Electrospray Ionization Mass Spectrometric Analysis of Microcystins, Cyclic Heptapeptide Hepatotoxins: Modulation of Charge States and $[M + H]^+$ to $[M + Na]^+$ Ratio

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Electrospray ionization mass spectrometry was used to develop a rapid, sensitive, and accurate method for determination and identification of hepatotoxic microcystins, cyanobacterial cyclic heptapeptides. To optimize the electrospray ionization conditions, factors affecting charge state distribution, such as amino acid components of sample, proton affinity of the additives, and additive concentration, were investigated in detail and a method for controlling charge states was developed to provide molecular-related ions for assignment of molecular weight and reasonably abundant precursor ions for MS/MS analysis. A procedure for identification of microcystins consisting of known amino acids was proposed: for microcystins giving abundant $[M + 2H]^{2+}$ ions, the addition of nitrogen-containing bases to the aqueous sample solution is effective to obtain an increased intensity of $[M + H]^+$ ions, whereas the addition of Lewis acids containing nitrogen can produce increased abundances of $[M + 2H]^{2+}$ ions for microcystins giving weak $[M + 2H]^{2+}$ ions. Microcystins possessing no arginine residue always give sodium adduct ions $[M + Na]^+$ as the base peak, and these are difficult to fragment via low energy collision-induced dissociation to yield structurally informative products; the addition of oxalic acid increases $[M + H]^+$ ion abundances, and these fragment readily. (J Am Soc Mass Spectrom 1999, 10, 1138–1151) © 1999 American Society for Mass Spectrometry

Microcystins, cyclic heptapeptide hepatotoxins, are some of the potent natural toxins produced by cyanobacteria (blue-green algae), which grow worldwide in fresh and brackish waters and cause animal and human water-based toxicoses [1]. Extensive efforts have been devoted by many researchers to studies on the chemical structures, biosynthesis, and structure-toxicity relationships of microcystins [2]. Up to now about 60 microcystins have been structurally characterized. Because new homologs with biological activities are being found [1], researchers still focus on their identification and characterization from a viewpoint of environmental toxicology.

In general, the structure of microcystins is described as cyclo(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) [3], where MeAsp is *erythro*-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and X and Z are two variable L-amino acids, with their one-letter abbreviations being used as the suffix of names [3]. Adda, (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*, 6*E*-dienoic acid, is a characteristic C_{20} amino acid which plays an important role in microcystin activity [2, 4–6]. Compositional analysis of about 60 known microcystins, however, confirmed the presence of only Adda and Glu in all of the samples and indicated that the other five amino acids are variable.

Due to the impact of microcystins as toxins in the aquatic environment, a rapid, sensitive, and accurate method to determine them qualitatively and quantitatively is required. Electrospray ionization mass spectrometry (ESIMS) has become a powerful method in structure determination of natural products, particularly peptides and proteins. The most attractive feature is the formation of multiply charged ions of large

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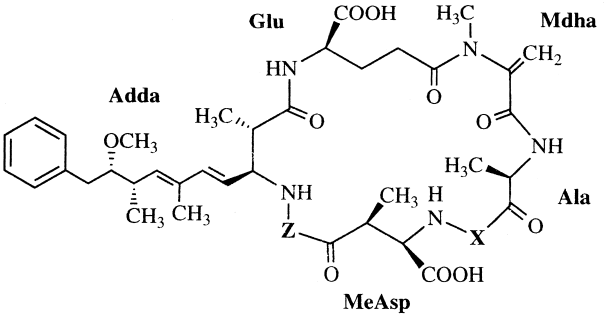
molecules that would provide critical information regarding the molecular masses [7]. ESIMS has been applied to the identification of several microcystins [8–10]. A potential advantage of the ESI technique is the formation of doubly charged ions of microcystins, which can be attractive precursor ions to determine the structure of microcystins by ESIMS/CID/MS in comparison with the singly charged ions generated by fast-atom bombardment [2].

To optimize the ESI conditions for the analysis of the microcystins, the solution and gas-phase ion chemistry of these specific species, such as mechanisms of ion formation and fragmentation, must be made clear. The ion formation processes of microcystin ESI are complex because of the special cyclic structure. Many parameters and the processes of solution equilibrium have an effect on the charge states of ions. In ESI mass spectra of the microcystins, the relative abundances of $[M + 2H]^{2+}/[M + H]^{+}$ ions vary with changes in amino acid residues [8]. Both $[M + H]^{+}$ and $[M + 2H]^{2+}$ ions are useful to assign molecular weights of new microcystins and can be important precursor ions for ESIMS/CID/MS analysis to afford structural information. Studies on the modulation of charge states and relative abundances of $[M + 2H]^{2+}/[M + H]^{+}$ of microcystins are, therefore, important for identification and structural analysis of new microcystins.

Mechanisms of ion formation as well as interpretation of charge state distributions have been hotly debated. Wang et al. [11] reported that the protonation of the cyclic peptide gramicidin S in solution was varied by adjusting pH, solvent composition, and sample concentration. Le Blanc et al. [12] found that the relative abundances of $[M + 2H]^{2+}$ to $[M + H]^{+}$ of gramicidin S did not correlate with the solution pH, but with the proton affinity of the nitrogen-containing bases that were added. It was reported that for compounds with $pK_a < 3$, gas-phase proton transfer reactions could be promoted by the addition of ammonium acetate to the sample solution to increase analyte electrospray response [13]. However, in these studies using linear proteins and gramicidin S that have different structural features from microcystins, these analytes have no such special amino acid residues that can provide directly important factors for efficient differentiation of cyanobacterial toxins in environmental toxicology by using ESIMS.

The doubly charged ions $[M + 2H]^{2+}$ were barely observed in the ESI mass spectra of microcystins without basic arginine residue [8]. Cationized species such as $[M + Na]^{+}$ and $[M + K]^{+}$ ions, derived from traces of alkali metal salts in sample or container, have always been observed in the spectra of these compounds. Although these adduct ions were beneficial to assign molecular weight, no structurally informative fragment ions could be found in their low energy collision-induced desorption (CID) mass spectra. Thus, it is also necessary to develop a simple way to adjust the abundance of $[M + Na]^{+}/[M + H]^{+}$ to measure CID mass spectra. Harada et al. succeeded in removal of sodium ion from the secondary ion mass spectra of saccharides and glycosides using a column packed with cryptand polymers [14]. Because this technique was effective for several samples but not applicable to trace amounts of the analyte, we examined an alternative method using simple additive(s) such as citric acid and oxalic acid for the analysis of trace amounts of microcystins.



	X	Z
Microcystin-RR	Arg	Arg
Microcystin-LR	Leu	Arg
Microcystin-YR	Tyr	Arg
Microcystin-LA	Leu	Ala

Figure 1. Structures of microcystins-RR, LR, YR, and LA.

In the present study, the effects of several factors have been examined, such as sample concentration, solution pH, proton affinity of the nitrogen-containing bases to be added to sample solution, and the amino acid composition. The relative abundances of $[M + 2H]^{2+}/[M + H]^{+}$ of cyclic heptapeptides, microcystins, in ESIMS have been examined and a method for controlling charge states of microcystins has been developed to obtain precursor ions that can give reasonably abundant fragment ions with structural information in ESIMS/CID/MS. A procedure for identification of known and new microcystins consisting of known amino acids is proposed.

Experimental

Microcystins-RR, LR, YR, and LA (Figure 1) were used. Solvents, nitrogen-containing bases, and organic acids were purchased from Wako Pure Chemical Industries (Osaka, Japan) and were used without further purification.

ESI mass spectra were recorded on a Finnigan TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT). The ESI mode (spray potential 4.5 kV) was used. Data analysis was controlled by a DEC 2100 data system. Full scan spectra were acquired in the ion peak centroid or profile modes over the mass/charge range of 50–1200 at 1.2–2.0 s. The mass spectra were obtained by direct infusion or flow injection method.

