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# First Identification of the Hepatotoxic Microcystins in the Serum of a Chronically Exposed Human Population Together with Indication of Hepatocellular Damage

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**Hepatotoxic microcystins (MCs) are the most commonly reported cyanotoxins in eutrophic freshwaters. In 1996, human intoxications by MCs caused deaths of 76 patients at Caruaru dialysis centers in Brazil. So far, there have been no direct evidences of MC occurrence in human tissue in consequence of exposure to MC. In this study, we improved cleanup procedures for detecting MCs in serum sample using liquid chromatography-mass spectrometry, and confirmed for the first time the presence of MCs in serum samples (average 0.39 ng/ml, which amounts to ca. 1/87 of the concentrations found in tissue samples of the Caruaru victims) of fishermen at Lake Chaohu. Daily intake by the fishermen was estimated to be in the range of 2.2–3.9 μg MC-LReq, whereas the provisional World Health Organization tolerable daily intake (TDI) for daily lifetime exposure is 0.04 μg/kg or 2–3 μg per person. Moreover, statistical analysis showed closer positive relationships between MC serum concentrations and concentrations of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase than between the MC concentrations and other biochemical indicators. Thus, the data raise the question whether extended exposure in the range of the TDI or up to a factor of 10 above it may already lead to indication of liver damage. The results also demonstrate a risk of health effects from chronic exposure to MCs at least for populations with high levels of exposure, like these fishermen.**

*Key Words:* **serum microcystins; serum biochemical indices; natural chronic exposure; fishermen; hepatocellular damage.**

In the past decades, increasing eutrophication has led to frequent outbreaks of cyanobacterial blooms in many freshwater lakes of the world, and in China, dense cyanobacterial blooms have occurred regularly in several large lakes (Lake Chaohu, Lake Taihu, and Lake Dianchi) in the warm seasons of each year (Xie, 2007). Such cyanobacteria (e.g., *Microcystis*, *Anabaena*) are causing serious environmental problems because they are able to produce a series of natural toxins (cyanotoxins), among which microcystins (MCs) are the most common and well studied (Chorus and Bartram, 1999). MCs, a family of cyclic heptapeptides, are called hepatotoxins because liver of animals is their primary target. Chronic animal experimentation suggests that MCs are tumor promoters (Falconer and Humpage, 1996) with possible carcinogens (Grosse *et al.*, 2006; Ito *et al.*, 1997).

The first animal intoxication episode caused by toxic cyanobacteria was documented more than 100 years ago (Francis, 1878). Although, human poisonings have, in the past, been suspected but not confirmed due to a lack of information regarding markers for exposure that would confirm the presence of cyanotoxins in human food or water supplies, plus a shortage of appropriate methods of detection (Chorus and Bartram, 1999). So the well-substantiated case reports of MCs-caused human toxicosis are rare, but anecdotal evidence includes gastrointestinal disturbance and respiratory and allergic reactions. Ueno *et al.* (1996) report that primary liver cancer in southeast of China is related with MCs concentration in drinking water. However, the direct linkage of the human hepatocellular carcinoma with MCs is difficult because of a lack of extensive epidemiological data regarding routes and circumstances of exposure (Chorus and Bartram, 1999). In 1996, an episode of human intoxications by MCs was first confirmed after an outbreak of acute liver failure that resulted in the deaths of 76 patients at two dialysis centers in Caruaru, Brazil (Azevedo *et al.*, 2002; Carmichael *et al.*, 2001; Jochimsen *et al.*, 1998; Pouria *et al.*, 1998).

In spite of great efforts in developing various techniques to analyze MCs in cyanobacteria and water, we still face challenging problems in detection and quantification of MCs in animal tissue samples: low concentration of target compounds, complex matrix due to high amounts of protein and other macromolecules, and covalent binding of the target compounds with proteins (Hilborn *et al.*, 2005; Soares *et al.*, 2006; Yuan *et al.*, 2006). A comprehensive review of the limited studies on human exposure to MCs (Azevedo *et al.*, 2002; Carmichael *et al.*, 2001; Hilborn *et al.*, 2005; Jochimsen

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*et al*., 1998; Pouria *et al*., 1998; Soares *et al*., 2006; Yuan *et al*., 2006) illustrates that the quantitative analysis of MCs in human serum still faces some technique difficulty, for example, several noise peaks were observed in the tandem mass chromatography (MS/MS) spectrum for the human serum samples, and that intensity of the fragment ions also had poor reproducibility compared with the pure standard compound (Yuan *et al*., 2006).

Toxic blooms may recur periodically in surface drinking and recreational water sources, and people may be exposed to cyanotoxins in a chronic manner at a relatively low dose. Nevertheless, so far there has been still no published study of MCs detection in human serum samples under natural chronic exposure situations, which prevents direct scientific evaluation on the healthy risks of humans frequently exposed to toxic cyanobacterial blooms. For these reasons, we targeted improvement of extraction and clean-up procedures to allow detection of trace analysis of MCs using electrospray ionization (ESI) liquid chromatography-mass spectrometry (LC/MS) in serum samples of fishermen, the high cyanotoxin exposure population, and investigated relationships between biochemical indices and MC contents in serum samples. Our main goal was to investigate—for the first time—direct evidence of the occurrence of MC in human serum in conjunction with serum enzyme levels whose elevation would indicate liver damage.

