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Determination of some physicochemical parameters of microcystins (cyanobacterial toxins) and trace level analysis in environmental samples using liquid chromatography

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Abstract

Some physicochemical parameters of three microcystin standards, known to be potent hepatotoxins produced by cyanobacteria, were determined using well defined chromatographic measurements. The logarithm of their retention factor on octadecylsilica (ODS) with water as the eluent, which is an estimation of the hydrophobicity of a molecule, was assessed at pH 7 at 3.9, 4.2 and 4.4 for microcystins-YR, -LR and -RR, respectively. Though being rather hydrophobic, microcystins also possess polar functions, namely carboxylic acids, amino and amido groups. The ionization of carboxylic groups occurs at pH values of 3.3–3.4. In environmental waters, microcystins are neutral or anionic. They are readily soluble in water, the solubility of microcystin-LR being higher than 1 g/l. Owing to their hydrophobicity and their polar functions, microcystins remain in the aqueous phase rather than being adsorbed on sediments or on suspended particulate matter. In a river water spiked with microcystins at 5 μg/l, only 10% was adsorbed on particles and 7% on the sandy sediment after three days. A method using solid-phase extraction on ODS followed by high-performance – or micro – liquid chromatography was optimized to detect microcystins at trace level in water. A clean-up was introduced to eliminate part of the interfering compounds coextracted during the sample percolation. Good recoveries (75–80%) were obtained. The method was linear, reproducible (with relative standard deviations ranging from 5 to 8%) and enabled the determination of microcystins at levels as low as 30 ng/l in drinking water and 100–200 ng/l in surface waters. The production of toxins by two strains of cyanobacteria was evaluated. Variations in the microcystin-LR content in the cells and in the medium of *Microcystis aeruginosa* PCC7806 were recorded over a five-week period. Toxin production was not correlated to the biomass but depended on the growth stage and was maximal at the end of the exponential growth phase. The release of toxin in water occurred essentially in old cultures where microcystin-LR was determined at concentrations of 170 and 280 μg/l in the media of *M. aeruginosa* PCC7806 and *M. aeruginosa* PCC7813, respectively. Other microcystins are likely to be synthesized by these strains. But owing to the lack of standards, mass spectrometric detection is required for further identification. This study points out the need of having other standards for water quality monitoring.

Keywords: Trace analysis; Physicochemical parameters; Microcystins; Cyanobacterial toxins; Toxins

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1. Introduction

Blooms of toxic microorganisms are commonly found in freshwater in several areas of the world. Freshwater poisonings are mainly caused by species of cyanobacteria, also known as blue–green algae [1,2]. They develop in eutrophic water wherever proper conditions for their growth are found, including a mild wind, a water temperature between 15 and 30°C, a neutral or alkaline pH and a rather high level of mineral nutrients. As a consequence of the extended use of nitrates and phosphates, blooms occur more and more frequently [3]. There is a risk that these blooms contaminate water supplies that are used as recreational areas or as drinking water reservoirs. This is a serious water quality problem because many of the cyanobacterial species are able to produce potent toxins.

The most frequently reported toxins are hepatotoxins. They are classified as microcystins and nodularins, the former representing the largest group. All microcystins have a common cyclic heptapeptide structure consisting of (–D-Ala–L–X–D-MeAsp–L–Z-Adda–D-Glu–Mdha), where MeAsp stands for erythro-β-methylaspartic acid, Mdha for N-methyldehydroalanine, Adda for 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and X and Z for the variable amino acids that give its name to the molecule [4]. Microcystins can be synthesized by various genera of cyanobacteria such as *Microcystis*, *Oscillatoria*, *Anabaena* or *Nostoc*. They have been responsible for the poisoning of fish, birds, wild and domestic animals in many countries [5,6]. Adverse effects on human health have also been recognized. Recent researches have actually shown that microcystins act as type 1, 2A and 3 protein phosphatase inhibitors, as well as tumor promoters when present in nanomolar concentrations [7–9]. Nowadays, no guidelines have been established for the monitoring of microcystins in water. Maximal values of 0.1 μg/l in drinking water for a long term exposure or 1 μg/l for a short term exposure have been proposed on the basis of laboratory experiments of toxicity on mice and pigs [10,11].

Usually, environmental behavior of compounds can be predicted from various physicochemical data which are generally measured in laboratory under well defined conditions, i.e., water solubility, vapour pressure, volatility, stability in water, photodegradation rate, water–octanol partition coefficient, etc. These data are usually known, since they are required for the registration of new organic chemicals. However, microcystins are natural products and these data are not available. The small amounts of available standards and their price may account for this lack of data.

