CHAPTER III

RESULTS

Identification of cyanobacteria samples

The species of cyanobacteria was identified (as described in Chapter II) and it was revealed in April 2003that the surface blooms in Huay Yuak reservoir, Chiang Mai was dominated by *Microcystis aeruginosa* Kütz. (over 95%).

M. aeruginosa cells were tightly aggregated under light microscope in a different-shape colony, e.g. subspherical and reticulated or lobated. The colony was enveloped with a gelatinous sheath. The gas vacuoles within the cells appeared blackish under the microscope. The colony and cells of *M. aeruginosa* are shown in Fig. 6.

Separation of microcystins in cyanobacterial extract

An HPLC chromatogram of the microcystin-LR standard is shown in Fig. 7. The calibration curve of microcystin-LR was constructed with 7 different concentrations of microcystin-LR, which ranged between 0.1-10 $\mu g.mL^{-1}$. The curve was linear with a correlation coefficient of 0.9837, as shown in Fig. 8. Fig. 9 shows the chromatogram resulting after injection of a standard mixture containing microcystin-RR, -YR and –LR (3.33 $\mu g.mL^{-1}$ for each toxin, 20 µL injection volume) and the retention time at 4.9, 9.7 and 11.8 min, respectively.

Cyanobacterial extract from the surface bloom samples and the laboratory cultures were rapidly separated by reversed-phase HPLC in 12 min. There were several peaks detected in the surface bloom extract. However, the main component was microcystin-LR, and its peak showed a retention time of 11.8 min, whilst microcystin-RR was also found in a small amount at the retention time of 4.9 min (Fig. 10). The microcystin-LR spiked sample was also operated to ensure that the peak at retention time of 11.8 min was exactly the same as microcystin-LR (Fig. 11). The quantity of microcystin-LR found in the surface bloom sample was 140 $\mu g.g^{-1}$ of dried cells. The purified microcystin-LR isolated from the surface cyanobacterial extract had almost

100% purity (Fig. 12), and was pooled for a confirmed identification by LC-MS-MS and the cytotoxicity test.

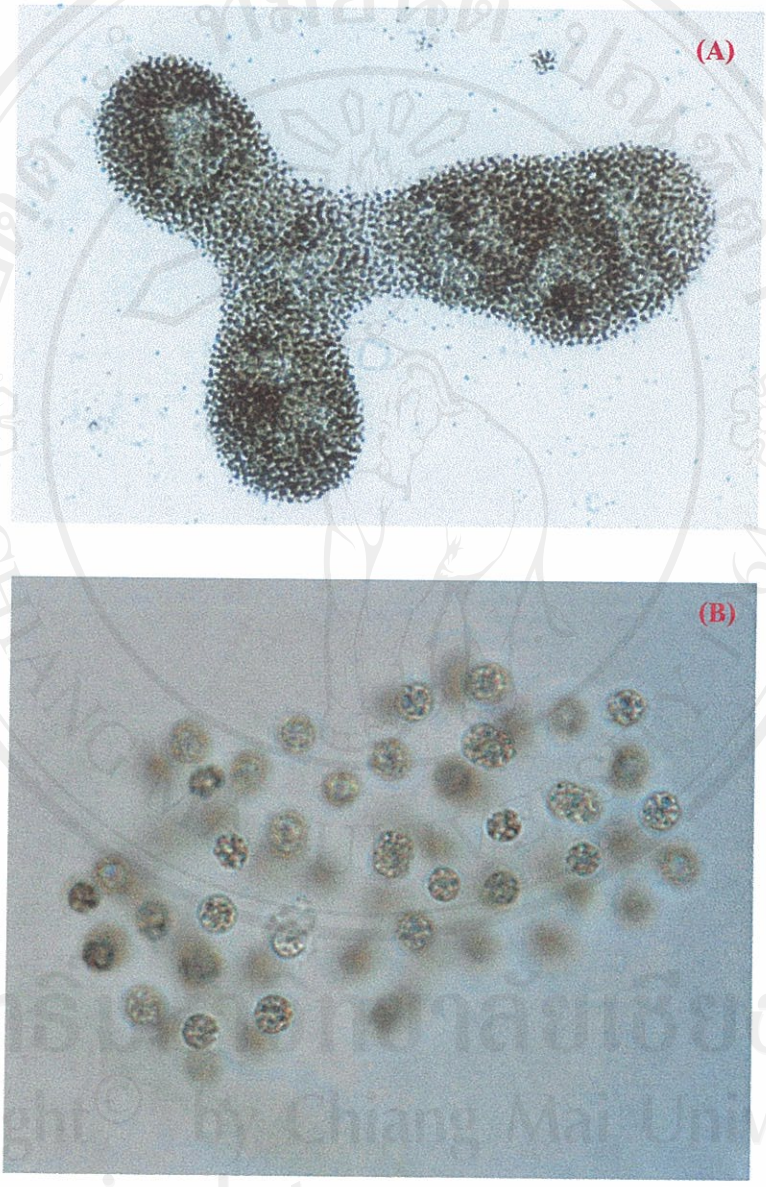

Figure 6 *Microcystis aeruginosa* Kütz. collected from Huay Yuak reservoir, Chiang Mai in April 2003. (A) A lobated colony covered with a gelatinous sheath (400X) and (B) cells contained many gas vacuoles (1000X).

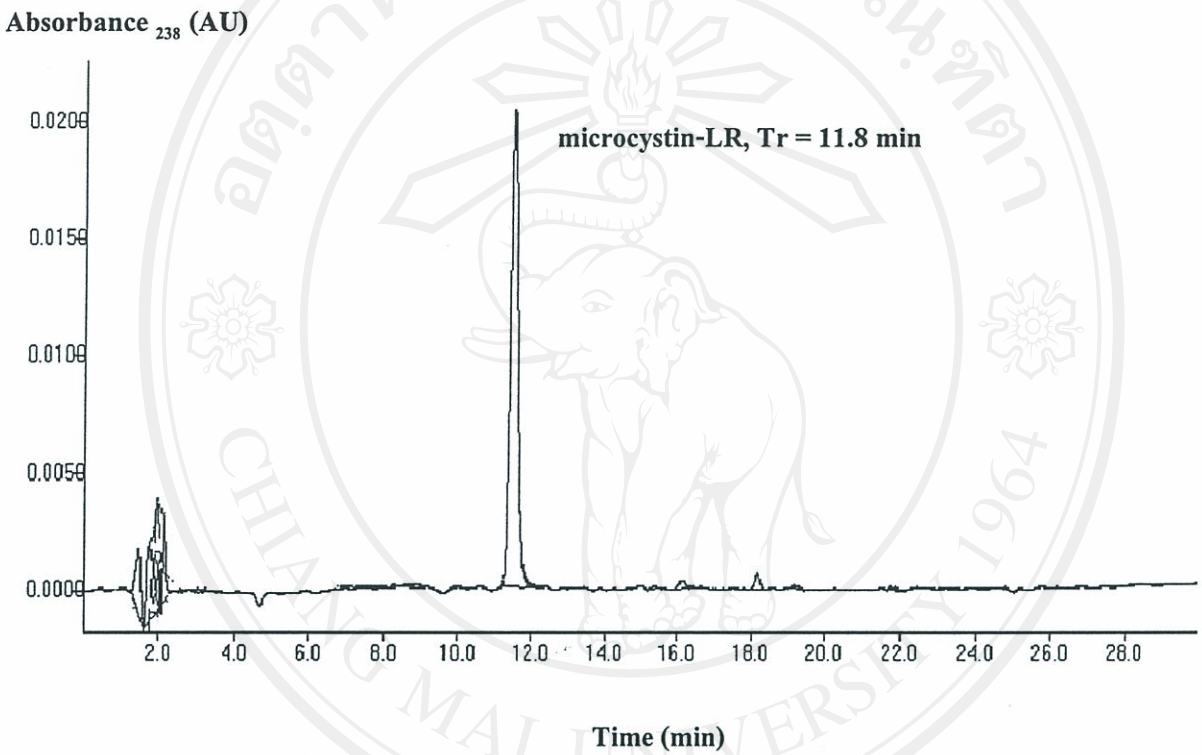

Figure 7 A typical HPLC-UV chromatogram of microcystin-LR standard, determined at 238 nm using a Mightysil RP-18GP column and linear gradient elution of 30-70% aqueous acetonitrile (0.05% TFA). The retention time of the microcystin-LR standard was at 11.8 min.

