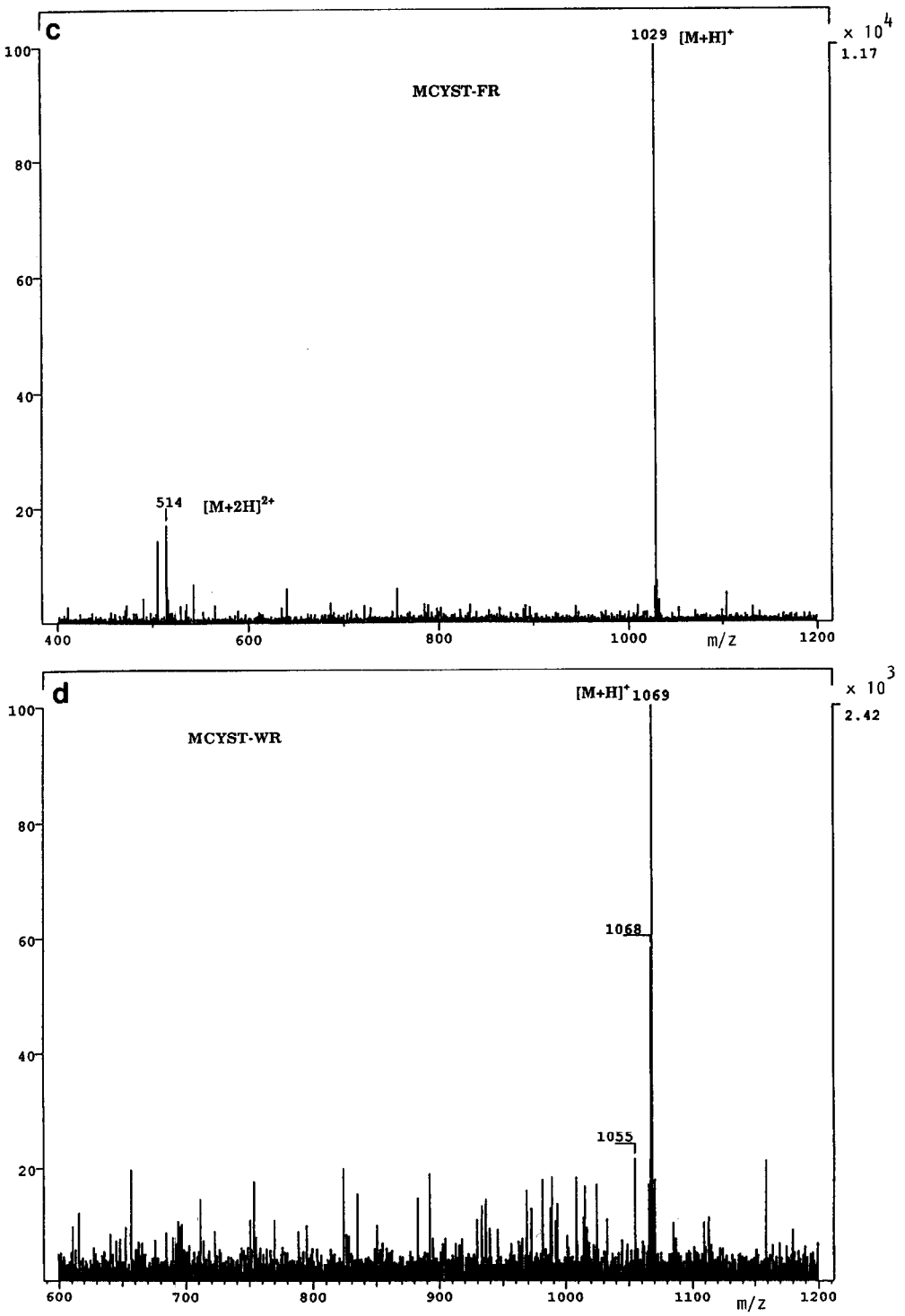
c
MCYST-FR
1029 [M+H]+
x 10^4
1.17
514 [M+2H]2+
100
80
60
40
20
400
600
800
1000
1200
m/z
d
MCYST-WR
[M+H]+ 1069
x 10^3
2.42
1068
1055
100
80
60
40
20
600
700
800
900
1000
1100
1200
m/z

Fig. 5.