In this work, we have used liquid chromatography (LC) under specific conditions to determine some of these parameters such as hydrophobicity, water solubility, ionisation state according to the pH, adsorption on particulate matter and on sandy sediment, that should control the behavior of microcystins in natural water. Furthermore, the trace-level determination of microcystins requires an enrichment step before the proper analysis and quantification using LC. This enrichment is often performed by solid-phase extraction (SPE) using ODS SPE cartridges [12,13], and it was shown that the knowledge of the water–octanol partition coefficient allows an easy optimization of the enrichment conditions [14]. Since both standards and natural extracts are found in small amounts, it was worthwhile to minimize the injected amount by using micro-LC [15]. A clean-up step could be introduced in the SPE sequence, so that the analytical procedures have also been successfully applied to the detection of some microcystins in environmental samples and in culture media of toxic cyanobacteria.

Under natural conditions, it is not easy to foresee the toxicity of a cyanobacterial bloom because blooms of a same species do not all have the same toxicity and the toxicity is likely to vary with time within a given species. Laboratory experiments showed that toxicity, measured by the mouse bioassay, was influenced by culture age, basic pH and temperature [16]. In other studies, the hepatotoxin production was also correlated with high nitrogen concentrations and low light intensity [17]. Toxin concentrations were usually highest under conditions which also favored growth. Many studies have concluded that toxicity [1,18] and toxin content of the cells [19] were maximum at the late exponential growth phase. In this study, micro-LC was used to determine the presence of a microcystin in toxic *Microcystis aeruginosa* strains. We also followed the

variations in the toxin production and its release in the surrounding medium during the growth phase.

2. Experimental

2.1. Materials

Microcystin-LR and microcystin-RR standards were purchased from Sigma (Saint Quentin Fallavier, France), microcystin-YR standard from Calbiochem Novabiochem (La Jolla, CA, USA). All reagents were of HPLC grade or analytical grade. Methanol was obtained from Carlo Erba (Milan, Italy), acetonitrile from Baker (Phillipsburg, NJ, USA). The 5 m*M* phosphate buffer was prepared in LC-quality water (Milli-Q filtration system, Millipore, Bedford, MA, USA) and adjusted to the working pH by addition of concentrated perchloric acid.

The culture media and the cellular extracts of two cyanobacteria strains were analysed. The strains, namely *Microcystis aeruginosa* PCC7806 and *Microcystis aeruginosa* PCC7813, were purchased from Pasteur Institute (Paris, France).

2.2. Cyanobacteria culture

Cultures of the two *Microcystis* strains were maintained in sterilized 250-ml Erlenmeyer flasks containing 100 ml growth medium at 22°C under white light (1070 lux, 12 h light/12 h dark). They were grown in $BG11_0$ medium [20]. To measure the growth and the amount of toxin produced by the cultures during the growth phase, 200 ml of *M. aeruginosa* PCC7806 culture (inoculum at day 0= $8.7 \cdot 10^5$ cell/ml) were collected at 3–4 days intervals, from the 5th day to the 22nd day of growth. The experiment was repeated twice. Cultures of *Microcystis aeruginosa* PCC7813 and PCC7806 were also harvested two weeks after the beginning of the decline phase (55th day). Free cell media were obtained by filtration of the cultures through 60-mm GF/C disks (pore size 1.2 μm) (Whatman, Maidstone, Kent, UK). Cellular components on the disks were extracted using methanol as described in Ref. [15]. Wet cellular mass on the filters were measured after squeezing and 15 min of air sucking through the disk.

2.3. Determination of physicochemical parameters

The ionic and the hydrophobic character of microcystins, as well as their solubility, were investigated using the micro-LC system. Each experiment was repeated twice. For pK_a measurements, a mobile phase containing a mixture of methanol–phosphate buffer (50:50, v/v) or a mixture of acetonitrile–phosphate buffer (40:60, v/v) were used. The pH was modified by addition of concentrated perchloric acid and retention factors (k) were measured according to the pH of the mobile phase.

Hydrophobicity was estimated at pH 7 using the retention factors of microcystins eluted with water (k_w). Microcystins were eluted using methanol–phosphate buffer in various proportions. The variations in the logarithm of the retention factor (log k) according to the percentage of organic modifier in the eluent were recorded. Values of log k_w were obtained by graphic extrapolation of these curves to a zero content in organic modifier.

Solubility of microcystins in water was evaluated at 25°C by comparison of peak areas obtained by high-performance liquid chromatography (HPLC) for the same amount of toxin dissolved either in methanol or in water. Standard solutions of microcystin-LR at different concentrations (0.2, 0.5, 1 and 2 mg/ml) were prepared by adding 100 μl of water to 20 μg of toxin, 100 μl to 50 μg, 500 μl to 500 μg and 250 μl to 500 μg.

The percentage of toxins adsorbed on particles in suspension was measured by spiking 500 ml of river water containing 8.4 mg of particles (dry mass) with microcystins at 5 μg/l. The sample was regularly shaken. After three days, it was filtered and the filtrate was analysed using SPE–micro-LC. The filters, which contained microcystins adsorbed on particles, were extracted with methanol. After evaporation of the solvent, the amount of toxins in the residue was assessed using micro-LC.