## MATERIALS AND METHODS

***Sampling site and study population.*** Lake Chaohu, located in Anhui Province in the southeastern China, is among the five largest freshwater lakes in China. It is a subtropical lake with a surface area of 760 $km^2$, a mean depth of 3.06 m, and a mean retention time of 136 days. During the past decades, the lake has witnessed a steady increase in eutrophication, characteristic of a regular occurrence of cyanobacterial surface blooms (mainly composed of *Microcystis* spp. and *Anabaena* sp.) in the warm seasons of each year (Deng, 2004).

From July 15 to July 24, 2005, we randomly chose 35 fishermen (14 [40%] males and 21 [60%] females) who were willing to cooperate with us for this study. Of these cases, 27 (77%) were living on the lake more than 10 years, whereas the rest (8, 23%) were living on the lake between 5 and 10 years. Their age, body height, body weight, anamnesis, and dwelling place were also collected.

***Serum biochemical and immunological indices.*** Venipuncture system was used by the trained nurse of The First National Hospital of Chaohu City to collect blood samples from the fishermen. Approximately 10 ml of blood was drawn per subject and left to clot. The clotted blood was centrifuged at 10°C for 15 min at 2000 × g to yield serum. Then the serum samples were stored and transported to the hospital immediately for analyses. Serum determinations were performed by Synchron Clinical System LX20 (Beckman-Coulter Diagnosis, Fullerton, CA). The following parameters were measured: alanine aminotransferase (ALT), aspartate aminotransferase (AST), ST/LT (AST/ALT), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), lactate dehydrogenase (LDH), cholinesterase (CHE) activity; total bile acids (TBA), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL); total protein (TP), albumin (ALB), globulin (GLB), A/G; blood urea nitrogen (BUN), creatinine (CREA), uric acid (UA); glucose (GLU), total cholesterol (TC), and triglyceride (TG).

Blood samples were also tested for serum hepatitis Bs antigen (HBsAg), hepatitis Bs antibody (HBsAb), hepatitis Be antigen (HBeAg), hepatitis Be antibody (HBeAb), and hepatitis Bc antibody (HBcAb) by real-time fluorescence quantitative PCR to measure the HBV DNA level of the subjects. And alpha-fetaprotein (AFP) index was also tested by AFP enzyme-linked immunosorbent assay kit for monitoring the hepatocelluar carcinogenesis.

***Extraction of MCs from serum samples.*** Lyophilized serum samples (ca. 0.2 g DW) were homogenized and extracted twice with 10 ml of BuOH:MeOH:$H_2O$ (1:4:15) for 24 h while stirring. The extracts were centrifuged at 36290 × g for 30 min (BR4, Jouan, Winchester, VA, France) at 4°C, and the combined extracts from the supernatants were mixed three times with an equal volume of hexane. Hexane layers were discarded, and the mixed solvent extract was diluted with deionized water. This diluted extract was loaded onto an Oasis hydrophilic-lipophilic balance (HLB) solid phase extraction (SPE) cartridge (500 mg/6 ml Waters, Milford, MA). The HLB SPE cartridge was conditioned with 1 column volume of MeOH and 1 column volume of deionized water. The cartridge was washed (20 ml of 20% MeOH) and eluted with 20 ml of 100% MeOH. The eluant collected from the HLB cartridges was evaporated to dryness and resuspended in 5 ml of 100% MeOH. This solution was passed through a Sep-Pak silica gel cartridge (2 g/12 ml, Waters, Milford, MA) which had been preconditioned by 100% MeOH. The cartridge was washed with 20 ml of 100% MeOH and then eluted with 70% MeOH (20 ml), and the toxin-containing fraction was also evaporated to dryness. The residue was dissolved with 100% MeOH (1.0 ml) and centrifuged at 10,000 rpm for 3.5 h through an YM-10 (Millipore, Bedford, MA) molecular weight cutoff filter. Finally, the extract was evaporated to dryness and redissolved in 100 μl of deionized water and transferred to high-performance liquid chromatography (HPLC) autosampler vials. The aliquots (10 μl) were injected into the LC-MS system.

All reagents were of HPLC or analytical grade. Three standards of MC variants (MC-RR, MC-YR, and MC-LR, where R = arginine, Y = tyrosine, and L = leucine) used in the detection were purchased from Wako Pure Chemical Industries–Japan.

***LC/MS analyses of serum sample extracts.*** Qualitative and quantitative analysis of MCs in human serum samples followed the $LC/MS^2$ method described previously (Chen *et al*., 2007).

***MC recovery from serum.*** To validate the above procedure, recovery of MC-LR spiked into control human sera samples was tested. The $LC/MS^2$ method (Chen *et al*., 2007) was used for MCs detection following the extraction procedure.

***Collection and MC analysis of water sample, seston sample, and aquatic products.*** Water samples were collected from two sites of Zhongmiao area in Lake Chaohu with Tygon tubing fitted with a one-way valve. Each integrated water sample was a mixture of two subsamples: one from 0.5 m below the surface and one from 0.5 m above the bottom. MC in the lake samples was fractionated to intracellular and extracellular toxins. The intracellular toxins were extracted from cyanobacterial cells filtered from 1 l of lake water on the GF/C glass fiber filter (Whatman, Brentford, UK). The filtrate (1 l) was used to detect the extracellular toxins. The extraction method was followed Park *et al.* (1998).