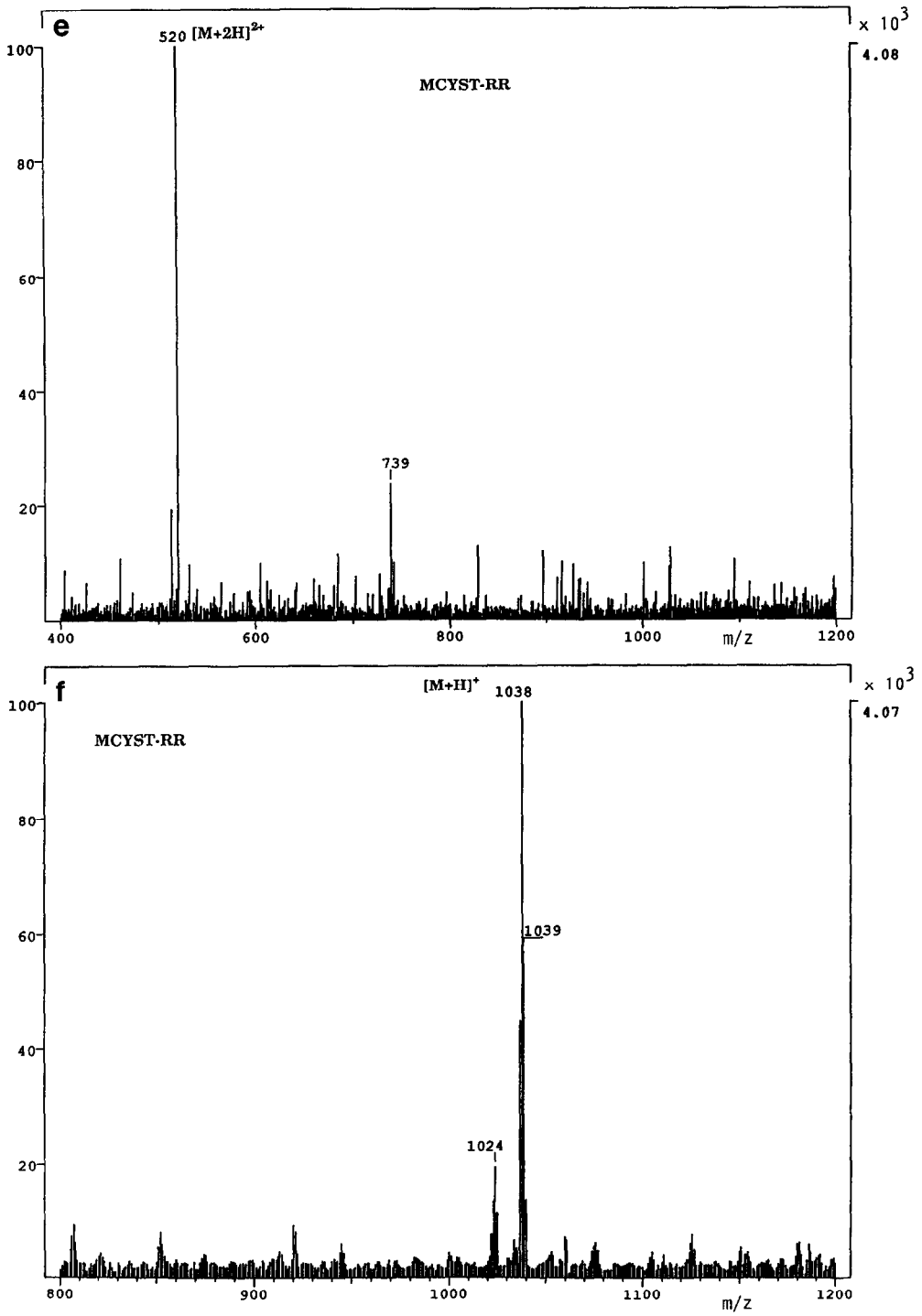

Fig. 5. Mass spectra of toxic peptides identified in bloom material from Hartbeespoort Dam including (a) mcyst-YR, (b) mcyst-LR, (c) mcyst-FR, (d) mcyst-WR and (e and f) mcyst-RR.