Table 1. Relative abundances of $[M + H]^+$ and $[M + 2H]^{2+}$ ions in the ESI mass spectra of microcystins-RR, LR, YR, and LA (50 ppm): amounts injected, 5 μL; solvent, MeOH/H_2O (1:1) containing 5% CH_3COOH. The ion abundance (%) was normalized to the base peak (100%) in each spectrum.

Sample	RR	LR	YR	LA[a]
$[M + H]^+$	1038.5 (21)	995.5 (100)	1045.5 (100)	910.5 (31)
$[M + 2H]^{2+}$	519.8 (100)	498.3 (11)	523.3 (28)	455.7 (0)

[a]Base peak: $[M + Na]^+$ (*m/z* 932.5).

For determining an ion abundance by the selected-ion monitoring (SIM) technique, the flow injection method was used. A sample solution was loaded into a sample loop and flushed into the ion source with MeOH/H_2O mobile phase containing CH_3COOH in positive mode, and 2-PrOH/H_2O containing NH_4OH in negative mode.

Tandem mass spectrometry experiments were based on CID occurring in the rf-only collision cell of the triple quadrupole at a collision energy of 15–25 eV. Argon was used as collision gas in the range of 1.5–2.5 mtorr (no calibration). The instrument was scanned at a rate of 2.8 s/decade through the required mass range. At least 15 scans were accumulated and averaged.

The semiempirical molecular orbital calculations were carried out by using WINMOPAC2.0 program (Japan, Fujitsu, 1998). WINMOPAC2.0 is a graphical user interface and full Windows implementation of MOPAC97 program. The primary structures of microcystins [2] were calculated, optimized geometries were given as the approximate starting structure for calculations of ions, and the global minima with the lowest energy were given.

Results and Discussion

Determination of Charge States in Positive and Negative Ion ESI Mass Spectra of Microcystins According to the Number of Arg Residues

Effect of amino acid residues in microcystins on positive ion charge states. The major positive ions in the ESI mass spectra of microcystins-RR, LR, YR, and LA obtained by flow injection method with MeOH/H_2O (1:1) mobile phase containing 5% CH_3COOH are shown in Table 1.

The base peak in the ESI mass spectrum of microcystin-RR was the doubly charged ion ($[M + 2H]^{2+}$), while microcystins-LR and YR gave the singly charged ion ($[M + H]^+$) as the base peak. In microcystin-LA containing no arginine residue, $[M + Na]^+$ was observed as the base peak.

The maximum charge state was generally identical to the number of basic sites (e.g., Arg, Lys, His, and *N*-terminus) [15–17], suggesting that the amino acid residues in microcystins have a strong influence on ESIMS charge states, especially the presence of arginine residues. Arginine having a guanidino side chain is the most basic among the 20 common amino acids. As a result, the abundance ratio of $[M + 2H]^{2+}/[M + H]^+$ ions increased with the number of Arg residues in microcystins, and the absence of such basic amino acid residues led to the formation of $[M + Na]^+$ ions such as microcystin-LA. These results suggested that the ESI mass spectra of microcystins are related to the number of basic amino acids, such as Arg.

Therefore, the charge state distribution of new microcystins in ESIMS may be predicted in terms of the amino acid composition given by amino acid analysis. The ESI mass spectra patterns revealed that the number of Arg residues in the molecules is a very important factor for efficient differentiation of microcystins by using ESIMS.

Although strong evidence has indicated that initial proton localization depends on the type and number of basic residues present in a given linear peptide [18], the initial protonation sites of cyclic peptides have not been investigated in detail. In microcystins it could be considered as follows. First, the amide nitrogens in cyclic peptide bonds can be protonation sites [19], and our results from the MS/MS [20] also support this possibility. Second, two side-chains of Arg and Adda residues in microcystins can be also protonation sites, because protonation on Arg residues has been recognized for linear peptides, and the particular neutral losses from these two side-chains have been observed. We have calculated the energy of the ions protonated in these proposed sites (Table 2) by using WINMOPAC 97. When protonations occur in the nitrogen of Arg residue's side-chain, the ion energy was the lowest in all the protonation ions.

Negative ions of microcystins in ESI mass spectra. Because arginine is a strong proton acceptor, the microcystins containing basic Arg residues would form an intramolecular salt with the carboxyl group in the Glu or MeAsp residue. The COO^- group could accept H^+ and Na^+ ions in acidic solution to form molecular ion species, such as $[M + H]^+$ and $[M + Na]^+$ ions. Because ESIMS can detect the ion in solution with a fair sensitivity [21], the sensitivity of analysis of microcystins containing Arg residues is high in positive mode, whereas negative ion detection has low sensitivity. For example, microcystin-RR in solvent MeOH/

Table 2. The calculated energies of protonation ions of microcystin-LR at different sites

	Side-chain		*N* of amino acid in cyclic peptide bond						
Protonation site	*N* of Arg	OCH_3 of Adda	Ala	Leu	MeAsp	Arg	Adda	Glu	Mdha
Energy (kcal/mol)	−191.16	−161.64	−157.06	−149.34	−146.15	−149.97	−156.73	−159.24	−148.37

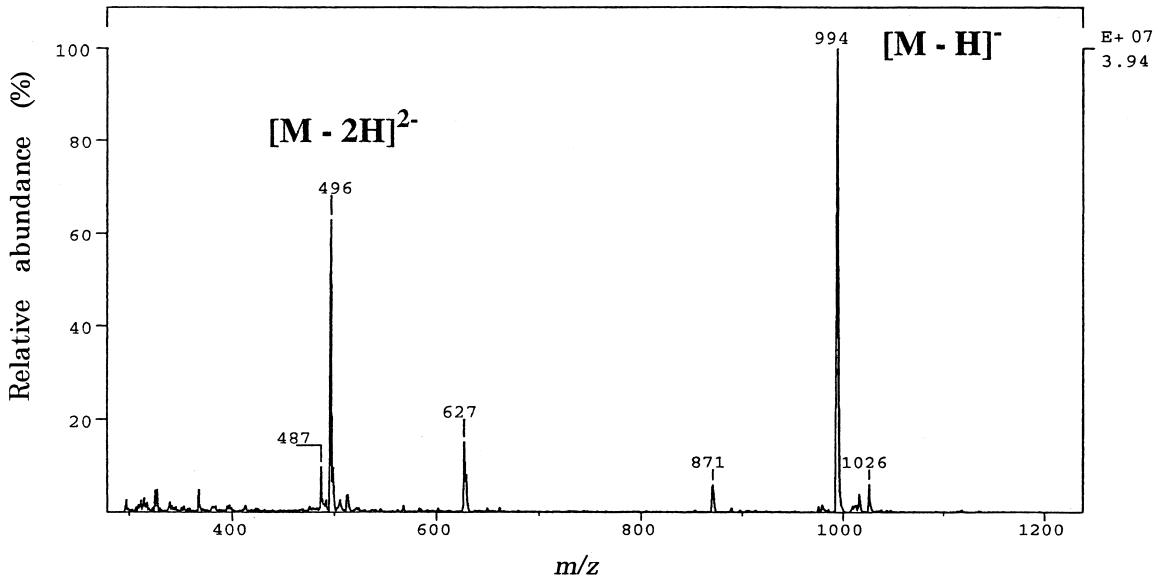


Figure 2. Negative ion ESI mass spectrum of microcystin-LR obtained by direct infusion in 2-PrOH/H_2O (1:1) mobile phase containing 1% NH_4OH: $[M - H]^-$, *m/z* 993; $[M - 2H]^{2-}$, *m/z* 496; $[M - 2H - H_2O]^{2-}$, *m/z* 487. Sample concentration: 50 μg/mL. Unknown ion peaks: *m/z* 627 and 871.

H_2O (1:1) showed no molecular ion species in negative mode. Conversely, microcystins containing no Arg residue, such as microcystin-LA, showed high sensitivity in negative ion ESIMS.