The proportion of microcystins adsorbed on a sandy sediment was measured by spiking 500 ml of river water containing 100 g of sediment with microcystins at 5 μg/l. The procedure was then identical to the previous one.

2.4. Aqueous sample preparation: SPE

Water samples (500 ml) were filtered through a 60-mm GF/C filter and methanol was added to the final concentration of 5%. The samples were then preconcentrated on a Bakerbond SPE cartridge (Baker, Deventer, Netherlands) packed with 500 mg ODS. The SPE procedure involved activation of the cartridge with 5 ml of methanol, conditioning with 10 ml of Milli-Q water, percolation of the sample at a flow-rate of 10 ml/min and clean-up with 5 ml of 30% aqueous methanol. Air was then sucked up through the cartridge for 2 min. Analytes were subsequently desorbed with 3 ml of methanol acidified with 1% (v/v) trifluoroacetic acid (TFA). The residue was evaporated to dryness at 45°C under reduce pressure and dissolved in 50 or 100 μl of acetonitrile–phosphate buffer (20:80, v/v) acidified to pH 2.

Culture media were processed as water samples, except that methanol was added to a final concentration of 1% and the sample volume was 130 ml.

2.5. HPLC analysis

Two HPLC techniques were employed for the analysis of aqueous samples. The first one used classical analytical columns of 4.6 mm I.D., the second one used FSOT micro-columns of 0.32 mm I.D. The apparatus was the same for both techniques, except that a flow splitter was added for micro-LC and the injection volume, the column and the detection cell were adapted to the technique. The micro-LC system coupled to UV–diode array detection (DAD) was described in a previous study [15]. The UV detector was set to 238 nm. In micro-LC, a volume of 200 nl was injected. Analytical separations were carried out on a 25 cm×0.32 mm I.D. column packed with 5 μm reversed-phase BioSil C_{18} (Bio-Rad, Hercules, CA, USA), at a flow-rate of 3.5 μl/min. In classical HPLC, a volume of 20 μl was injected on a 25 cm×4.6 mm I.D. column packed with 5 μm reversed-phase Hypersil BDS C_{18} (ColoChrom, Gagny, France). The flow-rate was 1 ml/min.

Analyses of microcystins in culture media and cellular extracts were performed using the micro-LC system with a mobile phase consisting of acetonitrile–5 m*M* phosphate buffer (PB) acidified to pH 2. The elution gradient employed solvent A [acetonitrile–PB pH 2 (10:90, v/v)] and solvent B [acetonitrile–PB pH 2 (90:10, v/v)] as follows: 10% of B from 0 to 10 min, 28% of B at 40 min, 33% of B at 60 min, 45% of B at 80 min and 100% of B at 90 min. The classical HPLC system was employed for the analysis of drinking and surface water. The mobile phase consisted of a mixture of solvent A [acetonitrile–PB pH 2.5 (25:75, v/v)] and solvent B (acetonitrile) as follows: 0% of B at 0 min, 20% of B at 35 min, 30% of B at 45 min and 100% of B at 50 min for surface water analyses, 0% of B at 0 min, 12% of B at 20 min, 12% of B at 40 min, 20% of B at 50 min and 100% of B at 60 min for the detection limit in surface water, 0% of B at 0 min, 17% of B at 35 min, 30% of B at 45 min and 100% of B at 50 min for drinking water analyses.

3. Results and discussion

3.1. Physicochemical parameters of microcystins in water

The chemical structures of microcystins have been well determined, but, owing to the small amounts of standards available, very little is known about their physicochemical properties until now. Reversed-phase LC has been extensively studied and it is now well established that this method, under defined and controlled experimental conditions, can lead to the measurements of some parameters such as ionization constants and water–octanol partition coefficients. Moreover, using micro-LC with narrowbore reversed-phase columns requires only a few nanolitres at each injection. Although more than 50 microcystins have now been identified, only three are commercially available. The experiments were conducted with these three standards, namely microcystin-YR, microcystin-LR and microcystin-RR. Their chemical structures are presented in Fig. 1.