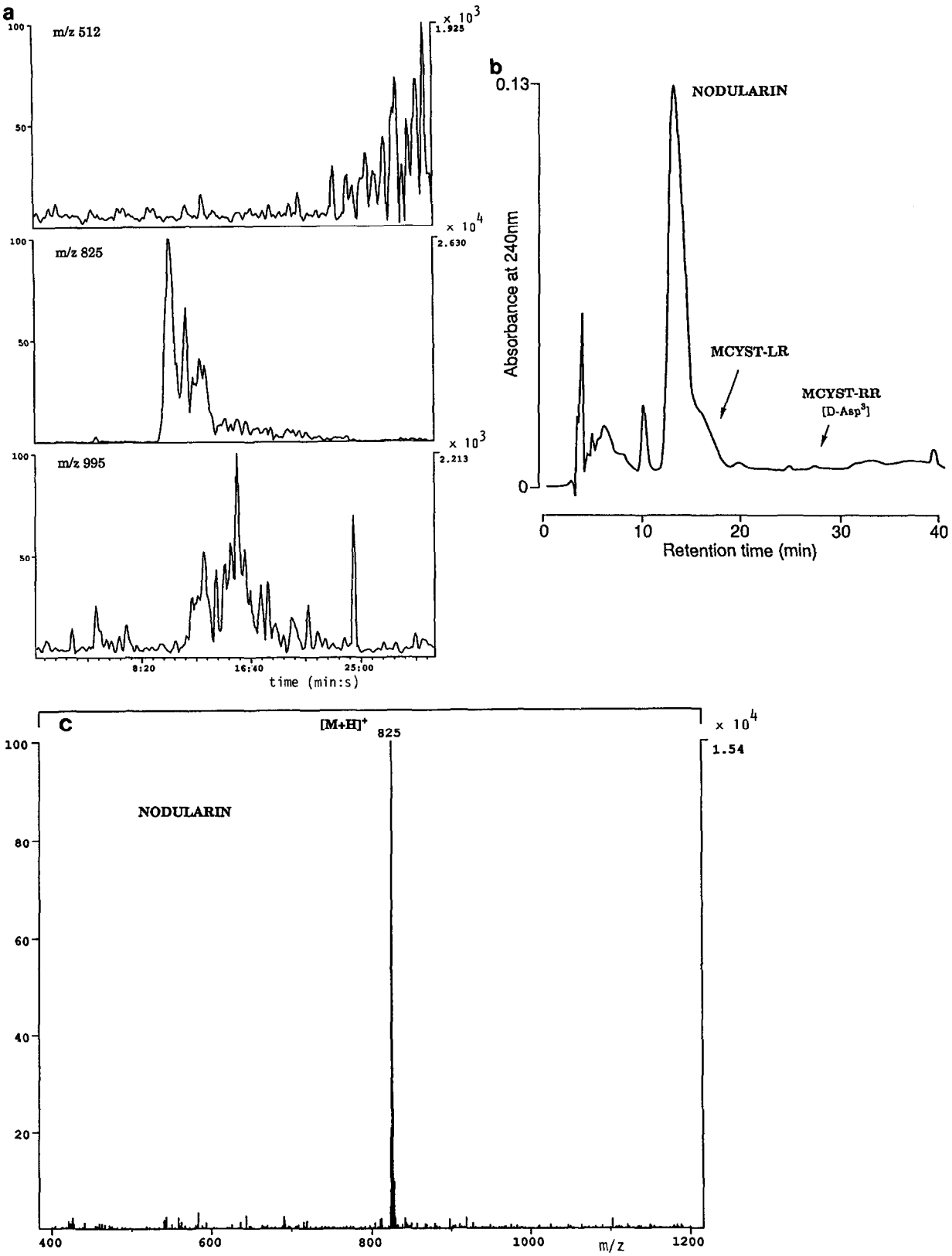
a
m/z 512
x 10^3
1.925
m/z 825
x 10^4
2.630
m/z 995
x 10^3
2.213
8:20
16:40
25:00
time (min:s)
b
0.13
NODULARIN
MCYST-LR
MCYST-RR
[D-Asp^3]
Absorbance at 240nm
0
0
10
20
30
40
Retention time (min)
c
[M+H]+
825
x 10^4
1.54
NODULARIN
400
600
800
1000
1200
m/z

Fig. 6.