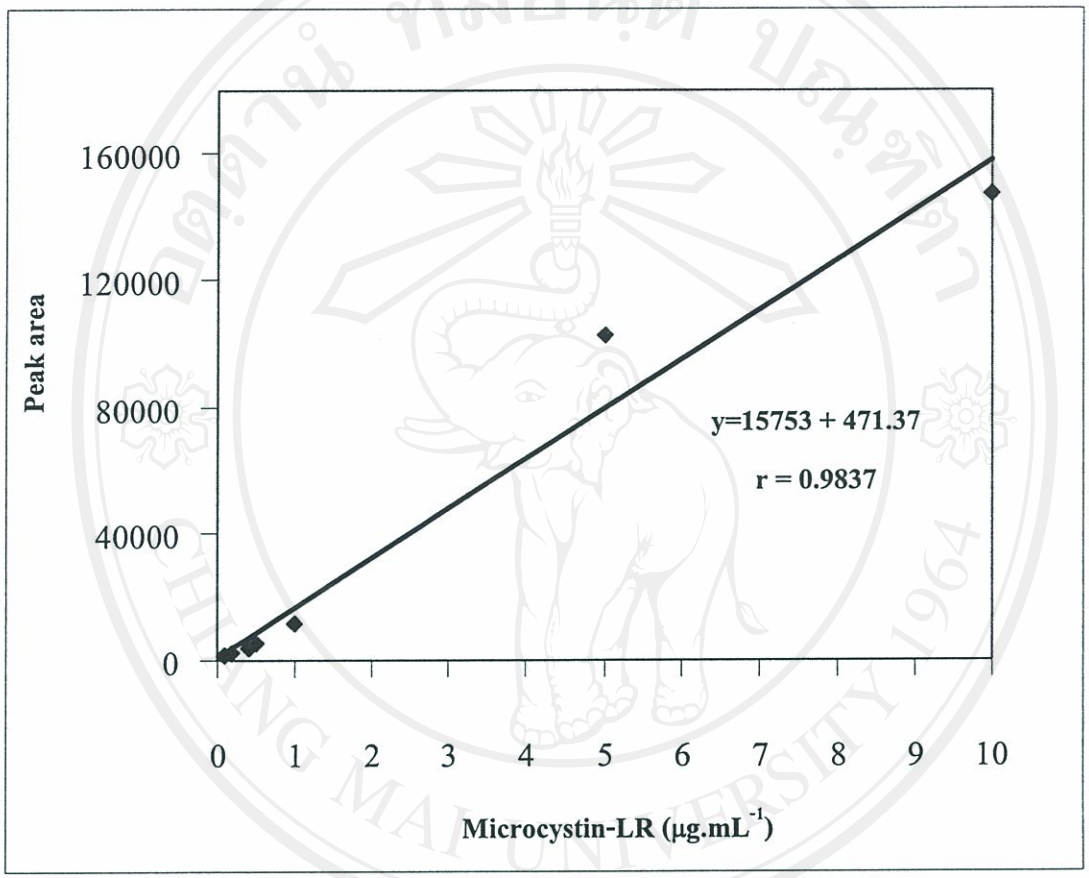

Figure 8 The calibration curve of microcystin-LR standard, plotted with concentrations that ranged between 0.1-10 $\mu g.mL^{-1}$ and the peak area (r = 0.9837).

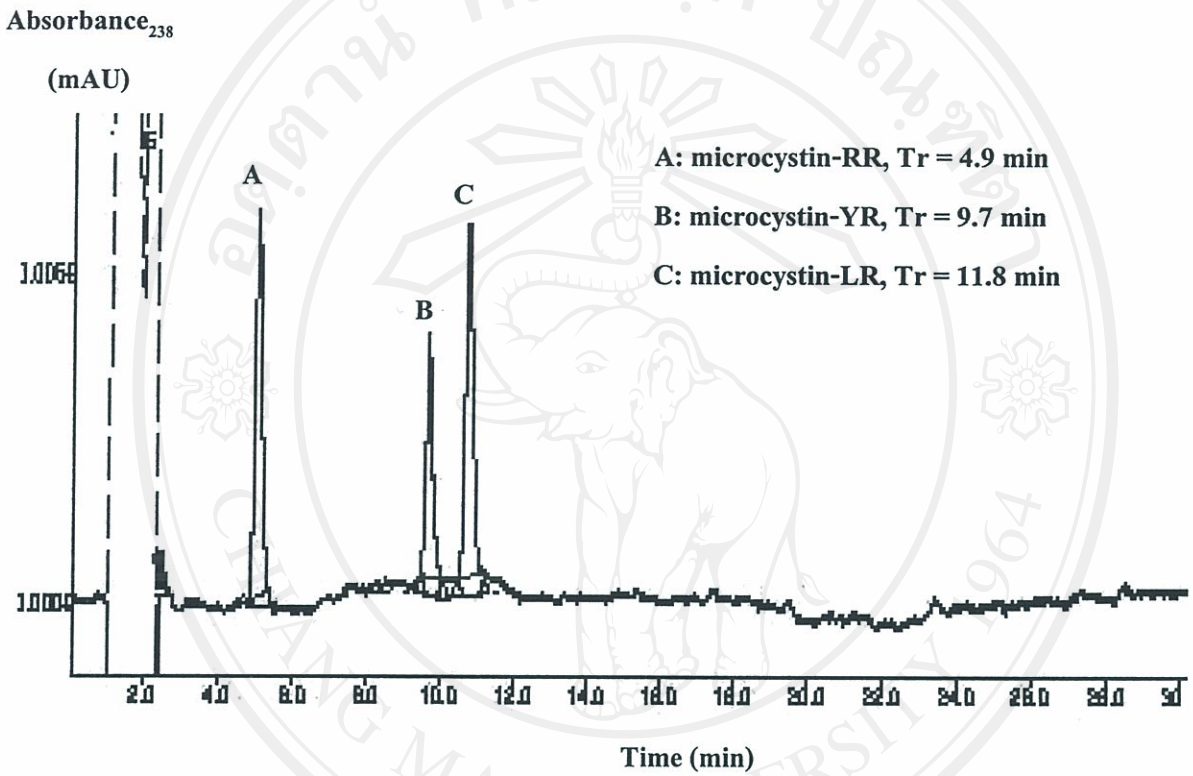

Figure 9 A typical HPLC-UV chromatogram of the standard microcystin mixture. The retention time of standard microcystin-RR (A), -YR (B) and –LR (C) was at 4.9, 9.7 and 11.8 min, respectively.