During the sampling period, edible parts (muscle) of four species of crustaceans (*Procambarus clarkii*, *Macrobrachium nipponensis*, *Palaemon modestus*, *Eriocheir sinensis*), three species of mollusks (*Bellamya aeruginosa*, *Cristaria plicata*, *Lamprotula leai*), and nine species of fish (*Hypophthalmichthys molitrix*, *Aristichys nobilis*, *Neosalax taihuensis*, *Carassius auratus*, *Cyprinus carpio*, *Misgurnus anguillicaudatus*, *Ctenopharyngodon idellus*, *Pseudobargrus fulvidraco*, *Culter erythropterus*) were also collected for analysis of MCs.

The collected muscle or foot samples were separately washed carefully by distilled water to avoid cross contamination, and then were immediately frozen at −20°C in the field. In the laboratory, the collected organs were frozen at −80°C prior to MC analysis. Samples were lyophilized using an Alpha 2-4 Freeze Dryer (Martin Christ, German). Extraction and analysis of MCs in the organs (ca. 0.5 g DW) of the study animals followed the method of Chen *et al.* (2007).

***Principal component and classifying analysis.*** Data were entered into Microsoft Excel version 2000 (Microsoft Corporation, Redmond, WA) and imported into and analyzed with STATISTIC for Windows statistical software (Version 6.0, StatSoft Inc., Tulsa, OK). We used principal component and classifying analysis (PCCA) to perform multivariate analyses, so as to characterize the relationships between serum MC concentrations and serum biochemical variables. To ensure that all variables were given equal weight, the data were standardized prior to the analysis.

PCCA removes redundancy from original data set so that only the first few principal-components scores are required to describe most of the information contained in the original data set. PCCA transforms a number of (possibly) correlated variables into a smaller number of uncorrelated principal-components. The first principal-component accounts for the most variability in the data and each accessorial component axis accounts for as much of the remaining as possible.

## RESULTS

### *Identification and Level of MCs in the Serum of 35 Fishermen Living on Lake Chaohu*

The presence of MC-RR, MC-YR, and MC-LR in the serum sample was confirmed by ESI LC/$MS^2$. The retention time and the main fragment ions corresponded with those of MC-RR, MC-YR, and MC-LR standards. The reproducibility was not always good, as not all of the characteristic fragment ions were obtained in every scan, especially at very low concentrations. A spectrum for a serum extract from a subject using the LC/MS/MS scan mode is shown in Figures 1A–C. The following ions were used as fingerprint ions for MC-RR due to their good reproducibility, *m/z* 452: [Ala-Arg-MeAsp-Arg-Adda-Glu-Mdha − $PheCH_2CHOCH_3$ + 2H]$^{2+}$ and *m/z* 887: [Ala-Arg-MeAsp-Arg-Adda-Glu-Mdha − $PheCH_2CHOCH_3$ − $NH_3$ + H]$^+$. Similarly, MC-YR, *m/z* 1017: [Ala-Tyr-MeAsp-Arg-Adda-Glu-Mdha + H]$^+$ − CO and *m/z* 916: [Arg-Adda-Glu-Mdha-Ala-Tyr + H]$^+$. MC-LR, *m/z* 553: [Mdha-Ala-Leu-MeAsp-Arg + H]$^+$; *m/z* 599: [Arg-Adda-Glu + H]$^+$ or [MeAsp-Arg-Adda + H]$^+$ and *m/z* 866: [Arg-Adda-Mdha-Ala-MeAsp-Leu + H]$^+$. Whereas LC/$MS^2$ analyses of the control sera samples did not support the presence of MCs (Figs. 1D–F).

As we could only collect approximately 10 ml of blood sample from each subject, the amount of serum was very limited for MC detection. All of the 35 serum samples were positive for MCs (Fig. 2), although not all of the MCs (MC-RR, -YR, and -LR) were detectable in every sample. Values below the limit of detection (LOD) were taken as zero, and total MC concentrations ranged from 0.045 to 1.832 ng/ml, with MC-RR ranging from 0.000 to 0.685 ng/ml (mean = 0.162 ng/ml), MC-YR from 0.000 to 0.316 ng/ml (mean = 0.052 ng/ml), and MC-LR from 0.000 to 1.221 ng/ml (mean = 0.175 ng/ml). The mean and median values for the 35 serum samples were 0.389 and 0.227 ng/ml, respectively. No peak could be observed in the control sera samples ($n = 3$) at the same retention time and mass as the standard, indicating absence of MCs in these samples.

To confirm efficiency of the MCs extraction procedure, a recovery experiment with MC-LR (0.01 μg/g) spiked into control human sera samples was performed. LC/MS/MS was used for MCs detection. The results show that our extraction and cleanup procedures had good recovery—the average recovery of MC-LR in spiked sera samples ($n = 6$) was 84.7% (range 79.3–90.4%).

LOD for MCs was also calculated by using quality control samples with low concentrations of MC-RR and MC-LR, and the LOD value was 0.002 μg/g DW for both toxin variants.

### *MC Contamination in Drinking Water and Aquatic Food of the Fishermen*

In Lake Chaohu, in July, MC-RR, -YR, and -LR concentrations in seston were 3.74, 0.48, and 2.34 μg/l, respectively, and MC-RR, -YR, and -LR concentrations in cyanobacterial scum were 351.6, 62.0, and 128.3 μg/g, respectively. In other words, the toxin content was 3.28 μg MC-LReq/l in seston, and 0.223 mg MC-LReq/g DW in cyanobacterial scum.