Straub et al. [22] reported that the solvent, 2-propanol (2-PrOH), was an ideal choice as a mobile-phase additive, and an increase in the solvent pH can increase the sensitivity in negative ion ESIMS operation. For microcystins containing Arg residues, the solvent 2-PrOH/H_2O containing NH_4OH (pH range from 9 to 11) was suitable for obtaining negative ion ESI mass spectra, such as the spectrum of microcystin-LR in Figure 2. As pointed out by Covey et al. [17] for linear proteins, the number of negative charges is related to the number of acidic groups. The experimental results for all microcystins showed that the maximum charge state in negative ion ESIMS is identical to the number of carboxylic groups in the molecule. Because such free carboxyl groups belong to the D-glutamic acid (Glu) and *erythro*-β-(methyl)aspartic acid (MeAsp/Asp), the number of charges in negative ion ESIMS can provide composition information on Glu and (Me)Asp residues.

Factors Affecting Charge State Distribution of Microcystins

Effect of sample concentration on ion intensities. The analyte ion current in ESIMS as a function of analyte concentration has been reported for singly charged nitrogen bases [23] and multiply charged proteins [24]. The ESI mass spectra of microcystin-LR in two concentrations obtained by the direct infusion method are shown in Figure 3. The trends observed in both singly and doubly charged ions of microcystin-LR were consistent with the results of previous studies [23, 24], showing increased responses for both $[M + 2H]^{2+}$ and $[M + H]^+$ ions by increasing the sample concentrations. It is noteworthy that the ratio of R2+/R1+ (R1+ = abundance of $[M + H]^+$ ion, R2+ = abundance of $[M + 2H]^{2+}$ and $[M + Na + H]^{2+}$ ions) varied also with an increase in the concentration of microcystin-LR.

The ratio of $[M + 2H]^{2+}/[M + H]^+$ ions increased by lowering the sample concentration of microcystin-LR. The samples were, therefore, introduced into the ESI ion source by loop injection to confirm this result, and the peak areas of ion response in different concentrations were measured by SIM (Figure 4). There was a linear correlation between injected amounts and the areas of ion response of the singly charged ion (*m/z* 995) in the concentration range from 10^{-7} to 10^{-6} M with a correlation coefficient (r^2) of 0.99. These results showed that quantitative analysis of trace microcystins could be performed by ESIMS using SIM of the singly charged ion.

The abundance ratios of R2+/R1+ varied with the injected concentrations of microcystin-LR. The R2+/R1+ values in three different concentrations of (a) 10^{-5} M, (b) 10^{-6} M, and (c) 10^{-7} M were examined. The average values were (a) $\Sigma[M + 2H]^{2+}/\Sigma[M + H]^+ = 0.12$ and $\Sigma[M + Na + H]^{2+}/\Sigma[M + H]^+ = 0.32$, (b) $\Sigma[M + 2H]^{2+}/\Sigma[M + H]^+ = 0.48$ and $\Sigma[M + Na + H]^{2+}/\Sigma[M + H]^+ = 0.58$, and (c) $\Sigma[M + 2H]^{2+}/\Sigma[M + H]^+ = 0.48$ and $\Sigma[M + Na + H]^{2+}/\Sigma[M + H]^+ = 3.27$. The intensity of doubly charged ion at the lowest sample concentration was greater than that of singly charged ion $[M + H]^+$. As a result, both $[M + H]^+$ and $[M + 2H]^{2+}$ have been reported as the base peak in the ESI mass spectra of microcystin-LR [8, 9].

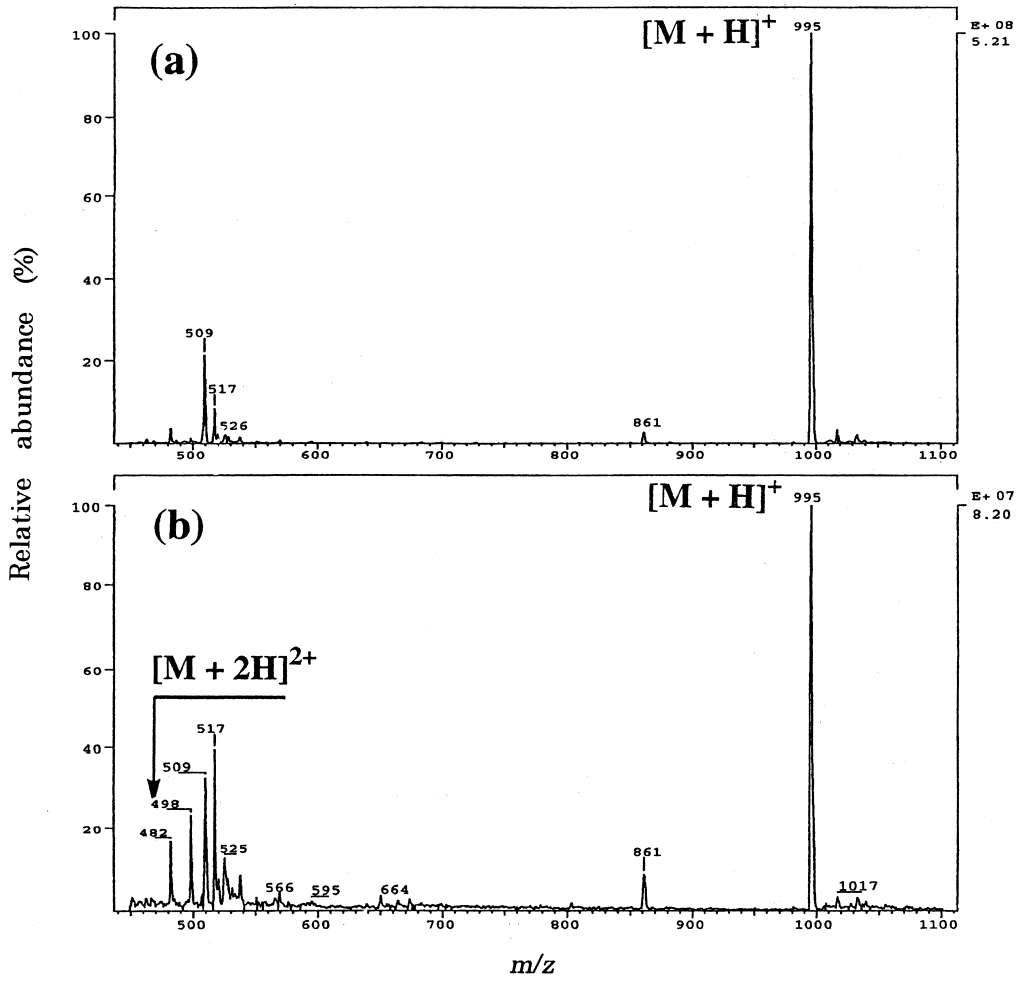


Figure 3. ESI mass spectra of microcystin-LR obtained by direct infusion in MeOH/H_2O (1:1) mobile phase containing 5% CH_3COOH. Sample concentration: (a) 100 μg/mL, (b) 10 μg/mL. *m/z* 995, $[M + H]^+$; *m/z* 498, $[M + 2H]^{2+}$; *m/z* 509, $[M + Na + H]^{2+}$; *m/z* 517, $[M + K + H]^{2+}$.

The present results were consistent with the observation for compounds bradykinin, gramicidin S, and 4,4′-bipiperidine by Wang and Cole [11, 25]. According to their view, at higher concentrations, the severe coulombic repulsion between neighboring charged species and the increase in abundance of cluster ions, $(M_x + H_y)^{y+}$ $(y < x)$, favoring the formation of ions with low charge states, would have caused the decrease in R2+/R1+ abundance ratios. To rationalize their results, Wang and Cole further presented a more relevant model including the concept of the ratio of the number of excess charges on all droplets produced by electrospray to the total number of analyte molecules in all droplets [25]. These findings have provided a mechanistic interpretation for the dependence of charge state distributions on analyte concentrations, and may be applied for the investigation of the quantitative analysis of microcystins.