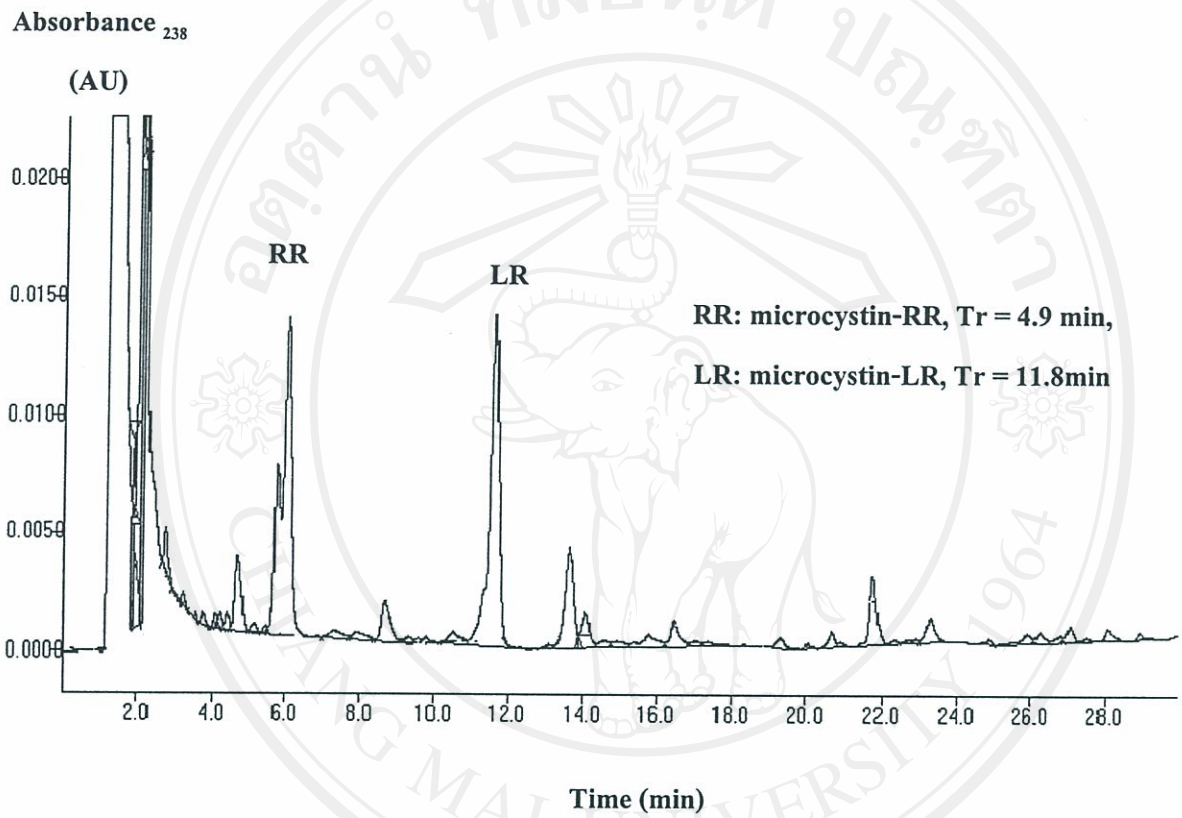

Figure 10 A typical HPLC chromatogram of cyanobacterial extract from a surface bloom sample. The retention time of microcystin-RR and –LR was at 4.9 and 11.8 min, respectively.

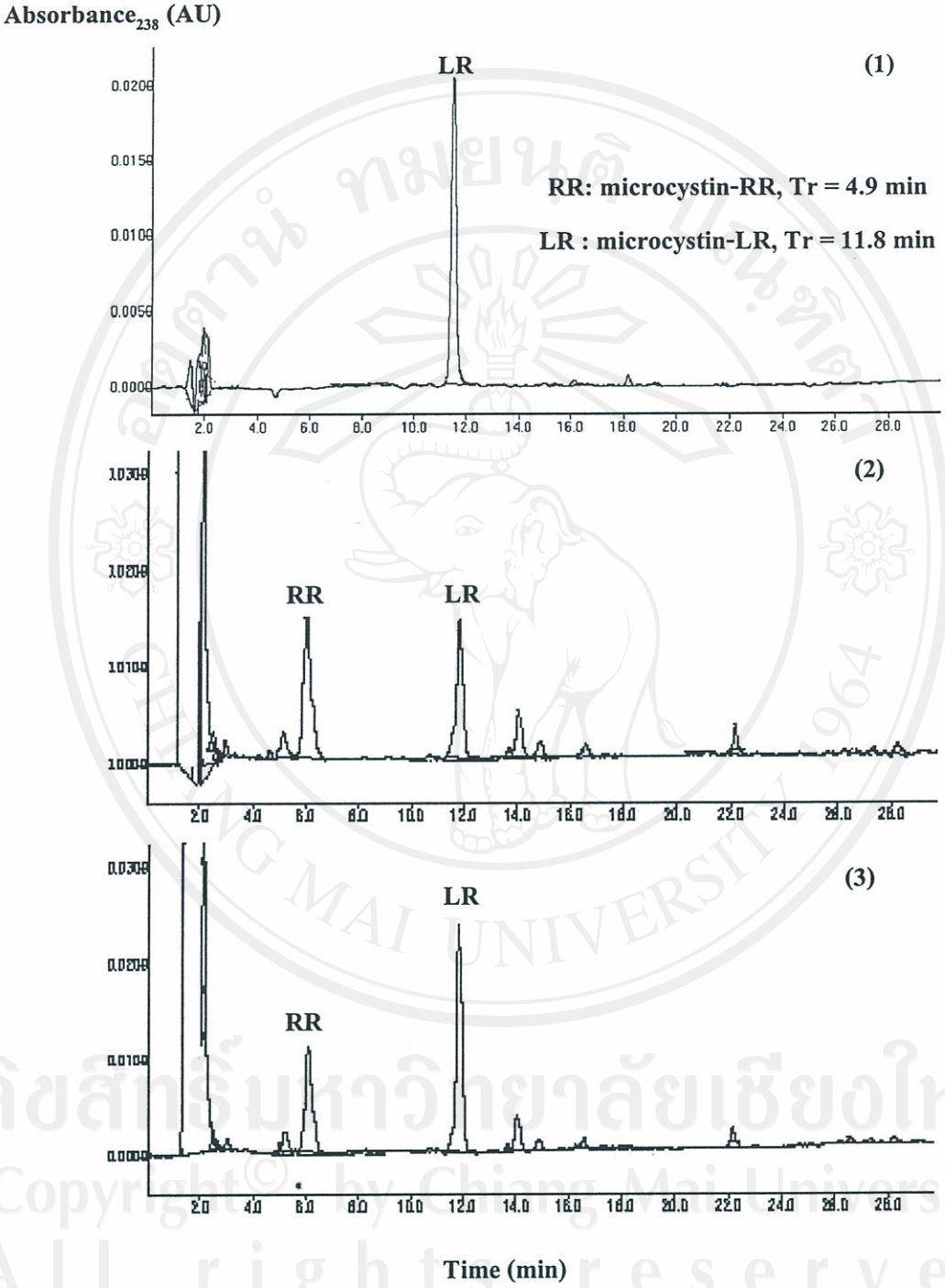

Figure 11 HPLC separation profiles of microcystin-LR: (1) standard mircoystin-LR; (2) the surface bloom sample; (3) the spiked sample.

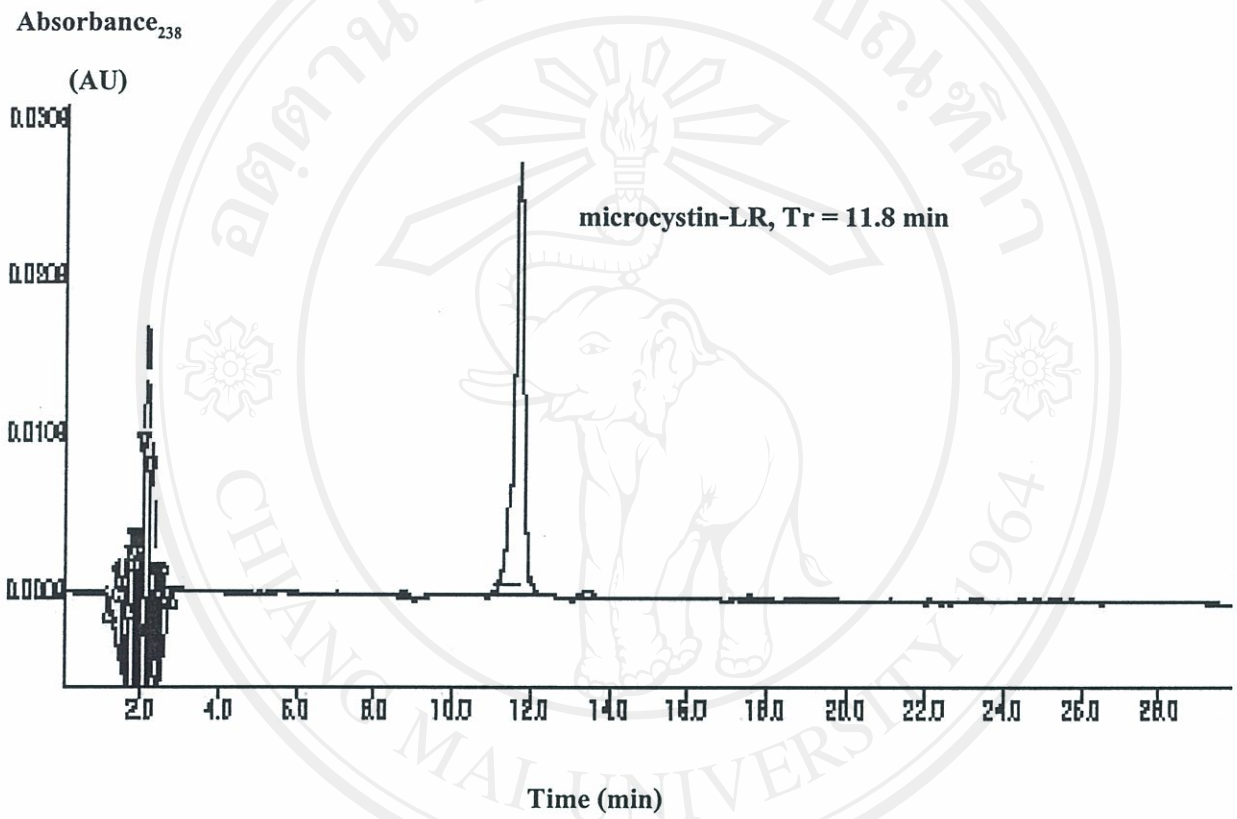

Figure 12 HPLC chromatogram of purified microcystin-LR isolated from surface cyanobacterial blooms extract. The retention time of purified microcystin-LR was at 11.8 min.