MC contents in muscle of 16 species of aquatic animals are showed in Figure 3. On the average, MC-RR, -YR and -LR contents in the muscle of the 16 species were 42.1, 3.3, and 33.1 ng/g DW, respectively. In terms of MC-LReq, this equals to 42.8 ng/g DW. Proportions of MC-RR, -YR, and -LR were 53.6, 4.2, and 42.2%, respectively. No MC was detected for the Chinese mitten crab (*Eriocheir sinensis*), and MC in muscle of icefish (*Neosalax taihuensis*) was also very low, whereas the highest MC content was observed in the muscle of snail (*Bellamya aeruginosa*).

### *General Characteristics of Serum Immunological and Biochemical Indices of the Fishermen*

Only three (female, 8.6%) subjects were positive for hepatitis B. 2*, [HBsAg(+), HBeAg(+), HBcAb(+)], had a family history of hepatitis B, and both her mother and uncle were hepatitis B sufferers. 4* was [HBsAg(+), HBeAg(+), HBcAb(+)], and 35* was [HBsAg(+), HBeAb(+), HBcAb(+)]. The rest 32 (91.4%) individuals were negative for hepatitis B, among which, HBsAg, HBsAb, HBeAg, HBeAb and HBcAb indices were all "−" for 25 individuals and other seven individuals had antibody with HBsAb display "+".

AFP detection revealed no evidence of hepatocelluar carcinogenesis. The index in all of the study subjects was < 10 ng/ml.

CREA for eight individuals (22.9%, two males, six females) was 40.75 ± 1.9 μmol/l, whereas TG for nine individuals (25.7%, four males, five females) was 0.47 ± 0.09 mmol/l. And nine individuals (25.7%, six males, three females) had lower concentrations of GLB (21.89 ± 1.69 g/l), whereas six individuals (17.1%, four males, two females) had higher concentrations of TC (up to 5.94 ± 0.65 mmol/l). Other serum biochemical indices were within the normal ranges for most subjects, except for a small quantity of the study population (Table 1).