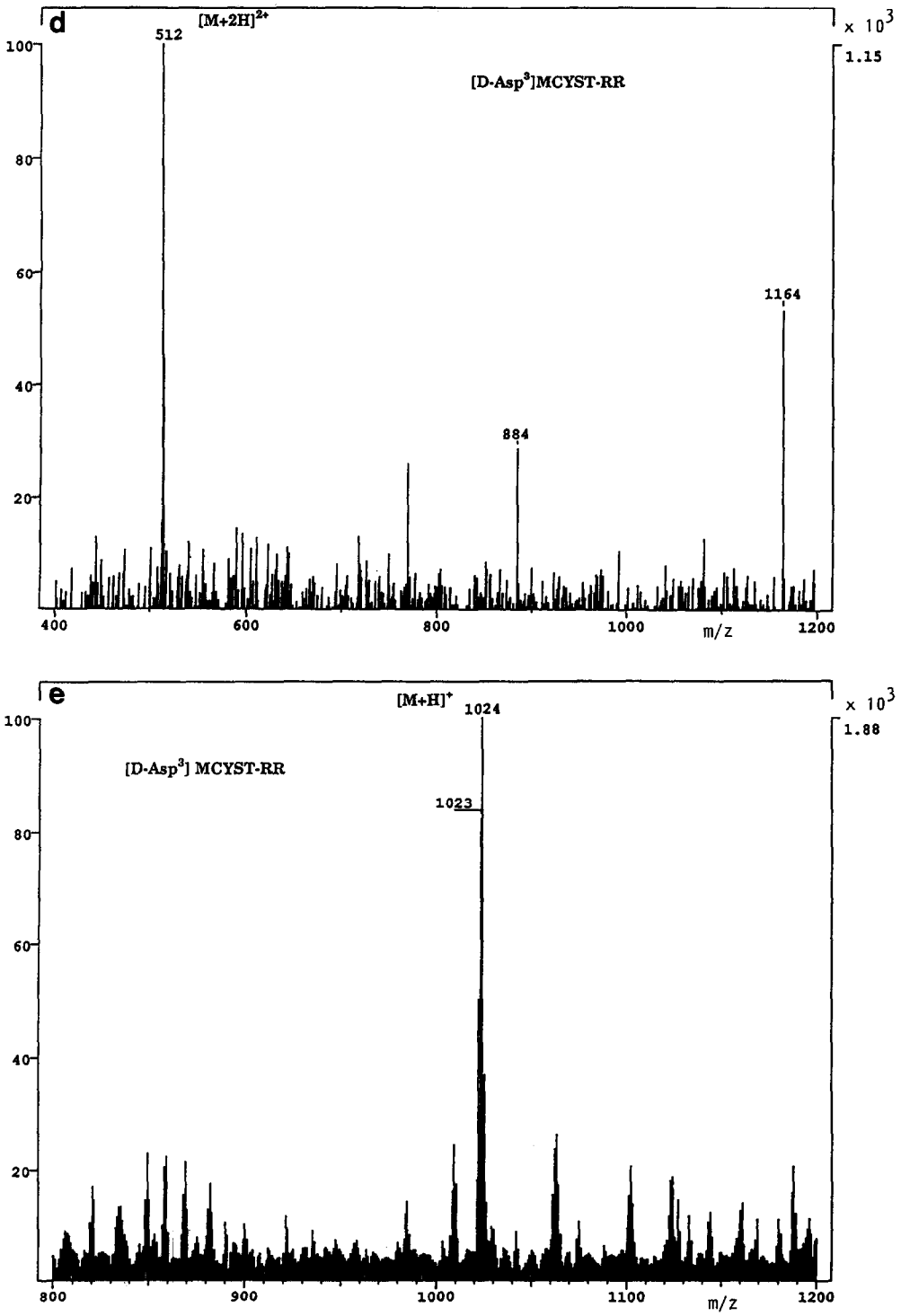

Fig. 6. (a) Reconstructed ion chromatograms; (b) reversed-phase LC chromatogram of extract from bloom containing *Nodularia spumigena*. Toxic peptides detected were (c) nodularin, (d and e) mcyst-LR and [D-Asp^3] mcyst-RR.

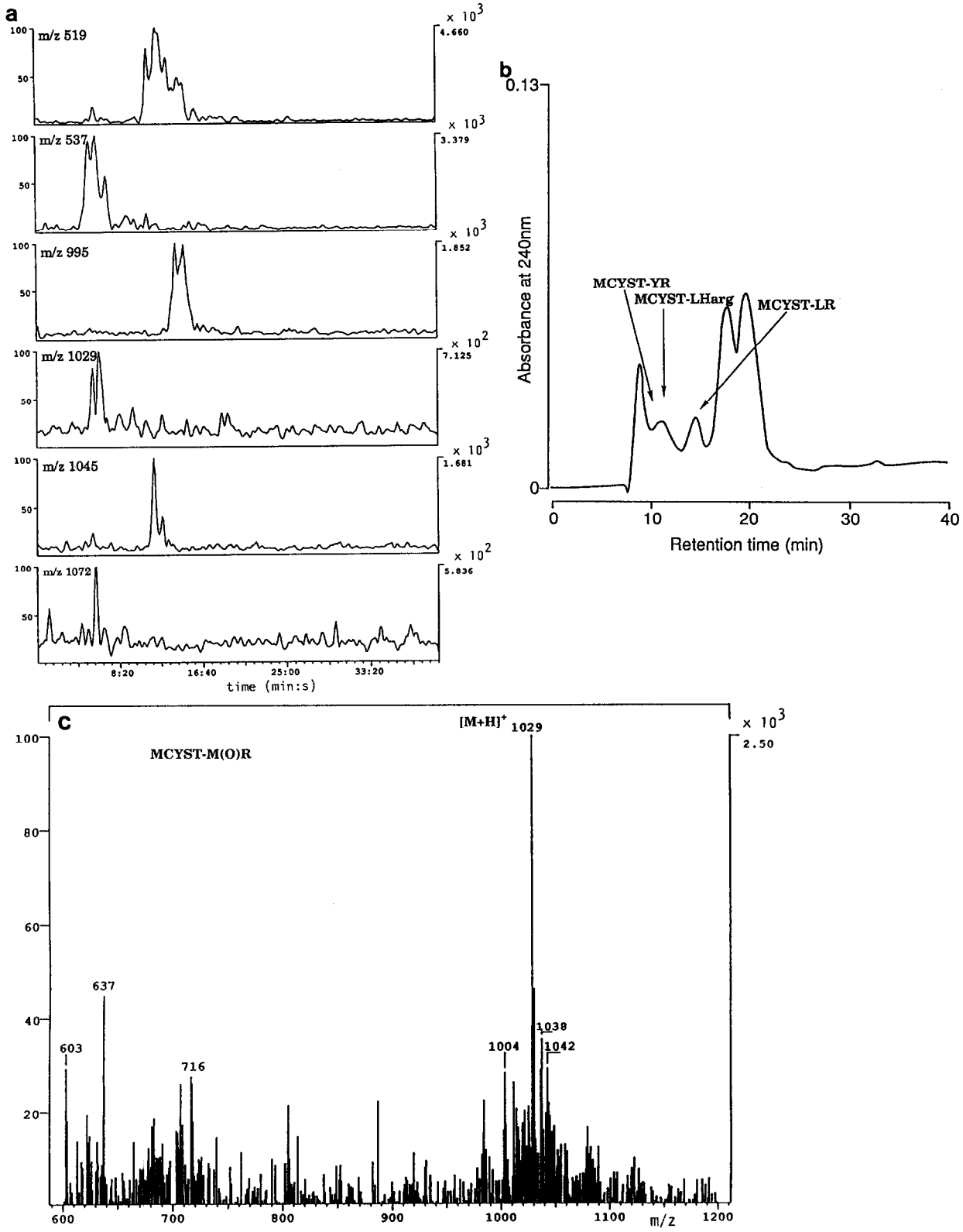
a
m/z 519
m/z 537
m/z 995
m/z 1029
m/z 1045
m/z 1072
time (min:s)
b
Absorbance at 240nm
MCYST-YR
MCYST-LHarg
MCYST-LR
Retention time (min)
c
MCYST-M(O)R
[M+H]+ 1029
637
603
716
1004
1038
1042
m/z