Microcystin-LR concentrations in the algae cells and the culture media

M. aeruginosa cells collected from Huay Yuak reservoir were grown in the 10 L batch-culture MA medium vessel for one month as described in Chapter II. The cultures were harvested and quantitated every 2 days for microcystin-LR concentrations in the algae cells and culture media. The growth phase of the *M. aeruginosa* culture was determined by cell counting at each sampling time. The growth curve of *M. aeruginosa* in the MA medium is shown in Fig. 13.

There were several peaks detected in the extract from the algae cells and culture media by HPLC-UV. However, the major component was microcystin-LR at the retention time of 10.8 min, and the unknown peak was at the retention time of 19.1 min (Fig. 14).

The retention time of microcystin-LR in the cyanobacterial cultures shifted from 11.8 min to 10.8 min. To ensure this peak was exactly the same as microcystin-LR, the microcystin-LR standard was scanned to investigate the absorption spectra at 190 to 367 nm. The absorption spectrum of microcystin-LR demonstrated characteristics between spectra within the range of absorbance at 220 to 330 nm. The pattern of expected microcystin-LR (Tr = 10.8) from the algae cells was similar to the spectrum of microcystin-LR standard (Fig. 15).

The results are illustrated in Table 2 and Fig. 16. The high concentration of microcystin-LR in the algae cells (day 2) was found at the initial sampling extract (258.62 μg. g^{-1} of wet cells) and decreased to 173.15 μg.g^{-1} of wet cells at the last sampling extract (day 30). In contrast, microcystin-LR in the culture media had a very low concentration (0.81 μg.mL^{-1}) at day 2, but increased to 10.46 μg.mL^{-1} at day 30. Therefore, the intracellular microcystin-LR concentrations in the algae cells correlated negatively with the extracellular microcystin-LR concentrations in the culture media.

Comparison between the cell numbers and the microcystin-LR concentration in the algae cells is shown in Fig.17. Initially, the algae cells grew rapidly in the logarithmic growth phase (day 2-12), while the microcystin-LR was mostly contained in the cells. After day 12, the cells reached the stationary growth phase and the reduction of intracellular microcystin-LR was found.

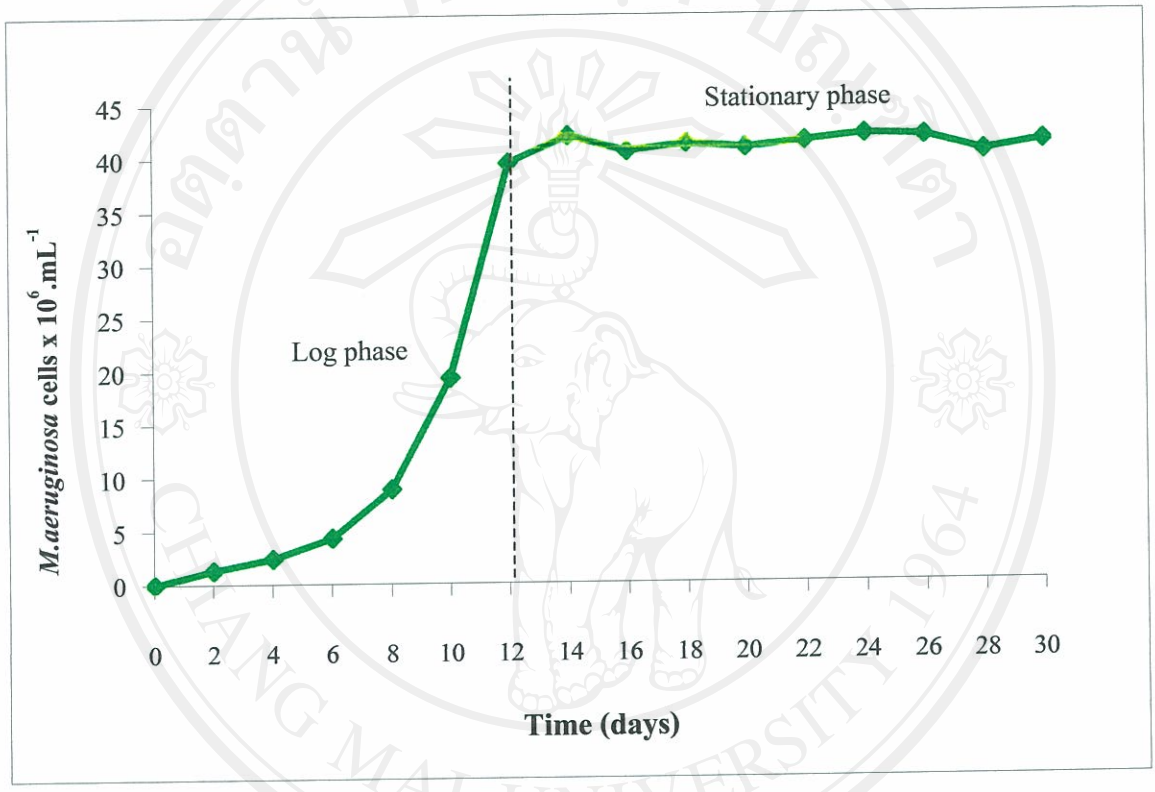

Figure 13 The growth curve of *M. aeruginosa* grown in the 10 L batch-culture MA medium vessel showing the logarithmic and stationary growth phase during 1 month of harvested *M. aeruginosa* cultures.