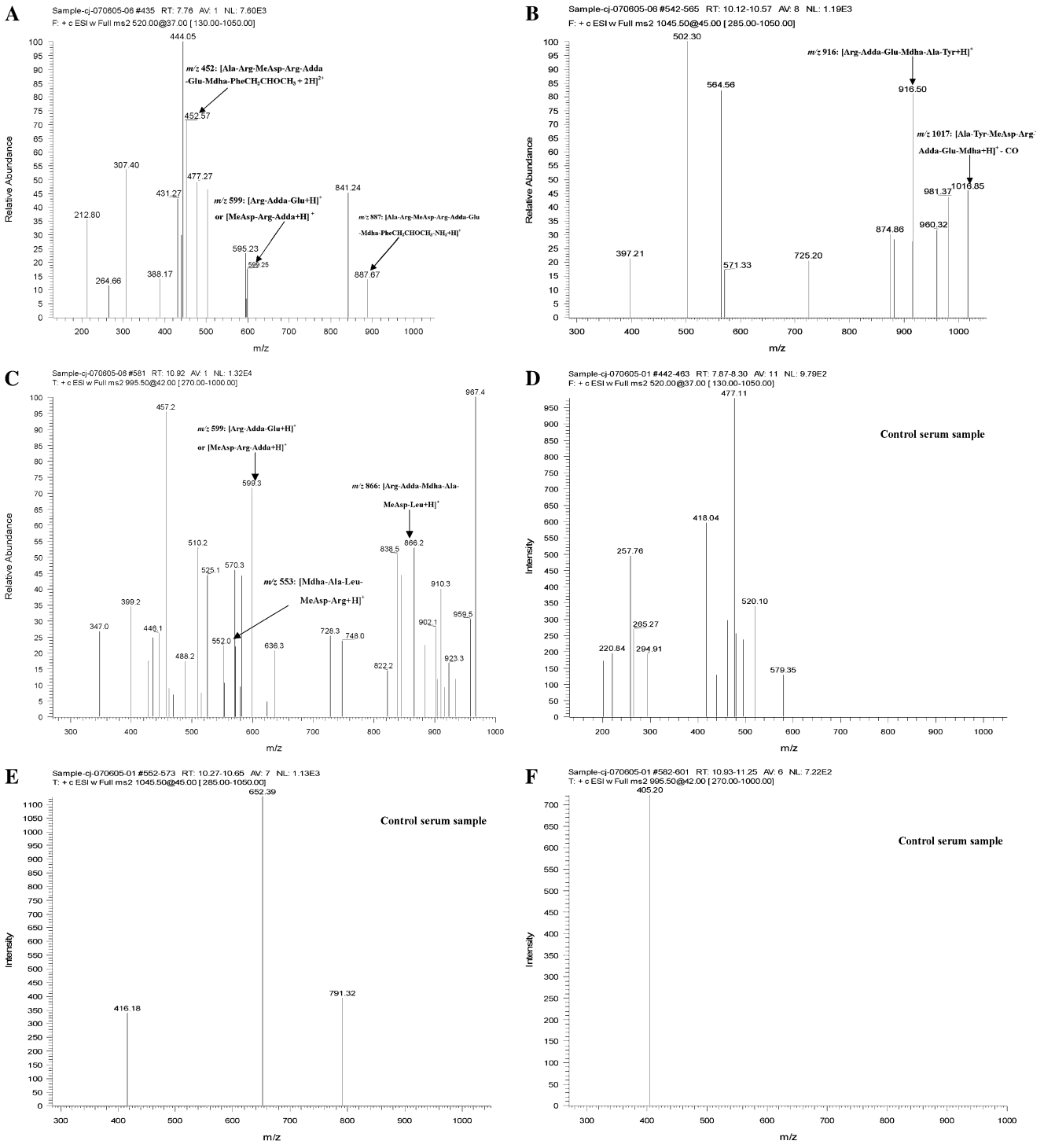


**FIG. 1.** LC/MS/MS spectrum of MC-RR (A), -YR (B), and -LR (C) from a fisherman serum extract (R = arginine, Y = tyrosine, and L = leucine) and spectrum from a human control serum extract, which did not support the presence of MC-RR (D), -YR (E), and -LR (F).

### *Relationship between Biochemical Indices and MC Contents in Serum Samples of Fishermen*

With the PCCA, more than 37% of the toxicological and biochemical variation in the data was explained by Component Axis 1 (22.19%) and Component Axis 2 (14.83%) (Fig. 4). Component Axis 1 had high positive weighting for some biochemical variables, such as UA, CHOL, CREA, and BUN, which mainly reflect the renal function, but negative weighting for MC concentrations and the indices (such as ALT, AST, LDH, ALP, DBIL, IBIL, TBIL, TP, and GLB) which mainly

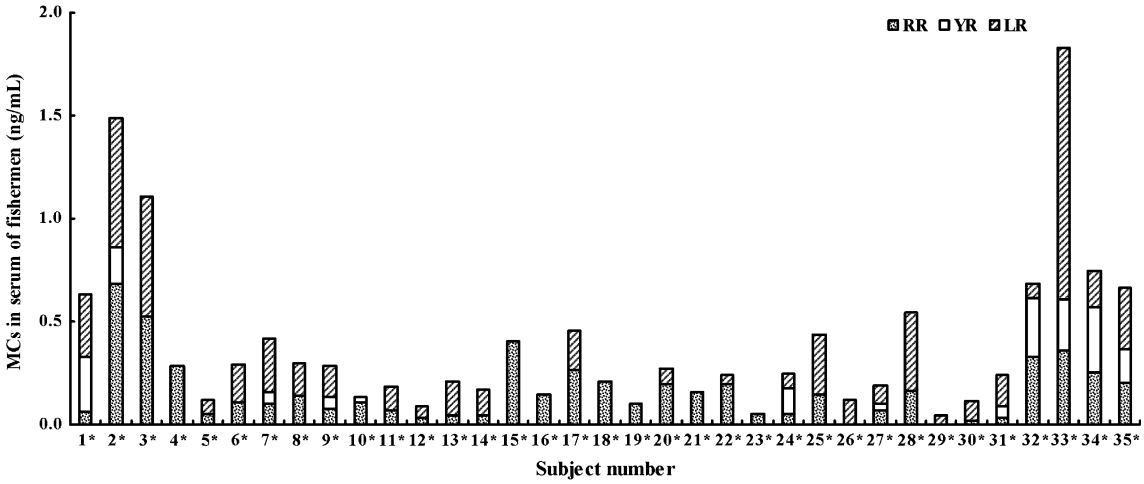


**FIG. 2.** LC/MS/MS results of the serum analysis of 35 randomly selected fishermen who were living on Lake Chaohu. (Serum samplings were collected from July 19 to July 22, 2005).

reflect the liver function and protein metabolism. Component Axis 2 had high positive weighting for MC concentrations and ALT, AST, LDH, ALP, but negative weighting for most other biochemical variables. MC concentrations, ALT, AST, LDH, and ALP had high negative correlations with factor 1, but positive correlations with factor 2.

## DISCUSSION

### *Method Improvement in Detecting MCs in Human Serum Samples*

The amount of serum sample used in the present study was 0.2 g in dry weight (DW), approximately equal to 4.8 ml of fresh serum specimen. This increased 4.8–24 folds compared with the earlier studies where 0.2–1.0 ml of serum sample was used (Carmichael *et al*., 2001; Hilborn *et al*., 2005; Soares *et al*., 2006). A significant increase in sample weight is quite important when the concentration of the target compound (MC) was very low with complex matrix, as sufficient samples enable us to do clean-up procedure.

After the sample preparation, a cleanup method utilizing a combination of HLB and silica gel cartridge was chosen to obtain clean extract of MCs. In this process, the suppression of ionization was evaluated by comparing the absolute peak areas of the extracts of serum spiked with a known amount of analytes to neat standard injected directly in the same reconstituted solvent. The results indicate that combination of HLB and silica gel cartridge improved the sample clean-up and thereby decreased the amount of matrix injected onto the column and the ion suppression effect was minimized better than separate use of the HLB cartridge (data not shown), although the HLB sorbent is a copolymer designed to have a hydrophilic-lipophilic balance that gives higher and more reproducible recoveries for MCs (Hilborn *et al*., 2005; Soares *et al*., 2006; Yuan *et al*., 2006) than C18 sorbent (Azevedo *et al*., 2002; Carmichael *et al*., 2001;