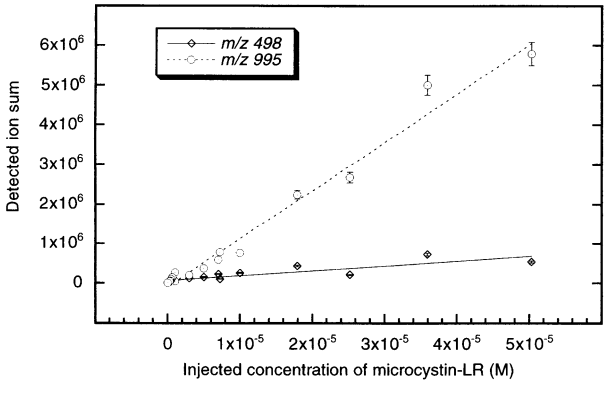


Figure 4. Variation of the detected sum of ions $[M + H]^+$ (*m/z* 995) and $[M + 2H]^{2+}$ (*m/z* 498) with the injected concentration of microcystin-LR. Mobile phase: MeOH/H_2O (1:1) containing 5% CH_3COOH. The sum of ions was determined by SIM. Samples were introduced into the ESI ion source by loop injection; flow rate, 200 μL/min; injected amount, 5 μL.

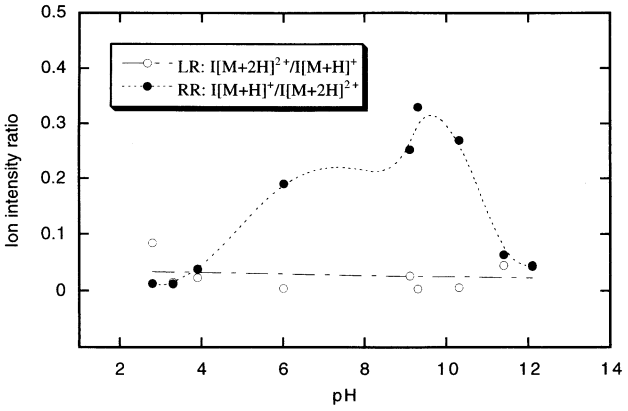


Figure 5. Relative abundances of molecular ion species of microcystins-LR and RR at different pH, adjusted by CH_3COOH (3–6), NaOH (9–11), and NH_4OH (11–12).

Effect of pH on ESIMS charge state distribution of microcystins

Figure 5 shows the variation of relative abundances of $[M + H]^+$ and $[M + 2H]^{2+}$ species in the ESI mass spectra of microcystins-LR and RR with different solution pH. The pH was adjusted by addition of acetic acid (pH 3–6), sodium hydroxide (pH 9–11), and ammonium hydroxide (pH 11–12).

Degrees of protonation modified by changing the solution pH have been reported for myoglobin protein [26, 27]. Although these studies might suggest that the ratios of singly to doubly charged ions in the ESI mass spectra of microcystins may vary with pH in a sample solution according to the liquid-phase acid–base equilibria, our results clearly showed different results. The abundance ratio of $[M + 2H]^{2+}/[M + H]^+$ ions for microcystin-LR did not change significantly at pH between 3 and 11. The intensity of $[M + H]^+$ of microcystin-RR increased significantly in changing from pH 3 to 6.

A reasonable explanation for these results may be that the microcystins containing basic Arg residues formed an intramolecular salt with the carboxyl group in the Glu or MeAsp residue, and therefore showed a buffer action dependent on the electrolytic dissociation constant pK. These results suggested that the proton affinity of solvent additives can be an important factor determining ESIMS charge state distribution of microcystins. Thus, nitrogen-containing bases were tested as additives.

Effect of the proton affinity of nitrogen-containing bases on charge states of microcystin-RR

The ESI mass spectrum recorded from acidic solution of microcystin-RR having two basic Arg residues showed the base peak at *m/z* 520, corresponding to the doubly charged ion $[M + 2H]^{2+}$, and only a weak singly charged species was observed at *m/z* 1038. To increase the intensity of the singly charged ion $[M + H]^+$, nitrogen-containing bases were added to remove a proton from the doubly charged ion $[M + 2H]^{2+}$ (eq 1).

$$[RR + 2H]^{2+} + :B \rightarrow [RR + H]^+ + HB^+ \quad (1)$$

Nitrogen-containing bases, such as NH_4OH, Et_2NH, and Et_3N, were found to be suitable to adjust the abundance of $[M + 2H]^{2+}/[M + H]^+$ ions of microcystin-RR. Several bases were examined to observe the correlation between the relative abundance of $[M + 2H]^{2+}/[M + H]^+$ and the proton affinity of the bases. Figure 6 shows the correlation between the charge state of ions and the proton affinity of the bases, indicating that the relative intensity (normalized to the main ion in each spectrum) of $[M + 2H]^{2+}$ ion of microcystin-RR

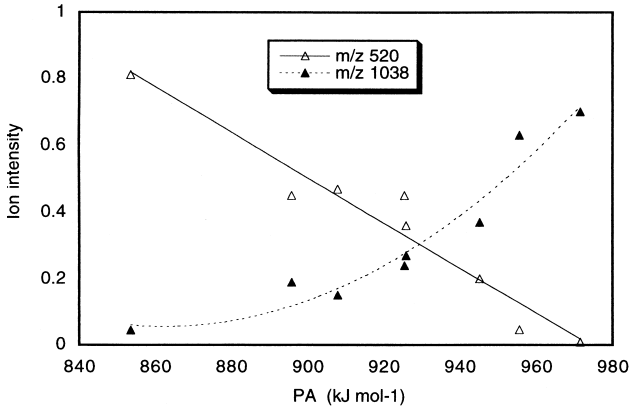


Figure 6. Effect of proton affinity (PA) on charge states of microcystin-RR. Ion intensity ratio was normalized to the main ion in each spectrum: $[M + 2H]^{2+}$, *m/z* 520; $[M + H]^+$, *m/z* 1038.

Base PA (kJ mol^{-1})	NH_4OH	$MeNH_2$	$EtNH_2$	Cyclohexylamine
	853.5	895.8	907.9	925.5
	Ethanolamine	Et_2NH	*n*-Bu_2NH	Et_3N
	925.9	945.2	955.6	971.5

linearly decreased with increase in the proton affinity of the bases ($y = 6.6161 + -0.0067924x$, $r^2 = 0.97$) and, on the other hand, the $[M + H]^+$ ion exponentially increased ($Y = M_0 + M_1x + \cdots M_8x^8 + M_9x^9$, $r^2 = 0.97$).

Inorganic bases such as sodium hydroxides were not suitable to remove a proton form $[RR + 2H]^{2+}$, because the carboxyl groups in the β-methylaspartic acid (MeAsp) and/or Glu residues would have been preferentially neutralized forming alkaline metal salts.

Effect of NH_4OAC on the charge states of microcystin-LR

The main ion formed in the ESI mass spectrum of microcystin-LR (100 μg/mL), recorded using MeOH/H_2O (1:1) mobile phase containing 5% CH_3COOH, was the singly charged ion $[M + H]^+$ (Figure 3a). The abundance ratio $[M + 2H]^{2+}/[M + H]^+$ in the ESI mass spectra of microcystin-LR did not change significantly when ammonium hydroxide was used to adjust the pH of sample solutions as described above (Figure 5). Several nitrogen-containing organic and inorganic bases and their salts were examined to control the ratio $[M + 2H]^{2+}/[M + H]^+$, and NH_4OAc was found to be the most effective additive. Figure 7 shows the ESI mass spectra of microcystin-LR measured with different concentrations of NH_4OAc, indicating that a solution of 0.5 M NH_4OAc gave the best spectrum, showing both $[M + 2H]^{2+}$ at *m/z* 498 and $[M + H]^+$ at *m/z* 995 with reasonable intensities. The small ion peak at *m/z* 1018 corresponds to $[M + Na]^+$. A decrease in the ion intensity of $[M + H]^+$ at *m/z* 995 was accompanied by increases in $[M + H + Na]^{2+}$ ion at *m/z* 509 and $[M + 2H - MeOH]^{2+}$ ion at *m/z* 482 as well as $[M + 2H]^{2+}$ ion at *m/z* 498.