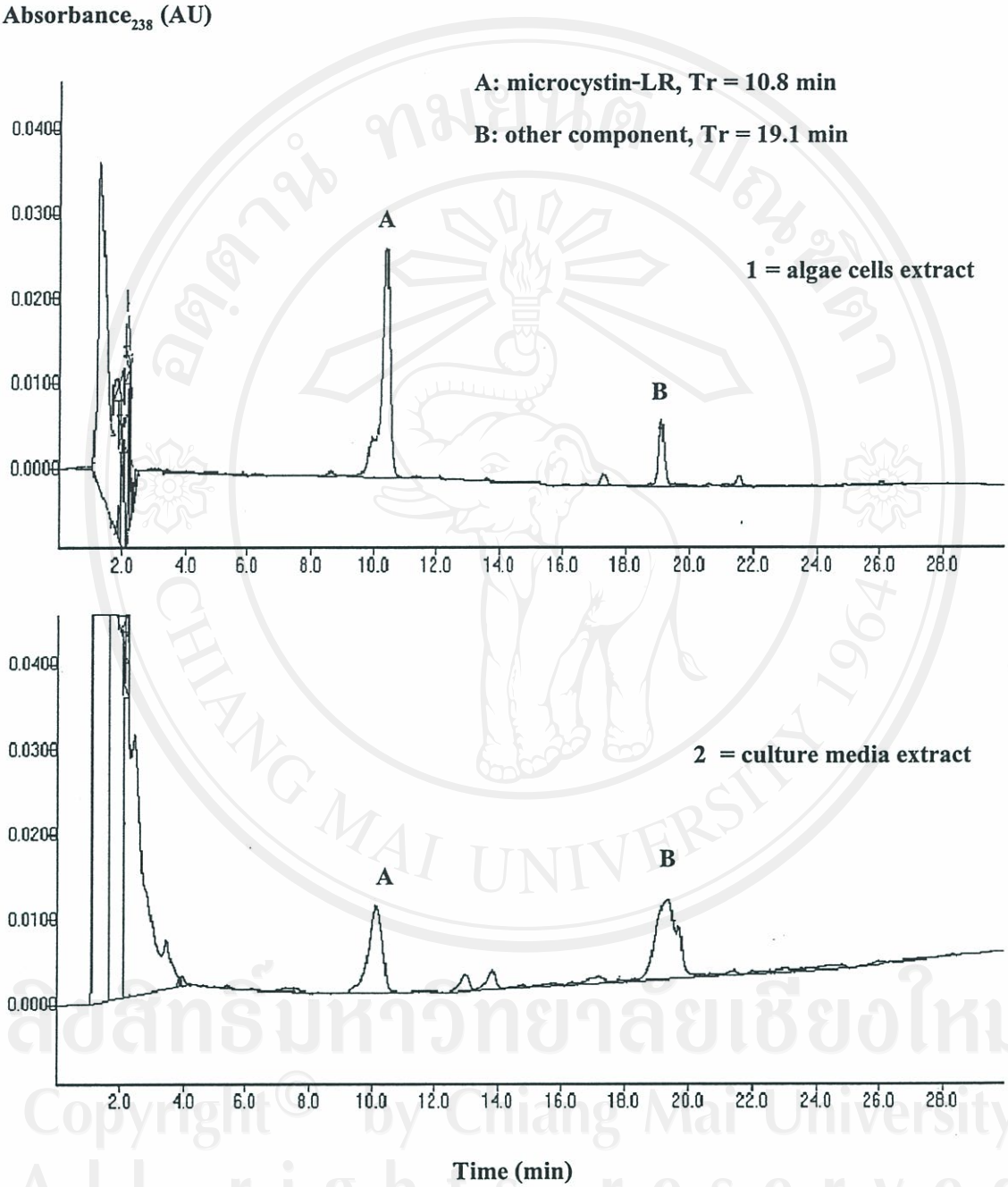

Figure 14 Typical HPLC chromatograms of extracted algae cells (1) comparison to the chromatogram detected culture media extracted from *M. aeruginosa* cultures (2).

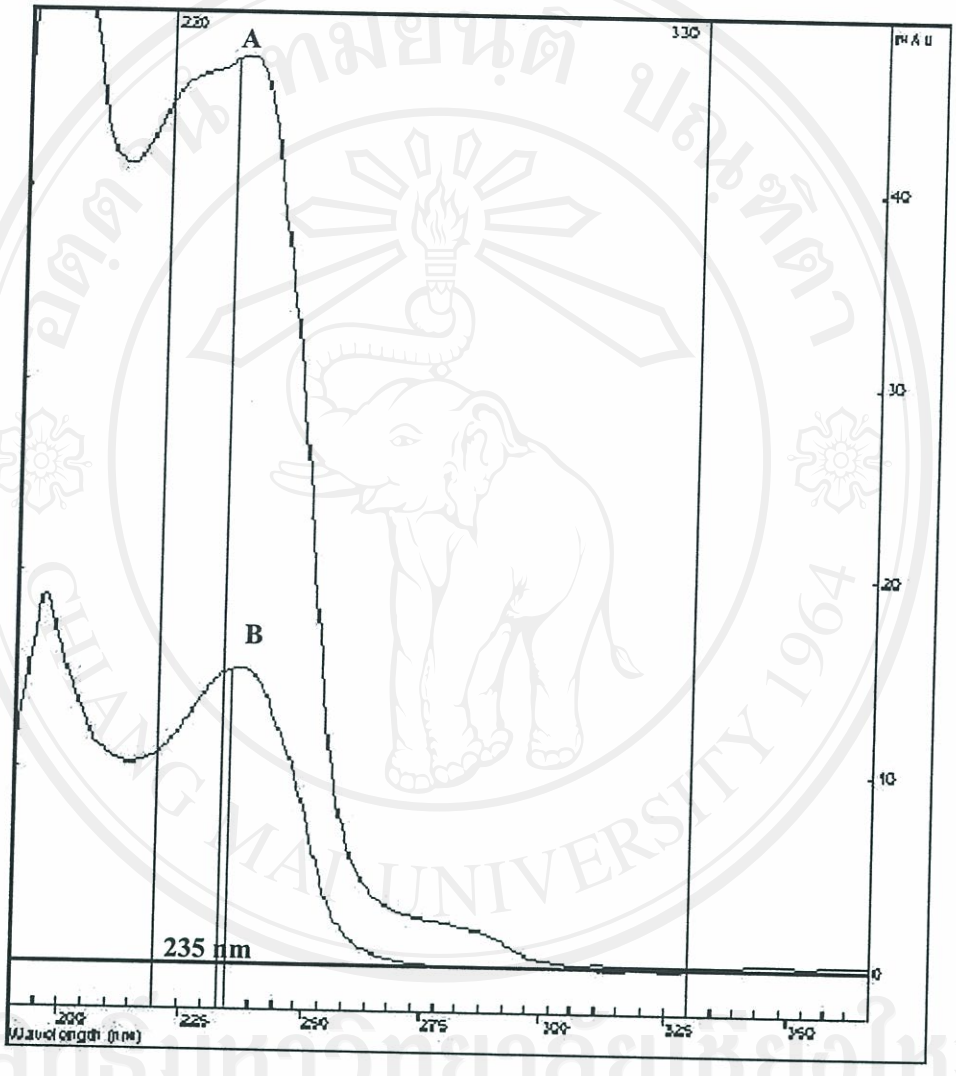

Figure 15 The absorption spectra of microcystin-LR scanning with an HPLC-Diode Array detector.

A = microcystin-LR in cyanobacterial extract from the algae cells

B = microcystin-LR standard.

Table 2 The concentrations of microcystin-LR in the algae cells and culture media obtained from laboratory *M. aeruginosa* cultures

Sampling time (days)	Microcystin-LR	
	Algae cells ($\mu g.g^{-1}$)	Culture media ($\mu g.mL^{-1}$)
2	258.62	0.81
4	435.9	0.67
6	298.12	3.37
8	317.6	2.95
10	353	1.87
12	458.59	3.78
14	425	7.37
16	158.85	10.35
18	188.57	9.25
20	212.82	8.81
22	161.36	8.31
24	120.09	7.62
26	138.61	8.54
28	141.4	9.17
30	173.15	10.46