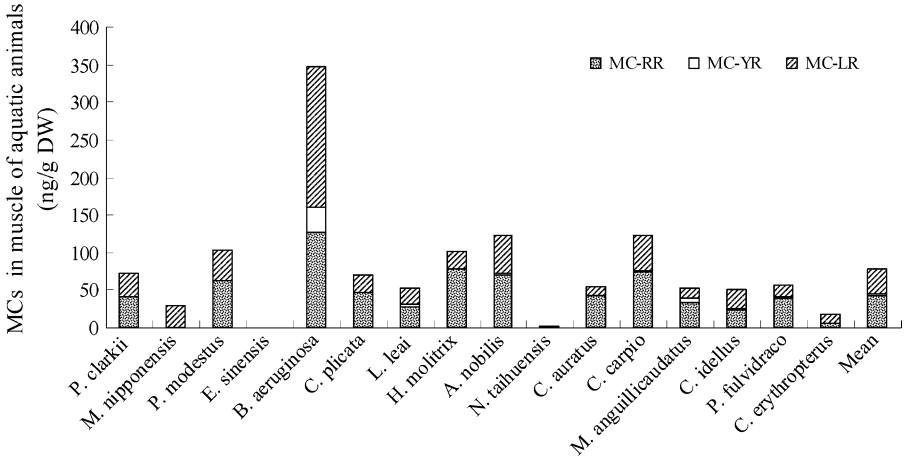


**FIG. 3.** MC contents in muscle of crustaceans, mollusks and fish from Lake Chaohu (samples were collected in July 2005).

**TABLE 1**
**Summary of the Serum Biochemical Indices of the Study Population ($n$ = 35)**

| Index | Normal range | % of subjects outside normal range | No. of the subjects (values measured above/below the normal range) |
|---|---|---|---|
| ALT | 7–40 U/l | ↑: 8.6 | 2*(156), 33*(55), 35*(67) |
| AST | 8–40 U/l | ↑: 5.7 | 2*(119), 35*(51) |
| ST/LT | 0–1.2 | ↑: 11.4 | 20*(1.24), 22*(1.26), 25*(1.45), 26*(1.25) |
| ALP | 26–117 U/l | ↑: 14.3 | ↑: 2*(168), 4*(130), 6*(161), 7*(176), 32*(142) |
| | | ↓: 14.3 | ↓: 1*(23), 15*(24), 16*(16), 19*(21), 27*(25) |
| LDH | 114–240 U/l | ↑: 11.4 | 1*(266), 2*(332), 8*(275), 18*(244) |
| GLB | 25–45 g/l | ↓: 25.7 | 5*(22), 7*(22), 8*(21) 10*(23), 16*(19), 17*(24), 19*(24), 20*(22), 31*(20) |
| A/G | 1.3–2.5 | ↓: 14.3 | 2*(1.2), 11*(1.2), 30*(1.2), 32*(1.1), 33*(1.2) |
| CREA | 44–97.5 μmol/l | ↓: 22.9 | 4*(41), 6*(41), 7*(42), 8*(37), 9*(39), 12*(42), 19*(41), 24*(43) |
| GLU | 3.8–6.1 mmol/L | ↓: 5.7 | 16*(3.7), 30*(3.2) |
| TC | 3.2–5.3 mmol/l | ↑: 17.1 | ↑: 1*(5.7), 14*(5.45), 21*(5.56), 22*(5.42), 25*(6.47), 28*(7.01) |
| | | ↓: 5.7 | ↓:12*(2.87), 32*(3.14) |
| TG | 0.6–1.7 mmol/l | ↑: 5.7 | ↑:30*(2.85), 33*(2.05) |
| | | ↓: 25.7 | ↓: 4*(0.46), 12*(0.56), 13*(0.39), 15*(0.52), 16*(0.59), 17*(0.34), 26*(0.55), 27*(0.45), 31*(0.41) |
| UA | 115–412 μmol/l | ↑: 2.9 | 28*(511) |
| TBIL | 5.1–17.2 μmol/l | ↑: 2.9 | 34*(33.4) |
| DBIL | 0–5.1 μmol/l | ↑: 2.9 | 34*(9.8) |
| IBIL | 0–17 μmol/l | ↑: 2.9 | 34*(23.6) |
| GGT | 10–60 U/l | ↑: 2.9 | 34*(63) |
| BUN | 1.7–8.3 mmol/l | 0 | — |
| CHE | 3.5–13.2 ku/l | 0 | — |
| TBA | 0–20 μmol/l | 0 | — |
| TP | 60–86 g/l | 0 | — |
| ALB | 35–55 g/l | 0 | — |

*Note*. ↑: higher than maximum limit of the normal range; ↓: lower than the minimum limit of the normal range.

Jochimsen *et al*., 1998; Pouria *et al*., 1998). We also validated that Microcon Centrifugal Filter with cellulose membrane (YM10, Millipore, USA) is important for protein removal prior to LC/MS analysis (Hilborn *et al*., 2005; Yuan *et al*., 2006).

Because of its high specificity and sensitivity, LC/MS has become the method of choice for quantitative determination of analytes in biological samples (Chen *et al*., 2002). Because MCs are cyclic peptides with a high proton affinity, using LC/MS with an ESI ion source can obtain best sensitivity and accuracy. Several analysis applications of this have been reported in the literatures (Hilborn *et al*., 2005; Meriluoto, 1997; Yuan *et al*., 2006). Similarly, in the present study, we used ESI LC/MS to detect MCs in the serum extracts and obtained a cleaner MS spectrum with good reproducibility of the fragment ions.