3.1.1. Ionization state depending on the pH

In reversed-phase LC using alkyl bonded silicas as stationary phases, the retention factor, *k*, of a monoacidic ionizable analyte is expressed by the following equation:

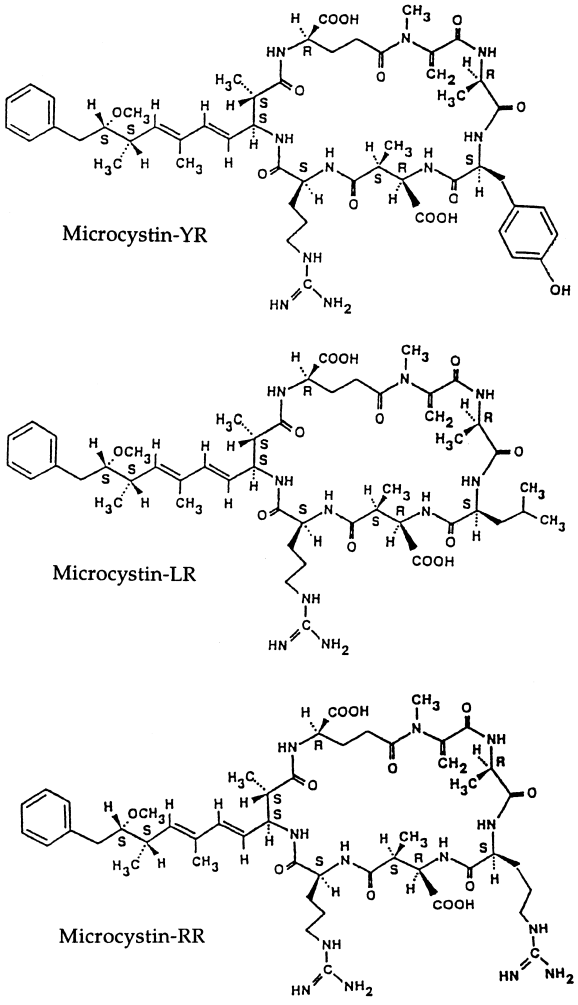

Fig. 1. Structure of microcystin standards.

$$k = k_1/\left[1 + 10^{(\mathrm{pH}-\mathrm{p}K_a)}\right] + k_2/\left[1 + 10^{(\mathrm{p}K_a-\mathrm{pH})}\right]$$

where pK_a is the negative logarithm of the acidic dissociation constant in the mobile phase and k_1 and k_2 are the limiting retention factors of the solute at $pH < pK_a - 2$ and $pH > pK_a + 2$. Therefore, the retention factor of a weakly acidic (or basic) solute as a function of the pH of the mobile phase can be represented by a typical sigmoidal (or antisigmoidal) curve because the retention factor of the ionized species is much lower than that of the unionized ones. For zwitterionic compounds, curves are made of a sigmoidal and antisigmoidal part and can be also predicted depending on the respective two ionization constants for anionic and cationic species. The retention factor of the amphoteric form is the largest one.

Microcystins have two carboxylic acid functions on the side chains of glutamic acid and aspartic acid, respectively, and one or two guanidine residues carried by the arginine amino acids. Therefore, one can expect different ionization states and different retention on ODS according to the pH. It is not easy to foresee the charge of each group as part of the whole molecule. Although the pK_a of each acid or basic function is well known for the amino acids alone, these values can greatly vary depending on the environment in the peptidic chain. We can only suggest that in very acidic medium, guanidine residues and the γ-carboxylic groups are protonated (NH_2^+ and COOH), resulting in a positive global charge for microcystins-YR and -LR and two positive charges for microcystin-RR. When the pH increases, the carboxylic functions evolve into their carboxylate form COO^-, which results in an ampholitic form, neutral and then anionic for microcystins-YR and -LR, cationic and then neutral for microcystin-RR. In basic medium, the guanidine residues become neutral (NH), so that, owing to the γ-carboxylates, microcystins carry two negative charges.

In order to examine the effect of the pH on the retention of microcystins on ODS, the pH of the eluent was fixed by a phosphate buffer initially at pH 7 and modified by the addition of concentrated acid. Variations in the retention factors (k) were recorded in the pH range 2 to 7. The curves obtained in water–methanol (Fig. 2A) and in water–acetonitrile (Fig. 2B) clearly show that the retention factors greatly depend on the pH and have a sigmoidal shape. Microcystins are strongly retained in acidic medium and almost not retained at pH above 5, in these mobile phases. Such a difference in k is difficult to explain by the predicted difference in ionization states according to the pH, since at pH 3.5, we can only expect the ionization of the carboxylic acids. However, as phosphate buffer was acidified with perchloric acid to adjust the pH of the mobile phase, the higher retention in acidic media is due in fact to an ion-pair retention mechanism. Ion-pairs occur between perchlorate and dihydrogenophosphate groups contained in the mo-