To investigate the structures of these doubly charged ions, and especially to understand the protonation sites, tandem ESIMS experiments and semiempirical molecular orbital calculations have been performed. In the ESIMS/CID/mass spectra of microcystins, microcystins-LR and YR showed fragment ions at *m/z* 482 and 507, respectively, generated by the neutral loss of MeOH, and the fragment ion series containing the (Adda + H − MeOH) unit was observed [20]. The corresponding fragment ions were not detected in the CID mass spectrum of microcystin-RR $[M + 2H]^{2+}$, whereas the fragment ions resulting from the neutral loss of ammonia were abundant. These differences are ascribable to the different attachment sites of protons in the $[M + 2H]^{2+}$ ions of microcystins-LR, YR, and RR; that is, the second site of protonation in the doubly charged ion of microcystins-LR and YR might be at the methoxy group in the Adda unit. The calculations using WINMOPAC 97 also showed that the energy of the structure protonated in the side-chains of Arg and Adda residues was the lowest of all the possible protonated structures (Table 3).

The NH_4^+ ion can provide a proton to form $[M + 2H]^{2+}$ and would also stabilize it as shown in Figure 8. Figure 9 shows the correlation between the abundances of singly and doubly charged ions of microcystin-LR and the concentrations of NH_4OAc, suggesting that the following processes might occur in liquid and gas phases (eqs 2 and 3).

$$[M + H]^+ + NH_4^+ \xrightarrow[\text{liquid phase}]{} [M + 2H]^{2+}$$

$$\cdot NH_3 \xrightarrow[\text{gas phase}]{-NH_3} [M + 2H]^{2+} \quad (2)$$

$$[M + H]^+ + Na^+ \longrightarrow [M + H + Na]^{2+} \quad (3)$$

The ESIMS/CID/mass spectrum of $[M + H + NH_4]^{2+}$ (*m/z* 507) of microcystin-LR showed fragment ion peaks at *m/z* 482 and 498 (Figure 10), which could be formed by the following neutral loss reactions (eq 4). The same product ions were observed in the absence of collision gas. These facts supported the proposed processes of ionization shown by eqs 2 and 4:

$$[M + NH_4 + H]^{2+} \ m/z\ 507 \longrightarrow \begin{cases} \xrightarrow{-NH_3} [M + 2H]^{2+} & m/z\ 498 \\ \xrightarrow{-NH_3,\ -CH_3OH} [M + 2H - CH_3OH]^{2+} & m/z\ 482 \end{cases} \quad (4)$$

The CID mass spectra of doubly charged ions $[M + H + Na]^{2+}$ (*m/z* 509) and $[M + 2H]^{2+}$ (*m/z* 498) of microcystin-LR under moderate collision conditions are presented in Figure 11. The ion at *m/z* 135 has been shown previously to be $PhCH_2CH(OMe)$ [2]. The main process was the dissociation of the doubly charged ion to produce a singly charged ion by loss of the singly charged Adda side chain (eqs 5 and 6) [20]. Therefore, the intensity of the ion peak at *m/z* 482 in Figure 7 also increased with that of doubly charged ions $[M + H + Na]^{2+}$ (*m/z* 509) and $[M + 2H]^{2+}$.

Because the decomposition of doubly charged ions mentioned above did not need strong collision conditions, these reactions would occur on the path from ion source of ESI to mass analyzer and give the diagnostic ions of microcystins in conventional ESIMS. These diagnostic ions include ion at *m/z* 135, $[M + H - 135]^+$, and $[M + H - MeOH]^{2+}$.

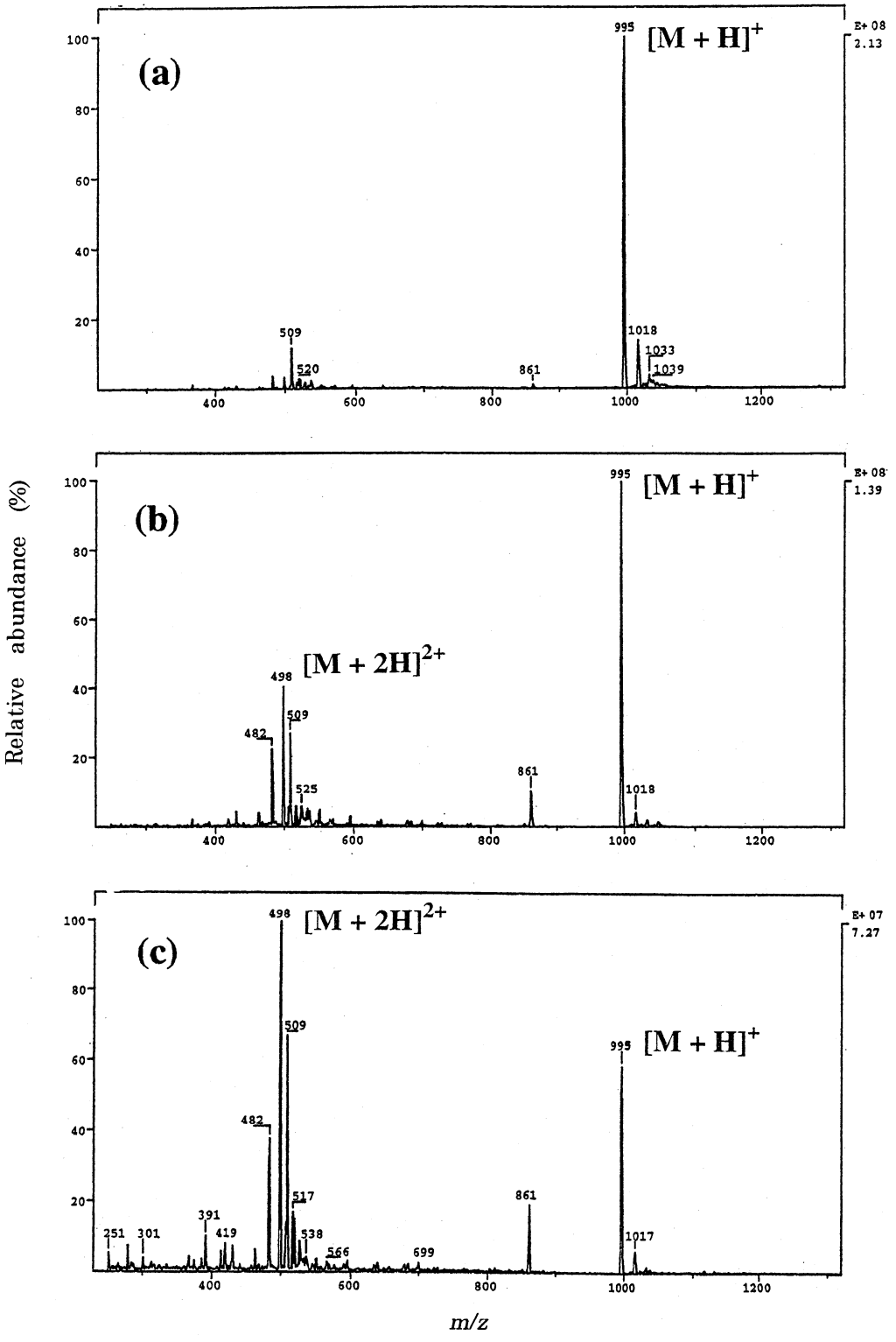


Figure 7. ESI mass spectra of microcystin-LR in MeOH/H_2O (1:1) mobile phase containing (**a**) 5×10^{-3} M, (**b**) 5×10^{-2} M, and (**c**) 0.5 M NH_4OAC solutions and 2.5% CH_3COOH.