Fig. 7.

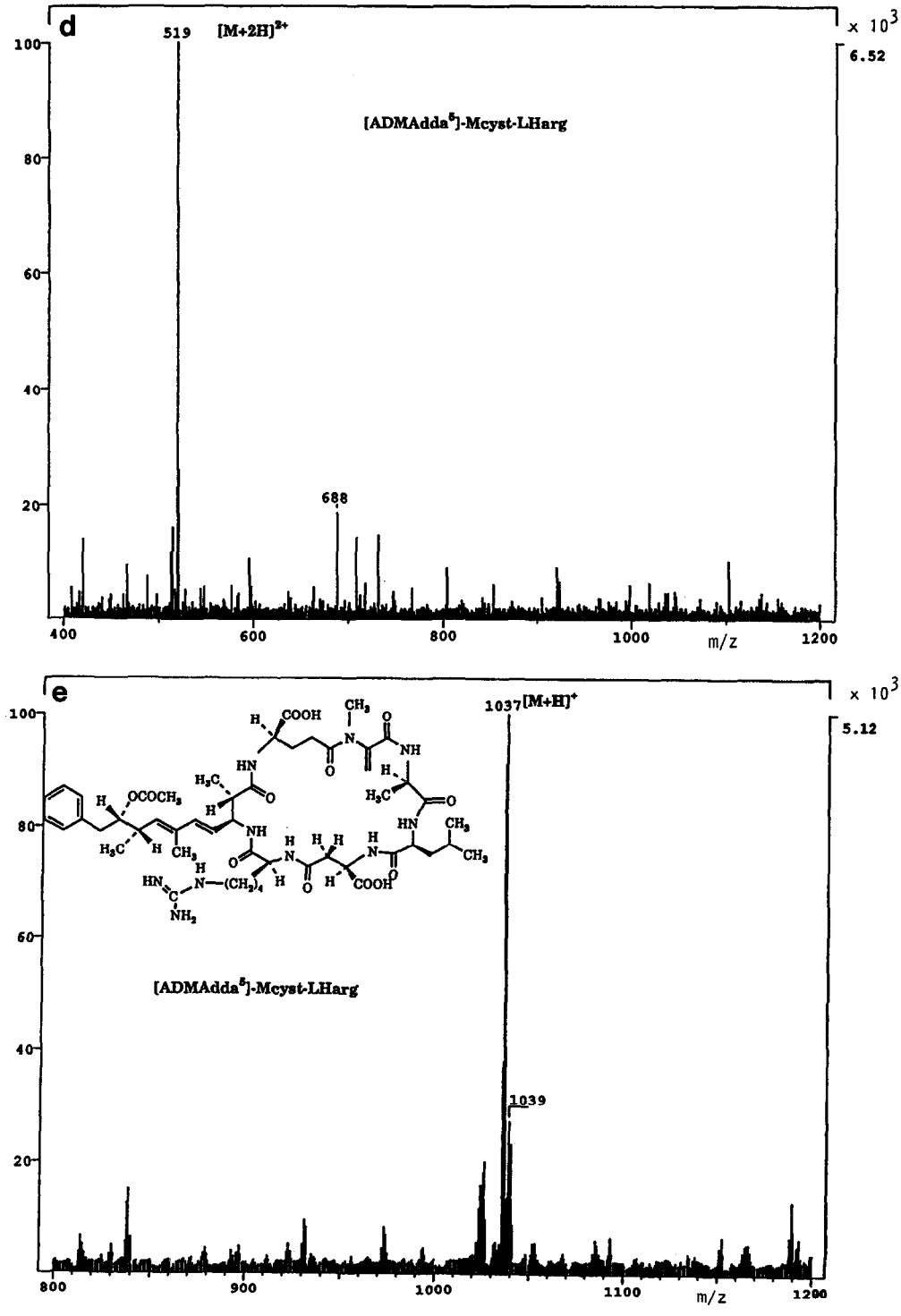

Fig. 7.