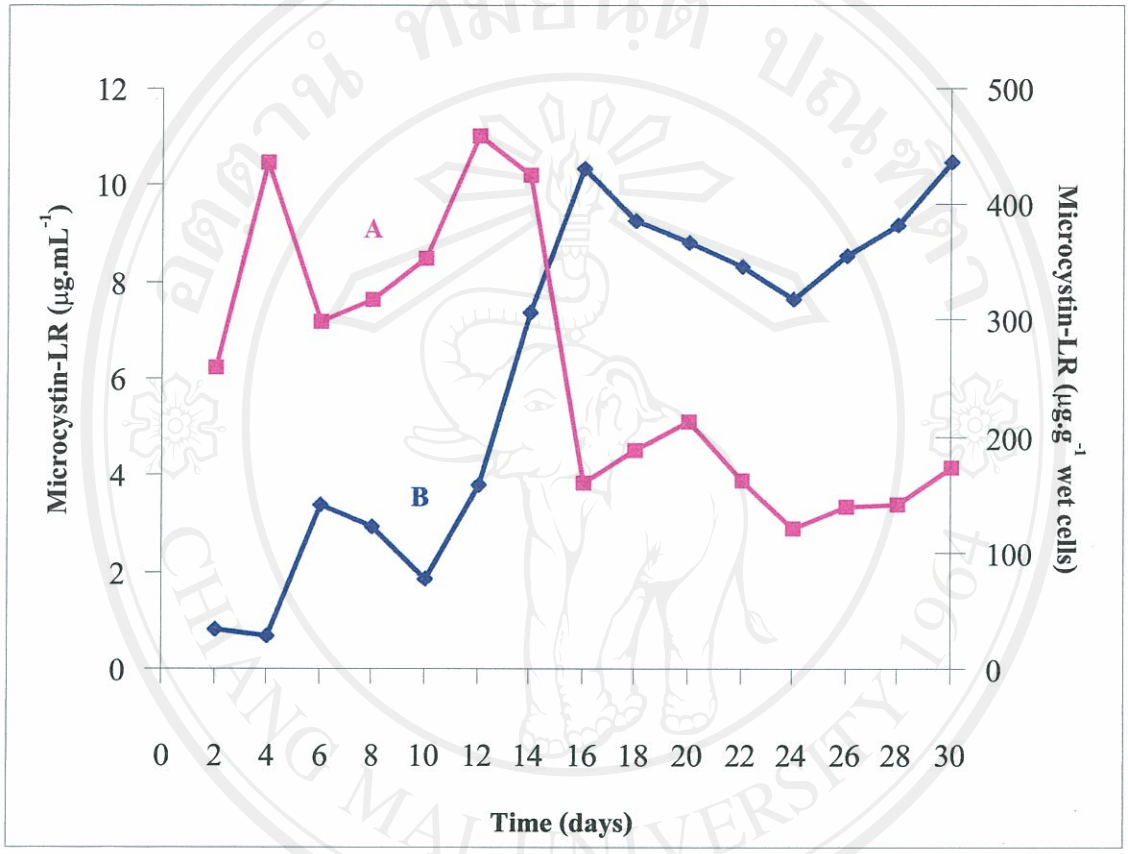

Figure 16 Relationship of microcystin-LR concentrations between in the algae cells (A) and culture media (B) from laboratory *M. aeruginosa* cultures.

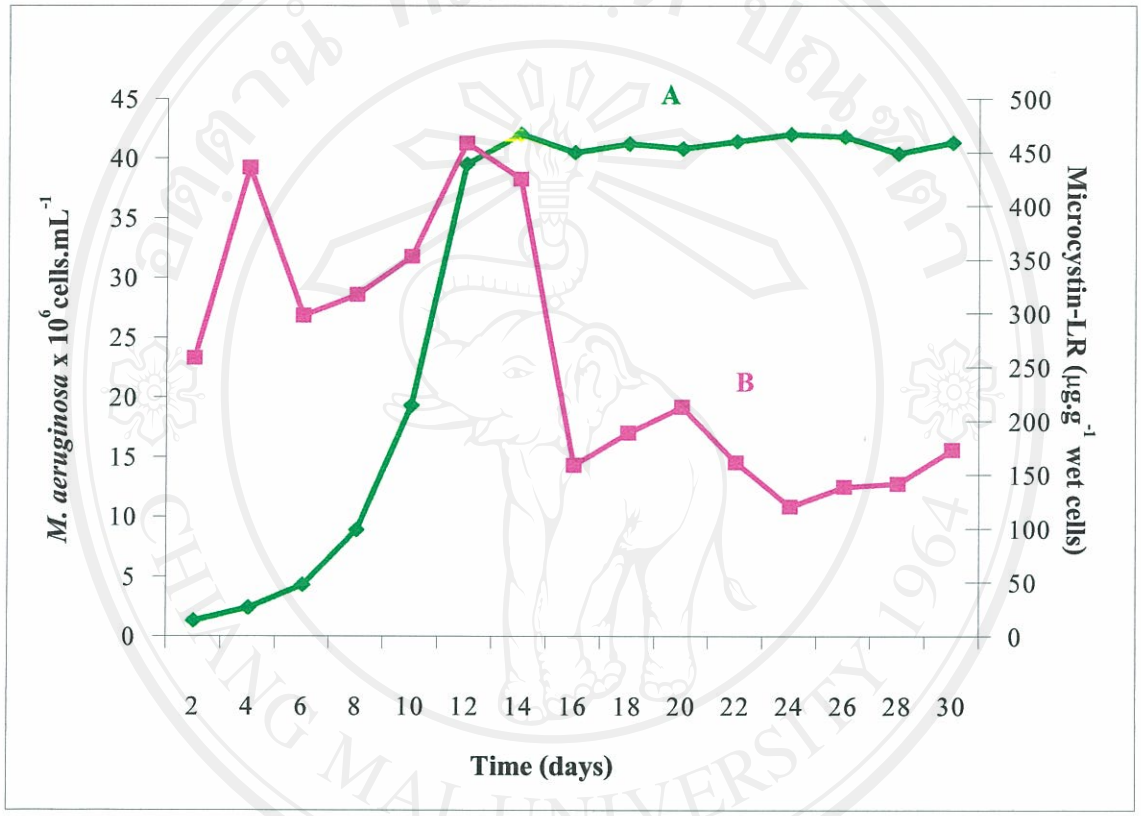

Figure 17 Relationship between the *M. aeruginosa* cell numbers (A) and microcystin-LR concentrations in the algae cells (B) from laboratory *M. aeruginosa* cultures.

LC-MS-MS Identification of microcystin-LR

The mass spectrum of microcystin-LR analyzed by the LC-MS-MS in positive scan mode for a mass range from m/z = 900-1100 revealed a molecular mass of 995 (Fig. 18), which was fully compatible with the standard microcystin-LR MS-MS spectrum (Fig. 19). The typical microcystin-LR fragment ions were presented at m/z 135 and 163 (Fig. 20), which corresponded to the Adda group of microcystin-LR.

Cytotoxicity of microcystin-LR on primary cultured rat hepatocytes

The purified microcystin-LR was collected from HPLC and pooled for the cytotoxicity test. Fig. 21 and 22 show the survival curve of primary cultured rat hepatocytes incubated with microcystin-LR concentrations that ranged between 0-125 $ng.mL^{-1}$ for 24 h. It revealed the prominent dose-dependent curve of the microcystin-LR and the primary cultured rat hepatotoxicity. The IC50 calculated from these curves was 10.34 $ng.mL^{-1}$.

Conclusion

The analysis of the microcystins produced by *M. aeruginosa* found in Huay Yuak reservoir, Chiang Mai province revealed that the most dominant cyanobacterial toxin was microcystin-LR. This result agreed with the MS-MS spectrum of the microcystin-LR standard. However, the quantity of the microcystin-LR was low. It was 0.14 $mg.g^{-1}$ of dried cells. The intracellular microcystin-LR concentrations in the algae cells correlated negatively to the extracellular microcystin-LR concentrations in the culture media. The isolated microcystin-LR had a cytotoxic effect on primary cultured rat hepatocytes with IC50 of 10.34 $ng.mL^{-1}$ at 24 h incubation time.