Modulation of Charge States to Protonated Ions of Microcystins

Le Blanc et al. [12] have postulated that a proportion of $[M + 2H]^{2+}$ and $[M + H]^+$ ions derives from adducts with nitrogen-containing bases in sample solution, and these adducts are desorbed into the gas phase during electrospray and dissociated in the lens region via CID to yield apparent proton attachment spectra. Their view could be used to explain the effects of NH_4^+ and

Table 3. The calculated energies of double protonation of microcystin-LR at different sites

Protonation site	*N* of Arg side-chain and OCH_3 of Adda	*N* of Arg side-chain and *N* of amino acid in cyclic peptide bond						
		Ala	Leu	MeAsp	Arg	Adda	Glu	Mdha
Energy (kcal/mol)	9.83	12.13	20.03	27.53	33.41	26.35	17.17	23.07

nitrogen-containing bases on the relative abundance of $[M + 2H]^{2+}/[M + H]^+$ in the ESI mass spectra of microcystins-LR and RR (eqs 7 and 8).

The charge states of protonated ions in the ESI mass spectra of microcystins could be controlled by the addition of nitrogen-containing bases or their Lewis acids. Nitrogen-containing bases such as NH_4OH, Et_2NH and Et_3N should be used for microcystins showing strong doubly charged ions to increase the abundance of singly charged ions (Figure 6). The Lewis acids containing nitrogen such as NH_4OAc were useful to produce abundant doubly charged ions in microcystins showing weak doubly charged ions in the absence of additive (Figure 7).

The addition of Lewis acids containing nitrogen was not successful in generating $[M + 2H]^{2+}$ for microcystins containing no Arg residue such as microcystin-LA or for cyclic peptides containing one strongly basic residue such as cyclo (Arg-Gly-Asp-D-Phe-Val).

Griffey et al. reported that the charge state distribution observed for oligonucleotides using negative ESIMS was a function of the concentration of ammonium ion from NH_4OAc added in the solution [28]. Our results in the positive ESIMS charge state distribution of microcystins show an analogous effect.

Control of $[M + Na]^+$ vs. $[M + H]^+$ of Microcystins Containing no Arg Residue by Addition of Oxalic Acid

Microcystin-LA containing no Arg residue showed $[M + Na]^+$ ion at *m/z* 933 as the base peak in the ESI mass spectrum measured in $MeOH/H_2O$ (1:1) mobile phase containing 5% CH_3COOH as mentioned above (Figure 12a). Cationized species such as $[M + Na]^+$ and $[M + K]^+$, derived from trace alkali metal salts in the sample or glass tube, were always observed in the ESI mass spectra of microcystins.

Analysis of microcystin-LA in $MeOH/H_2O$ (1:1) solution stored at −20 °C for one month in a glass tube clearly showed an increase in sodium adduct ions with no peak of $[M + H]^+$ (*m/z* 911) as shown in Figure 13a. It is reasonable to infer that the cationized ions were generated by attachment of trace Na^+ in the solution to carboxyl groups in microcystins. An increase in acidity of the sample solution was evaluated to remove Na^+ from these cationized ions. Figure 12 shows that the ratio of $[M + Na]^+/[M + H]^+$ ions varied with the addition of organic acids, indicating that the addition of oxalic acid effectively produced $[M + H]^+$ ion at *m/z* 911 as the base peak (Figure 13b). Oxalic acid was found to be the most effective reagent to decrease the alkali metal adduct ions of microcystin-LA (Figure 12c).

These results indicated that the addition of oxalic acid is useful to control the ratio of proton/metal adduct ions in the ESI mass spectra of microcystins, especially those having no Arg residue such as microcystin-LA. In our recent research, we have succeeded in increasing the intensities of $[M + H]^+$ ions of seven new microcystins [29] having no Arg residue but possessing two glutamic acid units by using the addition of oxalic acid, and the structures of these new microcystins were unambiguously assigned from the low energy CID mass spectra using reasonably abundant fragment ions.

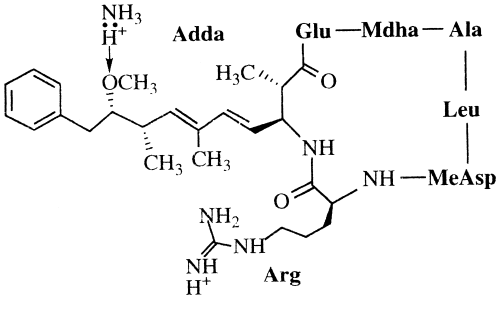


Figure 8. Proposed protonation sites and stabilization of the ions by the lone pair of nitrogen in microcystin-LR solution containing NH_4OAc.

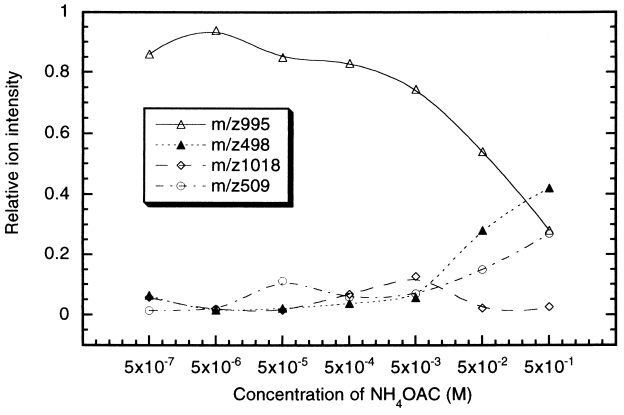


Figure 9. Relative abundance of molecular ion species in the ESI mass spectra of microcystin-LR measured with the different NH_4OAc concentrations. Ion intensity ratios were normalized to the main ion in each spectrum: $[M + H]^+$, 995; $[M + 2H]^{2+}$, 498; $[M + Na]^+$, 1018; and $[M + Na + H]^{2+}$, 509.

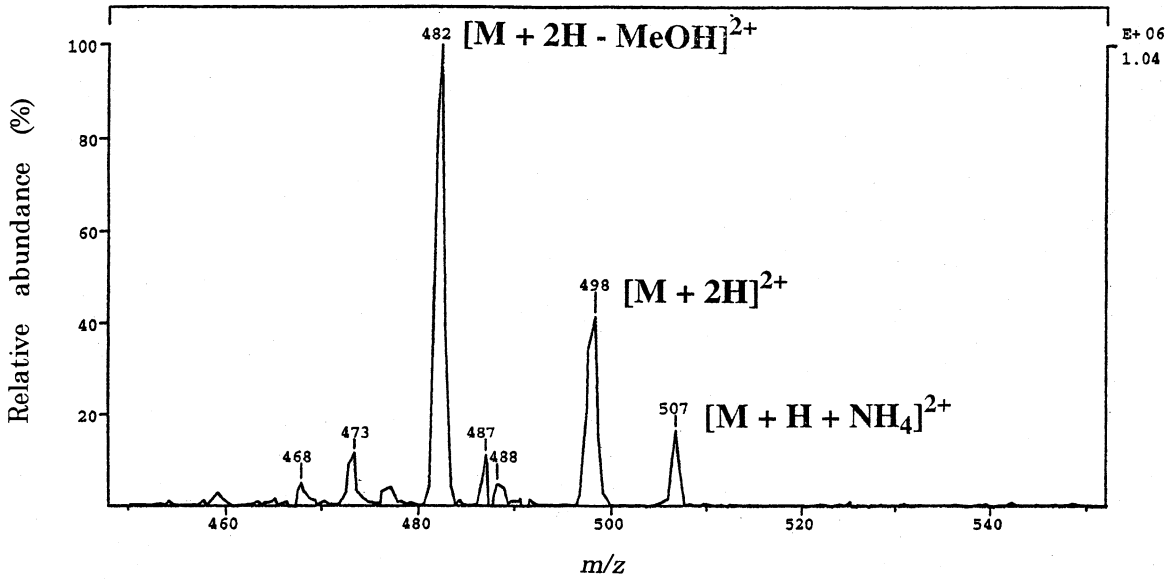


Figure 10. ESIMS/CID/mass spectrum at *m/z* 507 ($[M + H + NH_4]^{2+}$) of microcystin-LR. Collision cell offset voltage, 15 V; collision gas pressure, 2.5 mtorr. *m/z* 498, $[M + 2H]^{2+}$; *m/z* 482, $[M + 2H - MeOH]^{2+}$; *m/z* 473, $[M + 2H - MeOH - H_2O]^{2+}$; *m/z* 468, $[M + 2H - (NH_2)C{=}NH]^{2+}$.