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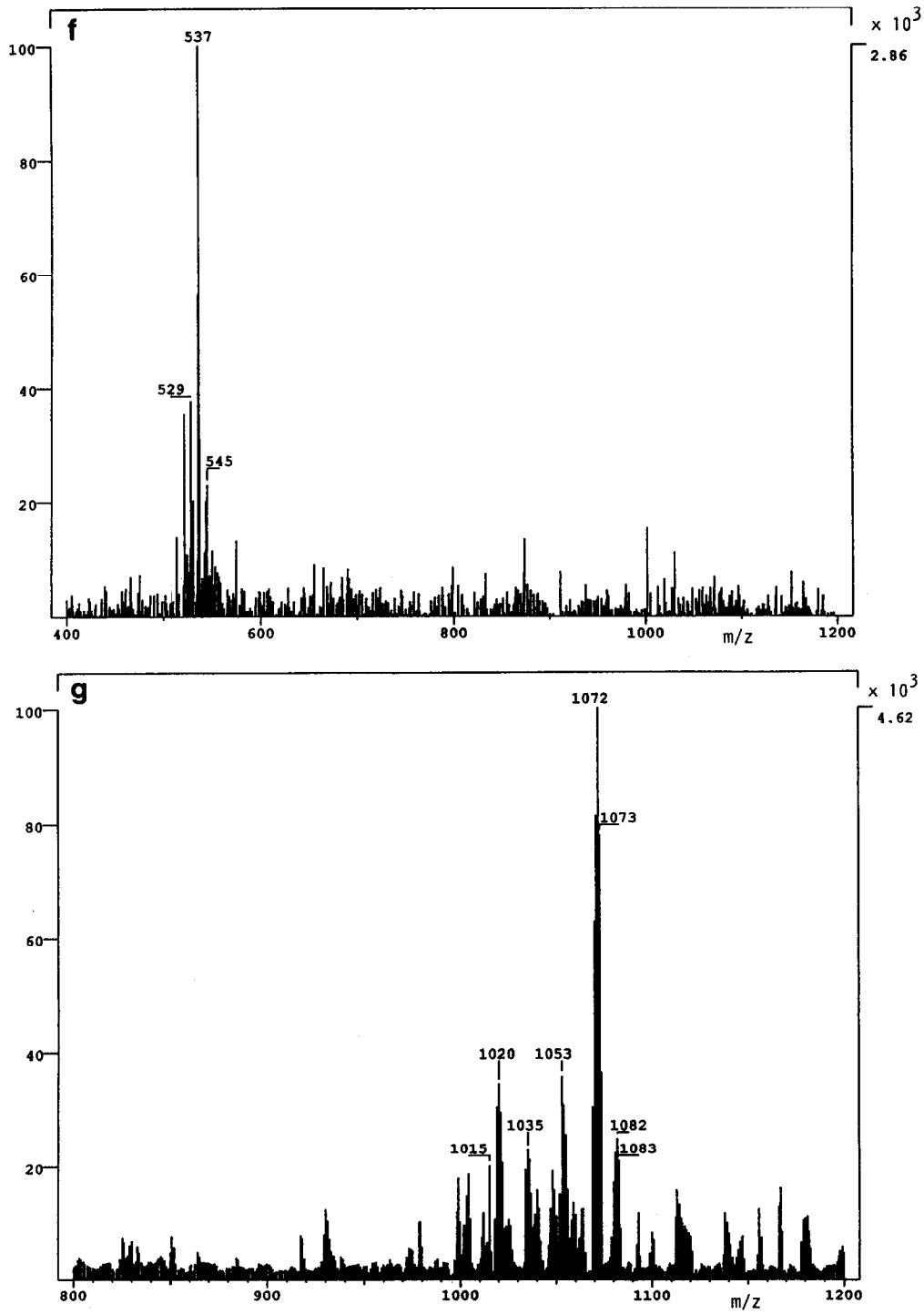

Fig. 7. (a) Reconstructed ion chromatograms; (b) reversed-phase LC chromatogram indicating toxins present in the extract from Water Lane Fish Farm. As well as mcyst-LR and -YR, two peptides were tentatively identified on the basis of their mass spectra as (c) mcyst-M(O)R and (d and e) mcyst-L-Harg. Another compound with $[M + 2H]^{2+}$ at m/z 537 and $[M + H]^{+}$ m/z 1072 (f and g, respectively) may be another peptide variant.

described by Namikoshi *et al.* [22]. A strong signal at *m/z* 537 with t_R 5.50 min could be the doubly charged ion of a toxin which has previously been observed but not characterised. The deconvoluted spectrum gave a protonated molecule at *m/z* 1072 (Fig. 7f and g). It is highly likely that this corresponds to another variant since it has previously been detected in extracts from laboratory cultures of *Microcystis aeruginosa* by FAB mass spectrometry.