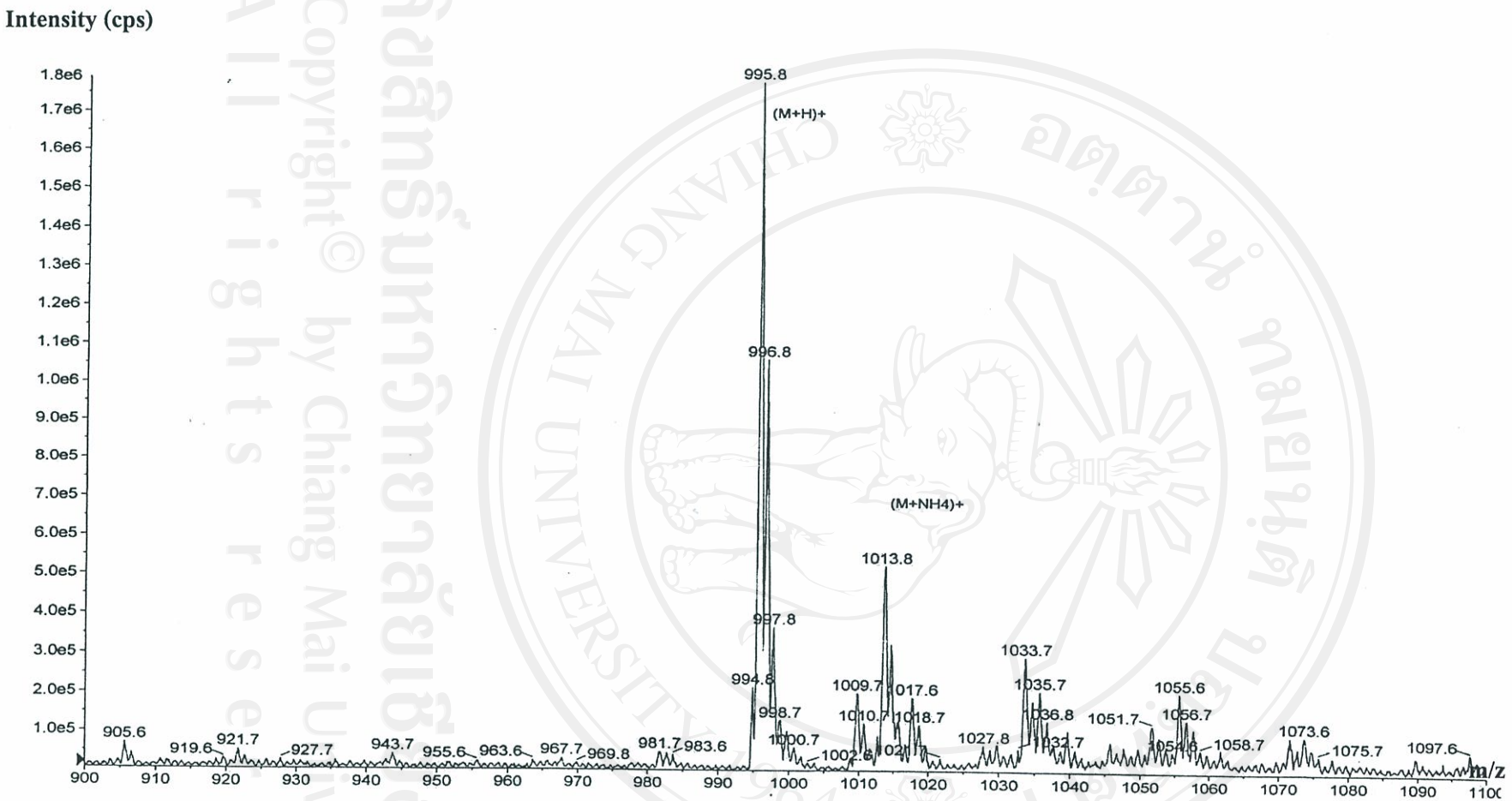

Figure 18 Mass spectrum of the purified microcystin-LR isolated from Huay Yuak *M. aeruginosa* cell extract identified by LC-MS-MS.

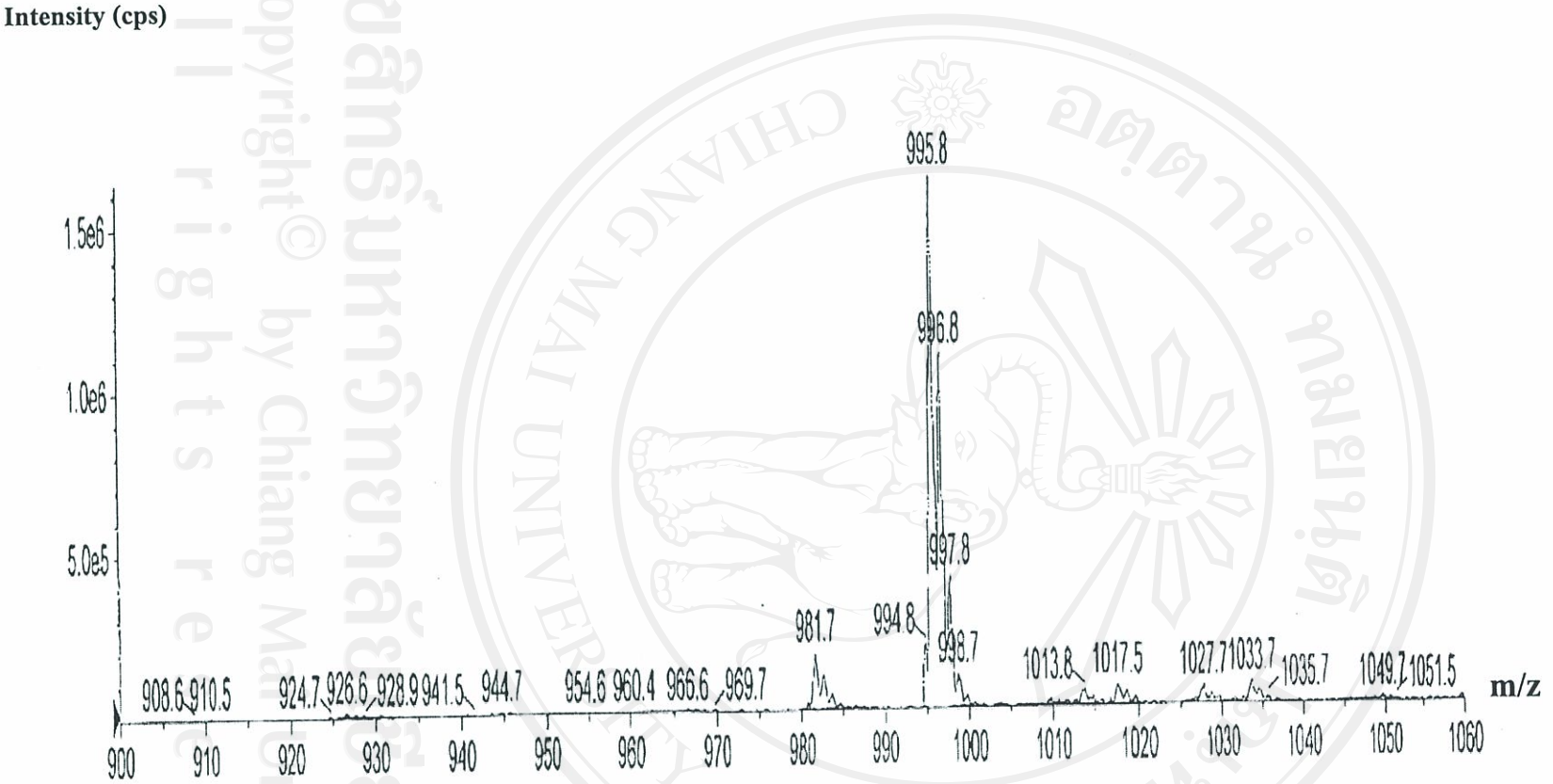

Figure 19 Mass spectrum of the standard microcystin-LR identified by LC-MS-MS showing the molecular ion at m/z 995.8.