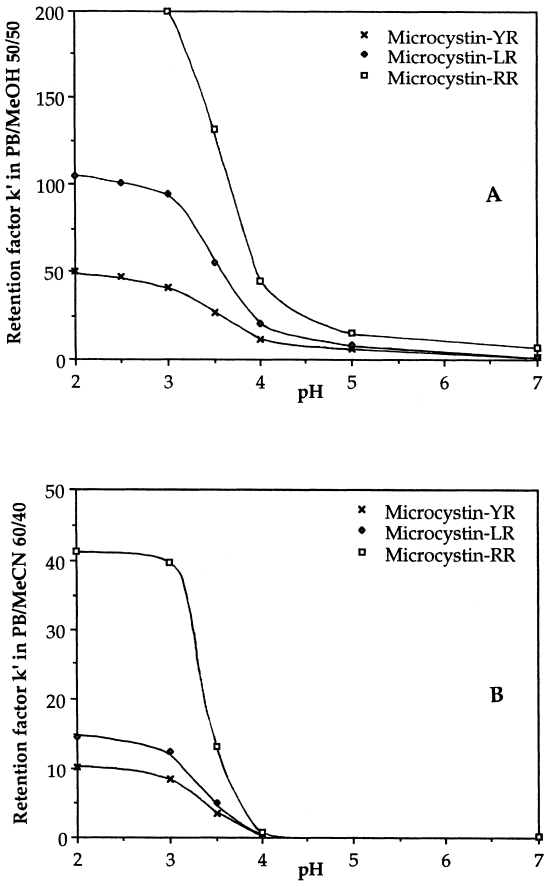

Fig. 2. Effect of pH on the retention of microcystins using ODS. Column: 20 cm×0.32 mm I.D. packed with 5 μm BioSil ODS; mobile phase: (A) methanol–phosphate buffer $5 \cdot 10^{-3}$ *M* (50:50, v/v) acidified with perchloric acid, (B) acetonitrile–phosphate buffer $5 \cdot 10^{-3}$ *M* (40:60, v/v) acidified with perchloric acid.

bile phase on one hand and the positive charges on the arginine groups of the toxins on the other hand. The partitioning of these ion-pairs between the stationary phase and the mobile phase contributes to the higher retention of the cationic species. The decrease in *k* is then explained by the ionization of the two carboxylic groups, which occurs at pH values around 3.5. Microcystins become then amphoteric or negatively charged and cannot be retained by the ion-pair mechanism. The pK_a values of the three microcystins determined from the curves in Fig. 2 are 3.4 for microcystins-YR and -RR and 3.3 for microcystin-LR, with an error of ±0.2. The similarity of both pK_a was also confirmed by calculating the partial charge carried by each COOH after geometric optimization by molecular mechanics and semi-empirical calculations. Both pK_a values were found close to 3.5 for the three microcystins.

Since the mobile phase consisted of a mixture of methanol or acetonitrile and phosphate buffer, the ionization constants thus determined are apparent constants and will be slightly different from those measured in pure water. It was impossible to use pure water as mobile phase, because microcystins are too strongly retained to measure their retention factors. Nonetheless, owing to the proportion of water in the mixture, the pH can be considered close to the pH of the aqueous phase. In order to verify the effect of the organic solvent content on the pK_a determination, the same process was applied to a well characterized compound, pentachlorophenol. The variations of *k* according to the pH yielded a similar bilogarithmic curve with an inflexion point at pH 4.6, corresponding to its pK_a and which is very close to the value of 4.5 reported in Ref. [21].

3.1.2. Hydrophobicity

This parameter, usually reported as the logarithm of the water–octanol partition coefficient, log K_{ow} (or log P_{oct}), is characteristic of the lipophility or hydrophobicity of a molecule. The concept of K_{ow} was developed by the pharmaceutical industry as a useful index of the drug's behaviour in the body because the partitioning between water and octanol roughly mimics the partitioning between water and biotic lipids. It is taken as an indication of the tendency of the compounds to accumulate in biological membranes and living organisms. Persistent organochlorine compounds withdrawn from the market were all characterized by log $K_{ow} > 4$. Microcystins however contain polar groups, such as carboxylic acids, amino and amido functions. Even if these compounds are hydrophobic, we therefore cannot expect them to behave like non-polar organochlorine compounds, since the combination of high hydrophobicity and polar functions leads to an easy formation of micelles and a possible formation of ion-pairs.

In reversed-phase chromatography, correlation exists between the retention factors measured on ODS with water as the eluent (k_w) and the water–octanol partition coefficient, for a range of com-

pounds having very different polarities and chemical properties [22,23]. Therefore, reversed-phase LC was endorsed by the OECD (Organisation for Economic Cooperation and Development) council in 1989 for indirect measurements of K_{ow} [24]. It is impossible to measure k_w values directly in water for hydrophobic compounds, so that k_w is extrapolated from the linear relation which exists between log k data in water–methanol mobile phases and the methanol content of this mobile phase.

The variations in the retention factors of microcystins depending on the content of organic solvent in the eluent have been measured and values of log k_w were obtained by extrapolation to a zero methanol content of the curves representing log k versus the methanol proportion in the eluent. Experiments were performed in neutral media. The results are shown in Table 1. Log k_w values were very close for the three toxins, about 4, with a margin of error estimated to ±6%. Microcystins are therefore rather hydrophobic, despite their neutral or anionic character (according to the structure) at pH 7.