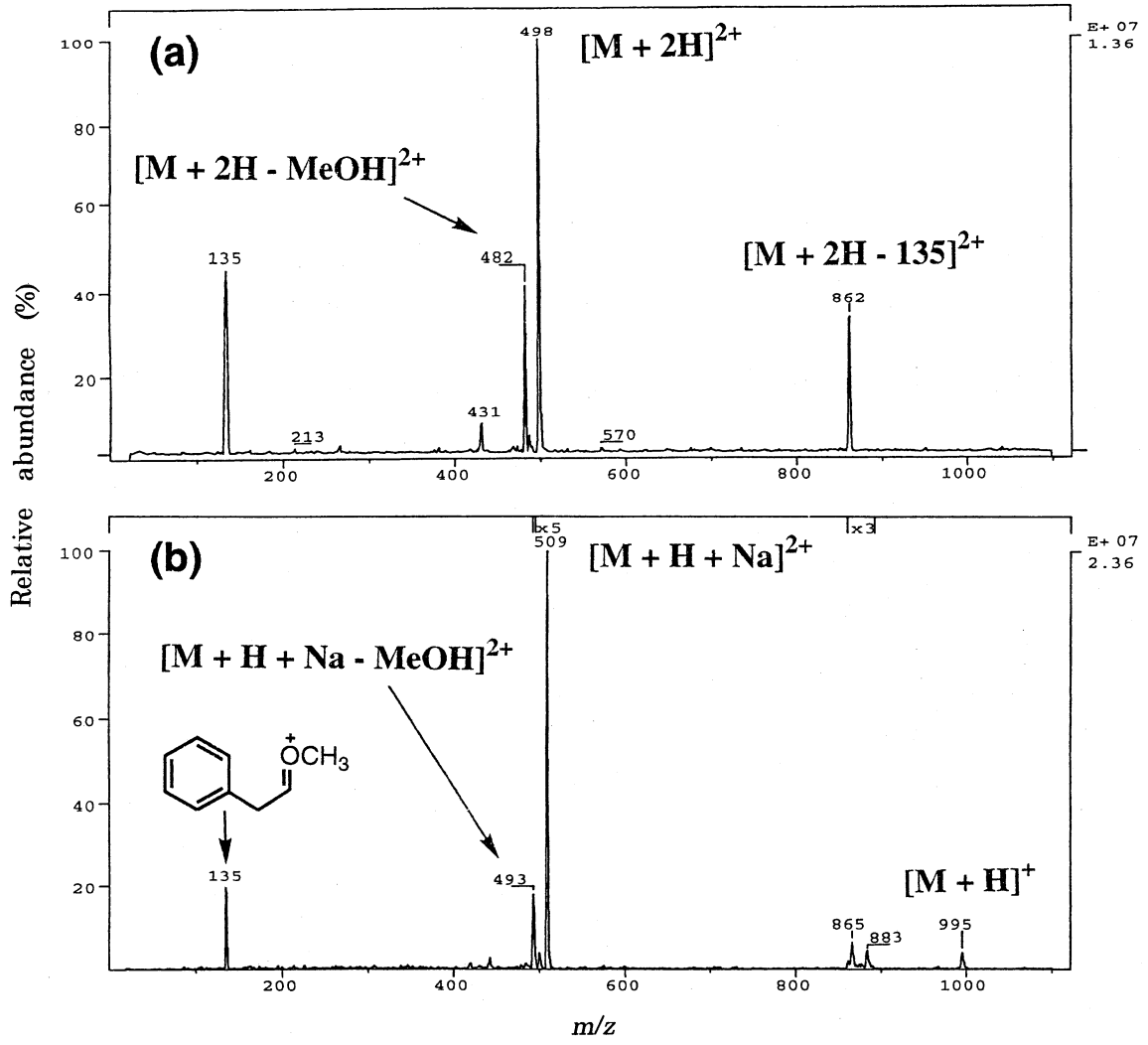


Figure 11. ESIMS/CID/mass spectra at *m/z* 498 ($[M + 2H]^{2+}$) and *m/z* 509 ($[M + H + Na]^{2+}$) of microcystin-LR. Collision cell offset voltage, 25 V; collision gas pressure, 1.5 mtorr.

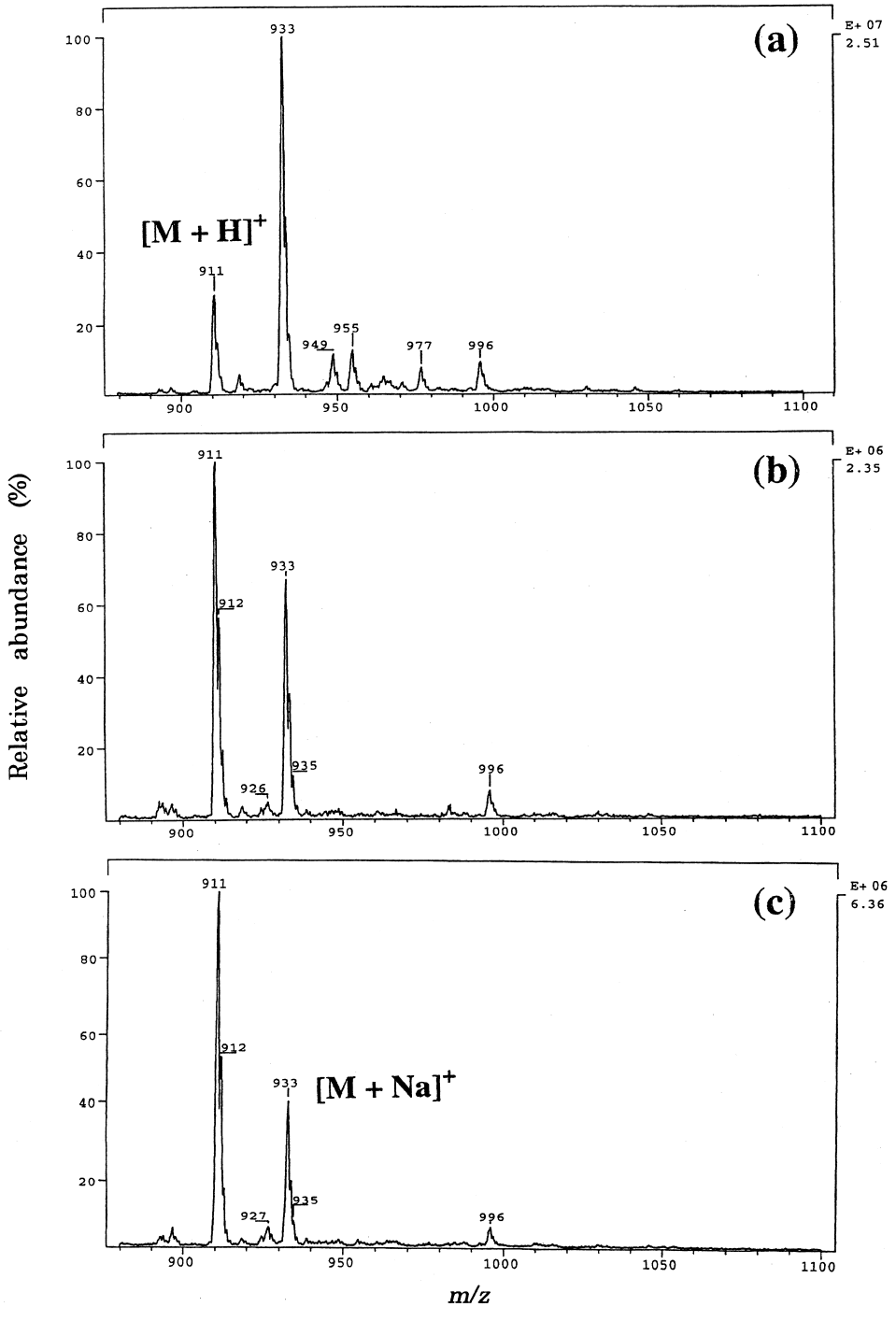


Figure 12. ESI mass spectra of microcystin-LA in MeOH/H_2O (1:1) mobile phase containing (**a**) 5% CH_3COOH ($pK_a = 1.8 \times 10^{-5}$), (**b**) 1% citric acid ($pK_{a1} = 3.1$), and (**c**) 1% oxalic acid ($pK_{a1} = 5.4 \times 10^{-2}$): *m/z* 911, $[M + H]^+$; *m/z* 933, $[M + Na]^+$; *m/z* 949, $[M + K]^+$; *m/z* 955, $[M + 2Na - H]^+$; and *m/z* 977, $[M + 3Na - 2H]^+$.