In the extract from the laboratory culture of *M. aeruginosa* mcyst-LR was the major component (Fig. 8a and b) but at t_R 19.10 min a second component, possibly mcyst-RR [D-Asp^3, D-Ha^7], was detected, giving an $[M + H]^+$ at *m/z* 1010 and an $[M + 2H]^{2+}$ ion at *m/z* 505 (Fig. 8c).

DISCUSSION

Traditionally different analytical methods such as UV detection (for toxins with UV chromophore), fluorescence detection for saxitoxin [11], GC–MS for anatoxin [8-10], static FAB-MS [7] and on-line CF-FAB-MS detection for microcystins [12,13], are required for studying different classes of cyanobacterial toxin. Mass spectrometry, unlike UV spectroscopy, has the advantage that it can handle compounds which lack the UV chromophore. Furthermore GC–MS is applicable to study anatoxin, although samples have to be derivatized before analysis. FAB-MS has been a method of choice to analyse microcystins. It requires a suitable matrix for sample ionization and subsequently, LC–CF-FAB-MS has to incorporate the matrix in the LC

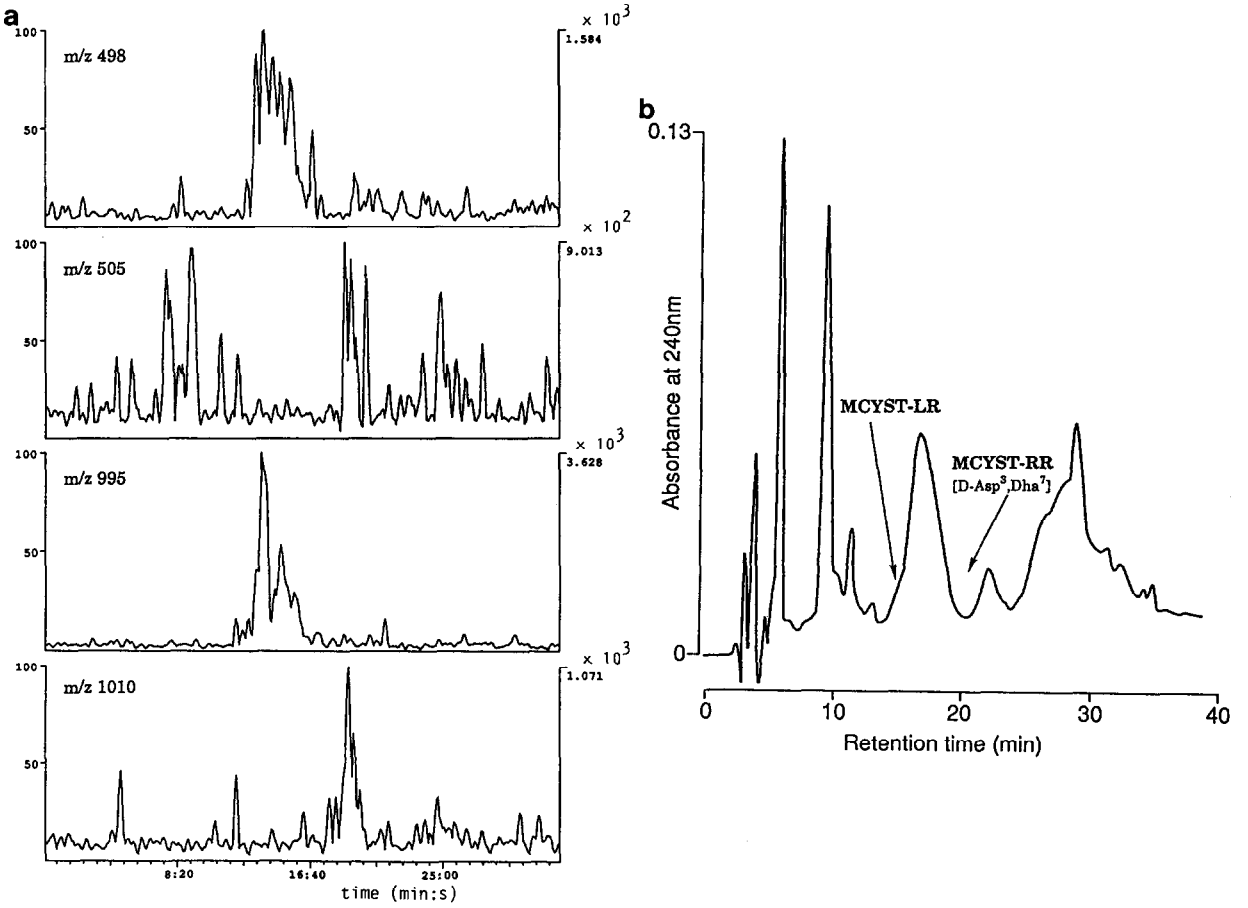

Fig. 8.