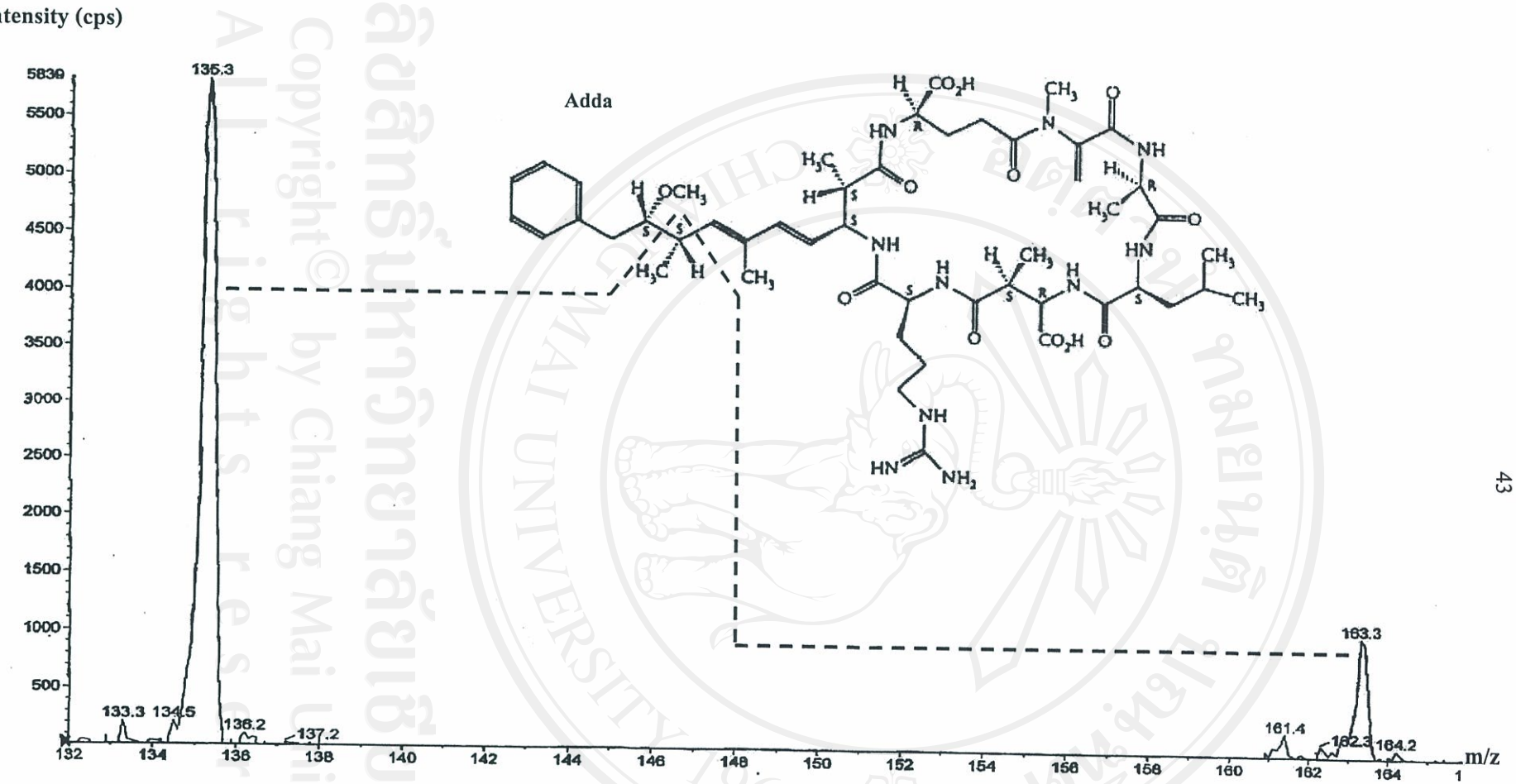

Figure 20 Mass spectrum obtained from the fragmentation of microcystin-LR showing the molecular ion at m/z 135 and 163.

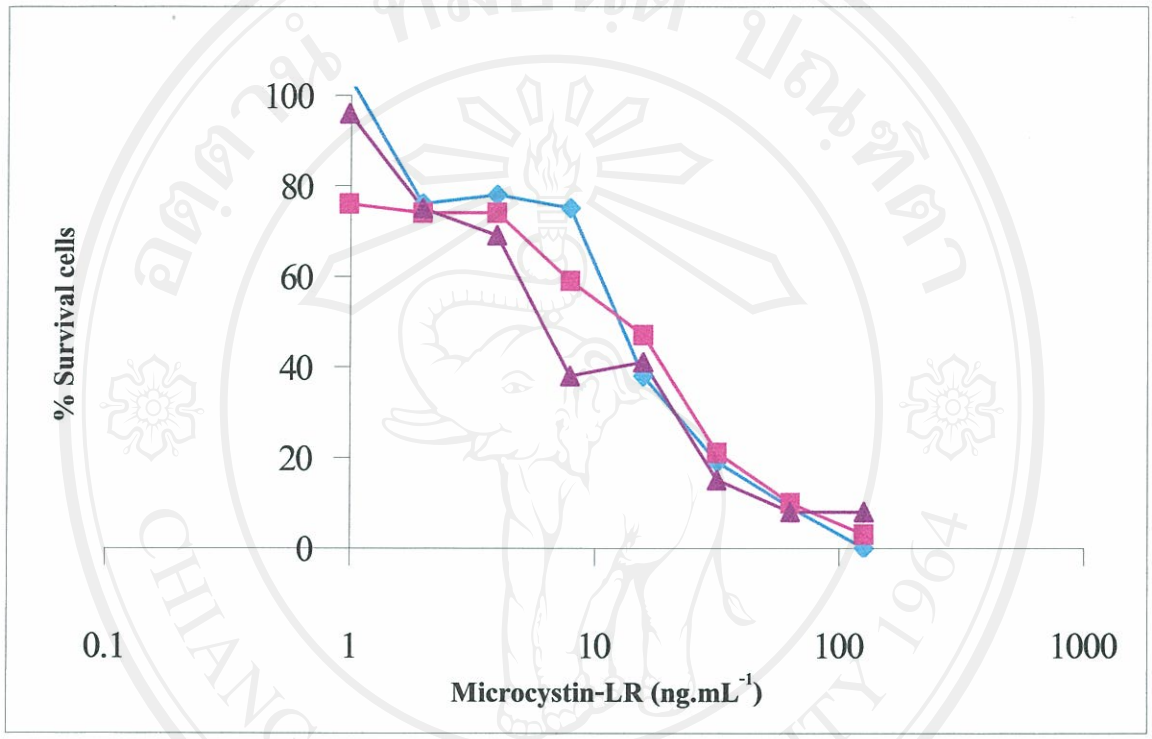

Figure 21 Survival curve of primary cultured rat hepatocytes incubated with different concentrations of purified microcystin-LR (0-125 ng.mL^{-1}) for 24 h. Values are in triplicate measurements.

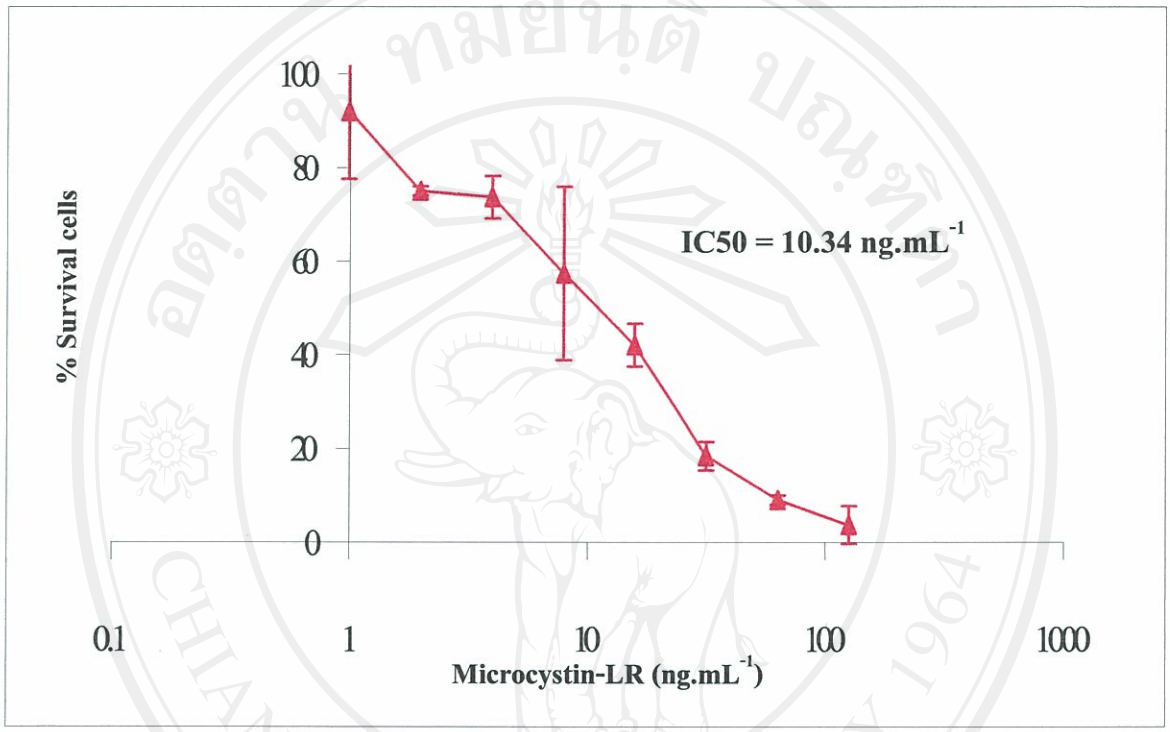

Figure 22 Survival curve of primary cultured rat hepatocytes incubated with different concentrations of purified microcystin-LR (0-125 ng.mL^{-1}) for 24 h. Values are the mean of triplicate measurements.