### *A Comparison of Serum MC Level between the Fishermen and the Victims of the Caruaru Hemodialysis Center in Brazil*

In the present study, we confirmed for the first time the presence of MCs in serum samples of fishermen who were chronically exposed to cyanotoxins by oral route. All of the 35 serum samples were positive for MCs by using ESI LC/MS

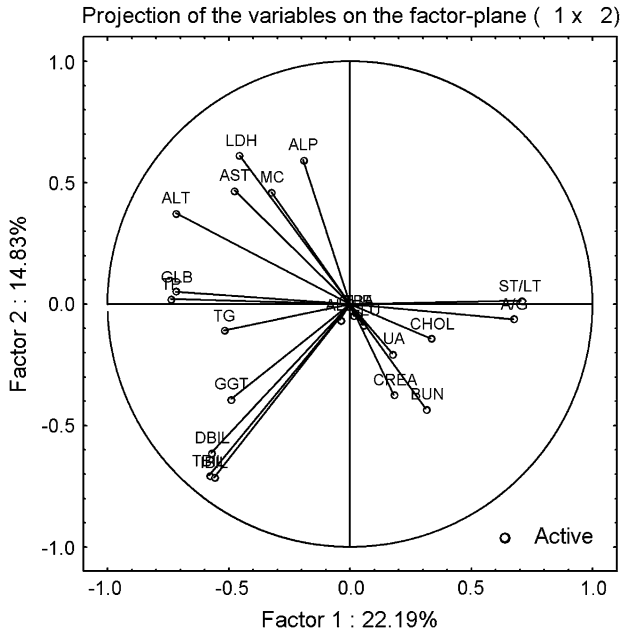


**FIG. 4.** Scatterplot of first two components from a PCCA performed with 21 biochemical variables and MC concentrations from 35 samples.

method. Total amount of MCs (-LR, -YR, -LR) ranged from 0.045 to 1.832 ng/ml (mean = 0.389, and median = 0.227 ng/ml), with a mean value of 0.228 MC-LReq/ml.

So far, there have been only very limited information on MC content in human serum. The average MC-LReq concentration in serum samples from the Caruaru hemodialysis patients who had suffered acute lethal exposure was estimated to be 2.2 ng/ml by ELISA method (17 samples from 12 victims) (Azevedo *et al*., 2002; Carmichael *et al*., 2001). This is about 10 times the mean MC concentration found in serum samples from the 35 fishermen in our study. In another study, Hilborn *et al*. (2005) reanalyzed 10 sera samples collected from the Caruaru dialysis patients, and concentration of MC-LReq detected by LC/MS ranged from 7.6 to 31.4 ng/ml (mean = 20 ng/ml). The average MC concentration in serum samples from this study was about 87 times the mean concentration of MC-LReq found in our study. Apparently, there was a difference of 9 times in serum MC content from the Caruaru dialysis patients between the detections by Hilborn *et al*. (2005) and by Carmichael *et al*. (2001), suggesting that different detection methods (ELISA vs. LC/MS analysis) might give extremely different values. It might be more reliable to compare our result with those using the same analytical method (Hilborn *et al*., 2005), and it is thus likely that mean serum MC level from the Caruaru hemodialysis patients was about 87 times that from the fishermen in our study.

It should be noted, however, that detection of MCs in human serum seems to be rather difficult sometimes. For example, although Soares *et al*. (2006) studied 97 serum samples from 12 hemodialysis patients treated at the HUCFF dialysis center that had received sublethal exposure to MCs by the intravenous route, and found that both the median and the mean MC concentrations of patients and samples were 0.34 ng/ml, and MC concentrations among all patients and samples ranged from $\leq$0.16 to 0.96 ng/ml by ELISA method, whereas the LC/MS/MS analyses of the serum samples did not support the presence of MCs. No clear peak could be observed in patient serum samples at the same retention time and mass as the standard. None but taken together the ELISA, LC/MS, and 2-methyl-3-methoxy-4-phenylbutyric acid results indicate that these renal dialysis patients were exposed to MCs.

### *Relation between MC Concentrations and Biochemical Indices of Serum Samples*

We established a relationship between serum MC concentration and biochemical indices in human using PCCA, which enables us to demonstrate chronic MC exposure, with potential consequences for human health. Our results indicate that among the biochemical variables, MC concentrations showed closer positive relationships with ALT, AST, LDH, and ALP than with other biochemical indices, suggesting that MC accumulation in bodies might have firstly influenced activities of these serum enzymes, which represent liver functions. It appears that once absorbed into the blood stream, MCs affected a target tissue like liver and induced lesions, consequently causing substantial alterations in levels of associated serum biochemical indices.

Increased serum activity of enzymes considered indicative of hepatocellular damage has been previously observed in toxicity studies on rats/mice treated with MCs (Table 2), and these results provided basis for understanding of MC toxicity to mammalians including human. In tendency, our results are comparable with these toxicity studies (Billam *et al*., 2008; Gehringer *et al*., 2004; Gupta *et al*., 2003; Moreno *et al*., 2003; Rao *et al*., 2004, 2005; Weng *et al*., 2007; Xu *et al*., 2007), and statistical evidence indicates that prolonged MC-LR exposure at a relatively low dose is also able to influence serum ALT, AST, LDH, and ALP activities of human being, although such increases were much smaller than rats/mice in the above toxicity studies or the Caruaru patients in Brazil.