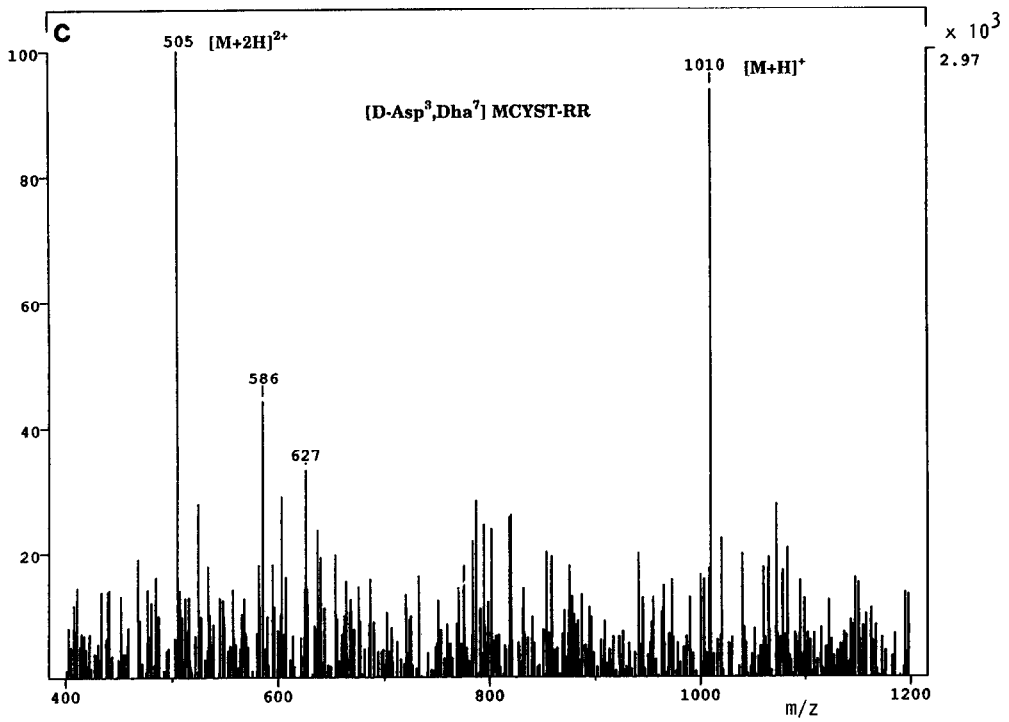

Fig. 8. (a) Reconstructed ion chromatograms; (b) reversed-phase LC chromatogram indicating the presence of toxins in an extract from a culture of *Microcystis aeruginosa* PCC 7813 as detected by (a) mass spectrometry and (b) UV absorption at 240 nm. As well as mcyst-LR another peptide, mcyst-RR [D-Asp^3, D-Ha^7] was identified on the basis of its mass spectrum (c) and comparison the retention time to that of the standard.

separation. Due to changes in polarity and viscosity of the mobile phase, the matrix will affect the chromatographic separation. In addition, not all matrices are amenable to LC–MS because they are not capable of forming a stable and consistent film on the FAB target. High matrix backgrounds often hinder the detection of compounds of interest and severely decrease the limit of detection. While various techniques are normally required to achieve efficient ionization for different classes of toxins, this study demonstrates that ESI-MS is practicable for all the compounds, especially when different classes of toxins are known to coexist in a bloom sample. ESI-MS experiences less ion suppression effect in comparison to FAB, hence it is a more attractive technique for analysing mixtures. Furthermore, on-line LC–MS analysis is less time consuming than off-line LC–MS and with a microbore LC system, less sample is required for the analysis in comparison to the conventional LC set up [13]. ESI-MS was developed primarily for molecular weight determination of proteins and other large biomolecules. This study shows the technique is also applicable for analysis of low-molecular-weight compounds with good sensitivity. Further studies are in progress to determine the suitability of this method for analysis of other cyanobacterial toxins.

These results show that developments and innovations in mass spectrometry require sophisticated (and sometimes expensive) equipment. However, the combination of LC–MS for the identification of peptide toxins exploits the separation capability of liquid chromatography with the molecular weight information provided by the mass spectrometer, and offer a much wider scope in the analysis of algal toxin. However in a system where authentic reference compounds are not available, tandem mass spectrometry (MS–MS) [23] can be considered and thereby further information on the identity of these compounds could be obtained.

Although LC–MS of other cyanobacterial toxins was not examined in this study their ability to be ionized by electrospray and to be separated on reversed-phase systems would suggest this is feasible.

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