3.1.3. Solubility

Owing to the very small amount of toxins, it was difficult to measure the solubility by introducing increasing amounts of toxin in a given volume of water until saturation and visible precipitation. We therefore added ultrapure water to known amounts of toxins introduced in a microvial and we assessed the dissolution by chromatographic measurement of peak areas, using a calibration in methanol as reference. At a theoretical concentration of 2 g/l, 90±3% of microcystin-LR was dissolved in water, and 96±3% was dissolved in water at 1 g/l. Although we are unable to provide a solubility value, we can conclude that microcystins are soluble in water, the solubility of microcystin-LR being higher than 1 g/l.

3.1.4. Adsorption on particulate matter and on sandy sediments

It is well known that non-polar pollutants with similar log P_{oct} values and non-polar structures such as polyaromatic hydrocarbons or some organochlorine pesticides tend to stick everywhere. In surface water, they are more found adsorbed onto the particulate matter than in the dissolved state. Their detection in sediments is very common. However, the polar moieties in the structure of microcystins – and the possibility of bearing a negative charge for some of them – can give rise to a different behavior. Thanks to hydrophobic interactions and interactions with the polar groups, microcystins can interact with humic and fulvic substances, suspended particulate matter or sediments. The proportion of microcystins adsorbed was evaluated using river water at pH 6.7 containing naturally suspended particles or with addition of sandy sediments. The samples were spiked with 5 μg/l of microcystins, which corresponds to a moderate contamination of water, since concentrations of 0.06 to 200 μg/l in lake or river waters have indeed been reported [25,26]. Measurements performed after 1 h showed that 1±0.1% of microcystin-YR and 0.9±0.1% of microcystins-LR and -RR was adsorbed on the sandy sediment. Owing to the slow kinetics of the adsorption phenomenon, the equilibrium was not reached. The proportion of toxins adsorbed after three days is reported in Table 1, the rest being found in water. It turns out that microcystins interact with suspended particulate matter to a small extent, since about 10% of the whole amount is adsorbed, but the major part remains in water. The same conclusion can be drawn from the adsorption of microcystins on the sandy sediment. This is consistent with the physicochemical properties measured, namely water solubility and hydrophobicity in combination with polar groups. Toxins released by cyanobacteria are suspected to

Table 1
Some physicochemical data for microcystins: extrapolated values of log k_w, pK_a and percentage of microcystins adsorbed on suspended particulate matter or on a sandy sediment ($n=2$)

	log k_w pH 7	pK_a	% Adsorbed (particles)	% Adsorbed (sandy sediment)
Microcystin-YR	3.9±0.2	3.4±0.2	13±2	7±2
Microcystin-LR	4.2±0.3	3.3±0.2	11±2	7±1
Microcystin-RR	4.4±0.3	3.4±0.2	9±3	8±2

remain in water, probably at low concentration depending on the size of the reservoir. However, accumulation risk exists owing cell sedimentation. It becomes significant when the microorganisms proliferate and form blooms.

3.2. Determination of microcystins in environmental aqueous samples

The direct HPLC analysis of aqueous samples does not allow the detection of microcystins at trace level. An enrichment of the sample in toxins is needed, which is achieved by a simultaneous extraction and concentration using SPE on an ODS cartridge. Two HPLC methods were employed thereafter, namely micro-LC for drinking water and cyanobacterial samples, and classical LC for drinking and surface water.

3.2.1. SPE optimization

The results obtained in the first part can help in determining the optimal conditions for trace enrichment on a SPE cartridge. A critical parameter in SPE is the breakthrough volume of the analyte V_b, which corresponds to the sample volume that can be percolated through the cartridge without loss of the analyte. It was shown that the extraction parameters using ODS can be predicted from k_w or from the octanol–water partition coefficient K_{ow}, since these values are correlated and very close [22]. As an example, the V_b value was evaluated and measured to 15 ml, 300 ml, 1.1 l and 12 l for compounds having log K_{ow} values of 2.1, 3.15, 4.1 and 5.0, respectively, on a cartridge containing 100 mg of ODS [27]. Therefore, there is no problem for the trace enrichment of microcystins on an ODS cartridge. Moreover, their high hydrophobicity enables the addition of a clean-up in the extraction sequence.

A small proportion of methanol was also added to the sample in order to avoid the simultaneous concentration of the more polar interferents on the cartridge. The retention factors of microcystins are then slightly reduced in comparison to the elution with pure water. With 5% of methanol in the sample, the logarithm of the retention factor at pH 7 can be estimated to 3.6, 4.0 and 4.2 for microcystins-YR, -LR and -RR, respectively, which remains considerable.