### *Estimation of MC Uptake by the Fishermen*

The fishermen were living on fishing ships on Lake Chaohu during most time of a year, and not only used the lake water for drinking, but also collected aquatic products (mainly fish, shrimps, and snail) as their main food. As the fishermen only used alum to remove algae from the lake water, we assume that 1/5 of the MC in the lake water was present in their drinking water, and that one adult drank 2 l of lake water per day, the daily intake of MC from drinking water by a fisherman could be 1.31 μg MC-LReq. It is assumed that 100–300 g fresh fisheries product was consumed daily by a fisherman, and a coefficient of 5 was used to convert dry weight to wet weight, and then the daily intake of MC from fisheries product by a fisherman could be 0.86–2.57 μg MC-LReq. Totally, daily intake of MC by a fisherman could be 2.2–3.9 μg MC-LReq, close to exceeding the tolerable daily intake (TDI) of 2.4 μg for an adult (body weight 60 kg) proposed by WHO (Chorus and Bartram, 1999).

In the present study, as mean MC-RR, -YR, and -LR concentrations in serum of the fishermen were 3.89, 1.24, and 4.20 ng/g dry weight (DW), respectively, the proportions of MC-RR, -YR, and -LR were 41.7, 13.3, and 45.0%, respectively, close to the proportions of MC-RR, -YR, and -LR in the muscle of aquatic animals. This may suggest the major contribution of the fisheries products to the daily uptake of MC by the fishermen, which is generally in agreement to the above estimation.

Ibelings and Chorus (2007) reviewed accumulation of cyanobacterial toxins in freshwater "seafood" and its consequence for public health. From the provisional WHO TDI per kg body weight, they calculate a TDI of 3 μg/day for a 75 kg adult and 0.4 μg/day for a 10 kg child. They then relate this to different allocation factors between food and other exposure routes to give Guideline values for food that range from 0.08 to 30 μg/kg food fresh weight—depending on age and allocation factor. The results of the present study suggest that either exposure was higher than our estimate, or that damage occurs at lower levels than assumed by Ibelings and Chorus (2007). Therefore, there is also a

**TABLE 2**
**A List of MCs-Induced Changes of Serum Biochemical Indices in Rats/Mice Reported in Literatures**

| Species | Toxin | Treatment | Dose | Biochemical indices | Reference |
|---|---|---|---|---|---|
| Swiss albino female mice | MC-LR, RR and YR | i.p. | 43 (LR), 235.4 (RR), and 110.6 (YR) μg/kg BW | Serum levels of AST, ALT, and GGT showed significant increase* compared with control as early as 30 min postexposure in all three toxins and was further enhanced at MTD. Similarly increased LDH levels were noticed at 30-min post-treatment. There was no significant change in profile of serum TP, ALB and A/G ratio of all three MC variants at both time points. | Gupta *et al*., 2003 |
| Male Wistar rats | MC-LR | i.p. | 100 μg/kg BW | ALT, AST, LDH, and ALP were significantly elevated (22-fold***, 8.5-fold***, 3.8-fold***, and 1.9-fold**, respectively). | Moreno *et al*., 2003 |
| Female Balb/c mice | MC-LR | i.p. | 75% $LD_{50}$ | ALT was significantly elevated after 8 h* pe and dropped at 16 h*, 24 h* and 32 h* pe when compared with the 8 h pe group. LDH was significantly elevated at 8 h* pe and remained significantly higher than the control values throughout the study period. | Gehringer *et al*., 2004 |
| Swiss albino female mice | MC-LR | i.p. | 100 μg/kg BW | ALT and LDH significantly increased (8-fold* and 6- to 7-fold*, respectively) in MC-LR group over control, respectively. | Rao *et al*., 2004 |
| Swiss albino male mice | MC-LR | i.p. | 43 μg/kg BW | LDH, AST and ALT showed significant increase* (three- to sevenfold) in levels compared with respective controls in all the age groups. | Rao *et al*., 2005 |
| | | Oral | 3.5 g/kg BW | LDH, AST, and ALT showed significant increase* (two- to fivefold) in treated animals compared with respective controls. | |
| Male ICR mice | MC-LR | i.p. | 60 μg/kg BW | ALT and AST were significantly elevated (about 26-fold* and 2-fold*, respectively) compared with control mice. (12 h pe). | Weng *et al*., 2007 |
| Kunming male mice (SPF grade) | MC-LR | i.p. | 10 μg/kg/day | ALT, AST, and ALP showed obvious increases (3.8-fold**, 1.5-fold**, and 1.5-fold**, respectively) in levels compared with respective controls. | Xu *et al*., 2007 |
| Male Fisher 344 rats | MC-LR | i.p. | Three doses: 50, 100, and 150 μg/kg | ALP, AST, ALT significantly increased ** in all doses, GGT, BUN, and CREA significantly increased ** only in the third dose, whereas levels of GLU, and TC significantly decreased** only in the third dose. TP, GLB and A/G showed no changes. | Billam *et al*., 2008 |

*Note*. (R = arginine, Y = tyrosine, and L = leucine); i.p. = intraperitoneal injection; BW = body weight; A/G = albumin/globulin; ICR = Institute of Cancer Research; pe = postexposure; MTD = mean time to death; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the control group.

pronounced need for more in-depth human exposure studies from settings such as that of the fishermen at Lake Chaohu.

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