General Method Utilizing ESIMS for Qualitative and Quantitative Analysis of Microcystins

A general method utilizing ESIMS for determination and identification of hepatotoxic microcystins, cyanobacterial cyclic heptapeptides, was proposed. The scheme consists of

1. Isolation and purification of toxins from cyanobacteria [30].
2. Qualitative analysis of toxins utilizing positive/negative ion ESIMS, including (a) assignment of molecular weight, (b) observation of diagnostic ions, such as peaks at *m/z* 135 [Ph-CH_2-CH(OMe)], $[M + 2H - 135]^+$, $[M + 2H - MeOH]^{2+}$, and (c) amino acid

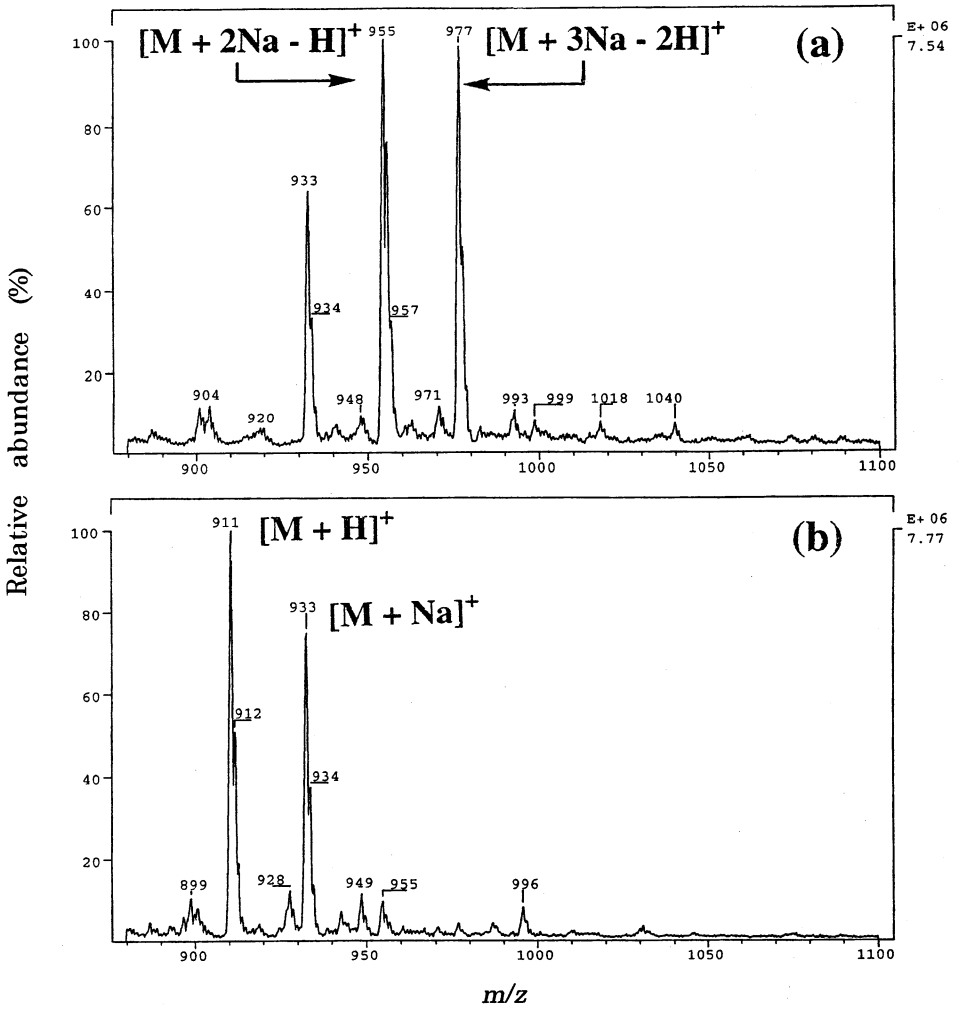


Figure 13. (**a**) ESI mass spectrum of microcystin-LA stored in MeOH/H_2O (1:1) mobile phase for one month in a glass tube. (**b**) ESI mass spectrum of the above sample measured after the addition of 0.3% oxalic acid. For assignment of peaks see Figure 12.

analysis, if necessary. The relative abundances of ions retaining the intact molecules vary according to amino acid composition, and can be modulated according to the method mentioned above. For microcystins having no Arg residue, the addition of oxalic acid is useful to produce obvious molecular-related ions $[M + H]^+$.

3. Quantitative analysis of microcystins by using SIM mode.

The CID mass spectra of analytes must be obtained for detailed qualitative analysis. By using these methods, the qualitative and quantitative analysis of known or new microcystins containing known amino acids can be completed rapidly, sensitively and accurately.

Conclusions

The effects of amino acid residues, sample concentrations, solution pH, proton affinities of nitrogen-containing bases and of Lewis acids containing nitrogen on ESIMS charge state distributions of microcystins were explored. The charge states of microcystins were significantly correlated with the number of basic Arg residues in the molecules. Microcystin-RR having two Arg residues showed $[M + 2H]^{2+}$ ion as the base peak and a weak peak of $[M + H]^+$. The ratio of $[M + 2H]^{2+}/[M + H]^+$ was controlled by addition of nitrogen-containing bases, such as diethylamine and triethylamine, to the aqueous sample solution, and showed a correlation with the proton affinity of these bases. The base peak of microcystins-LR and YR possessing one Arg residue was $[M + H]^+$ ion, and $[M + 2H]^{2+}$ ion was observed as a weak peak in each ESI mass spectrum. The intensity of $[M + 2H]^{2+}$ relative to the $[M + H]^+$ ion of microcystins-LR and YR can be increased by the addition of NH_4OAc to the sample solutions. The ratios of singly and doubly charged ions of these compounds did not change significantly by changing the solution pH.

The abundances of $[M + H]^+$ and $[M + 2H]^{2+}$ ions of microcystins-LR detected by SIM showed remark-

ably linear correlation with the sample concentrations injected. Therefore, ESIMS also provides a useful method for the quantitative analysis of trace microcystins. It is notable that the doubly charged molecular ion species of microcystin-LR became predominant at lower sample concentrations.

Microcystin-LA containing no Arg residue did not show doubly charged molecular ion species under the present experimental conditions. The base peak of this compound in the ESI mass spectrum measured in MeOH/H_2O (1:1) mobile phase containing 5% CH_3COOH was an alkali metal adduct ion ($[M + Na]^+$); $[M + H]^+$ ion formation was promoted by adding oxalic acid to the sample solution.

Thus, the molecular ion species of microcystins in their ESI mass spectra can be modulated by addition of suitable additives to sample solution, allowing the assignment of molecular masses of microcystins very easily, and the control of singly and/or doubly charged ions for ESIMS/CID/MS analysis also contributes to the identification and determination of amino acid sequences of new microcystins.

Acknowledgments

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