The sample volume percolated on the cartridge (V_p) can be determined according to the detection limits required. In order to get a quantification limit related to the toxicity, namely 0.1 μg/l, a detection limit of 0.01 μg/l (C_{lim}) in the aqueous sample is required. The detection limit (q_{lim}) of the chromatographic system, defined for a signal-to-noise ratio equal to three, is about 10 pg for each microcystin in the micro-LC system and 2 ng in the classical LC system. The minimal volume (V_{min}) of a sample containing microcystins at C_{lim} that must be percolated has then to contain an analyte amount of at least q_{lim}. In our off-line SPE–LC procedure, only part of the whole sample extract obtained after enrichment is injected into the chromatograph. The minimal volume is then equal to $(q_{lim}/C_{lim})\cdot(V_{extract}/V_{inj})$, the term $V_{extract}/V_{inj}$ representing the ratio of the volume in which the extract obtained after preconcentration is dissolved ($V_{extract}$) to the injection volume of the chromatograph (V_{inj}). For the micro-LC analysis, the extract is dissolved in 50 μl and only 200 nl are injected, so that the minimal percolated volume must be 250 ml. For classical LC analysis, the extract is dissolved in a minimum volume of 100 μl for a 50 μl injection, so that the minimal percolated volume must be 500 ml. The benefit in the injection ratio due to a higher loop volume in classical LC makes up for the lower detection limit in the micro-LC system, which results in V_{min} volumes of the same order. In practice however, the effect of the sample matrix must be taken into account because the presence of interfering compounds makes the method less sensitive and increases the C_{lim}. We chose to preconcentrate 500 ml of sample.

The selection of the pH of the sample is important. Higher retention factors will be obtained when acidifying water samples with perchloric acid. However, river water, and, to a lesser extent, drinking water, contain organic matter consisting mainly of humic substances. At acidic pH, humic and fulvic acids are coextracted on the cartridge, thus resulting in a large hump of polar interferents at the beginning of the chromatogram. At neutral pH, humic substances are not coextracted, probably because they form colloids that are excluded from the stationary

phase and because they become ionic. The effect of the sample pH is illustrated by the chromatograms A and B in Fig. 3, obtained for surface water at pH 2 and 7. The preconcentration of humic substances was partly avoided by the choice of a neutral pH for the percolated sample. The retention of microcystins remains largely sufficient at this pH (see the log k_w above) to percolate a 500 ml sample without loss.

The desorption conditions of microcystins from the cartridge must be optimized. The SPE sorbent was chosen to enable the percolation of a large aqueous volume (100 ml to 1 l) without loss of analyte during the extraction step and to allow the analyte desorption by a small volume (some millilitres) of a suitable solvent during the desorption step. ODS, selected because it strongly retains hydrophobic compounds in presence of water, weakly retains these compounds in presence of an organic solvent. In order to minimize the desorption volume so as to obtain a high enrichment factor, pure organic solvents such as methanol or acetonitrile are generally employed. According to the literature which describes the SPE using cartridges packed with ODS, microcystins can be desorbed from 1 g of sorbent using 30 ml of 10% (v/v) water–methanol [13] or using 3 ml of methanol acidified with TFA [12]. In our study, 500 mg of sorbent was employed. Pure methanol, methanol basified with 0.06% of ammonia and methanol acidified with 1% of TFA were tested for the desorption of microcystins. The composition of each desorption milliliter was analysed using micro-LC. The recoveries in each fraction, defined as the ratio between the amount of analyte extracted in each fraction to the amount percolated on the cartridge, are reported in Table 2. The three solvents allow the complete desorption of microcystins in less than 5 ml. However, a 4 ml volume of methanol or basified methanol was needed to desorb all of the microcystins from the cartridge, whereas 2 ml of acidified methanol were sufficient to recover all of the toxins. This was certainly due to an increase in the solubility of microcystins in acidified methanol. In order to minimize the volume, microcystins were subsequently desorbed from the cartridge using 3 ml of methanol acidified with 1% of TFA.

Owing to the large k_w values of microcystins, a clean-up step can be included in the SPE sequence. For surface water samples containing a high level of

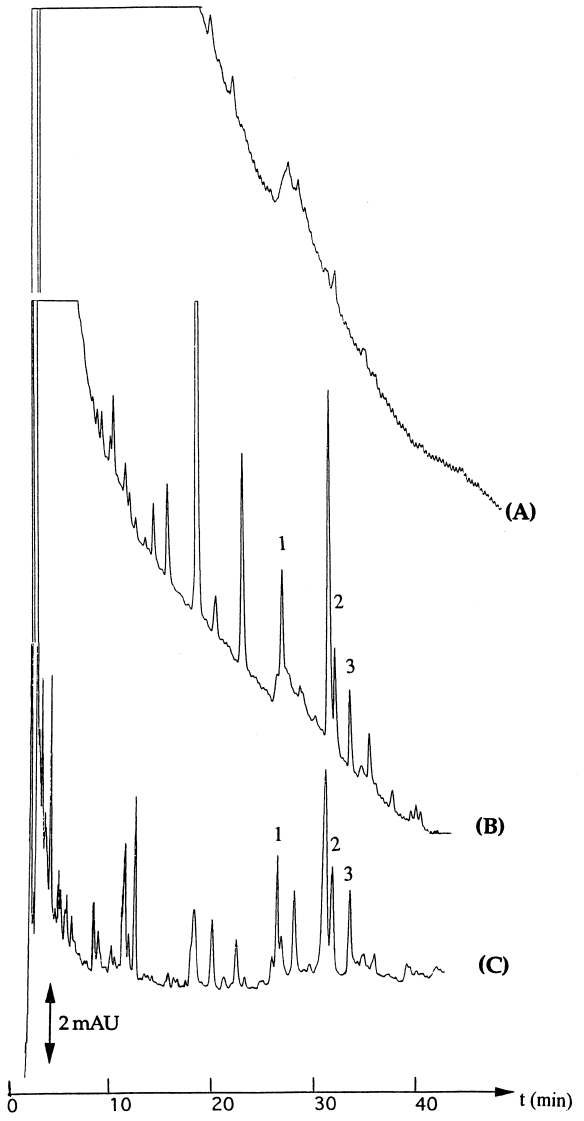

Fig. 3. Effect of the sample pH and the clean-up in the SPE for the analysis of 500 ml of surface water using SPE–HPLC: (A) pH 2, non-spiked, without any clean-up, (B) pH 7, spiked with 0.5 μg/l of each toxin, without any clean-up, (C) pH 7, spiked with 0.5 μg/l of each toxin, with a clean-up with 5 ml of 30% (v/v) aqueous methanol [microcystin-RR (1), microcystin-YR (2) and microcystin-LR (3)]. Preconcentration: Bakerbond SPE cartridge packed with 500 mg ODS; injection volume: 20 μl; column: 25 cm×4.6 mm I.D. packed with 5 μm Hypersil BDS ODS; mobile phase: gradient (see Section 2.5) of acetonitrile–phosphate buffer $5 \cdot 10^{-3}$ *M* acidified to pH 2; flow-rate: 1 ml/min; detection: UV at 238 nm.

Table 2
Microcystin recoveries, expressed as the percentage of the amount preconcentrated, in successive fractions eluted from a Bakerbond SPE cartridge using different eluents

Elution volume	1st ml	2nd ml	3rd ml	4th ml	5th ml	Total
Basicified methanol						
Microcystin-YR	27	54	11	2	–	94
Microcystin-LR	17	65	12	2	–	96
Microcystin-RR	6	48	28	5	2	89
Methanol						
Microcystin-YR	56	33	5	2	–	96
Microcystin-LR	39	47	6	3	–	96
Microcystin-RR	59	25	2	–	–	86
Acidified methanol						
Microcystin-YR	85	1	–	–	–	86
Microcystin-LR	84	1	–	–	–	85
Microcystin-RR	96	3	1	–	–	100

organic material, this additional step enables the elimination of some interferents before the analysis. After the percolation of the sample, the cartridge is washed with a small volume of an hydroorganic mixture of low eluent strength. Care must be taken that no loss in recovery occurs. Successive clean-ups with 10 ml of 10, 20, and 30% (v/v) methanol–water mixtures were employed by Lawton et al. [12]. The clean-up must be optimized so as to eliminate most of the interferents without desorbing the microcystins. We therefore evaluated the effect of separate clean-ups using 5 ml of 20, 30 and 40% (v/v) of methanol–water mixtures. Whatever the clean-up, no loss in recovery was noticed, as reported in Table 3. Recoveries of about 75% are satisfactory considering the number of steps implemented. The higher recovery observed for microcystin-YR with the 40% clean-up results from its coelution with an interferent. The chromatogram presented in Fig. 3C shows the efficiency of the 30% methanol–water clean-up. The hump of interferents is sufficiently reduced with this clean-up, which was then retained for all subsequent experiments.

3.2.2. Application to drinking and surface water

This method was applied to the analysis of microcystins in drinking water and various surface waters. Its performances were evaluated with respect to repeatability, linearity and detection limits and are reported in Table 4.

The repeatability of the SPE procedure was tested using the extraction recoveries obtained with three 500 ml drinking water samples spiked with 0.5 μg/l of each microcystin standard. The relative standard deviation (R.S.D.) ranges from 5 to 8, which is satisfactory considering the number of steps implemented.

Calibration curves were drawn near the detection limit for 500 ml of drinking water at concentrations ranging from 0.1 to 1 μg/l. The correlation coefficients are satisfactory.

The detection limits, defined for a signal-to-noise

Table 3
Microcystin recoveries (%) from the SPE extraction of 500 ml of drinking water spiked with 1 μg/l of each microcystin, followed by different clean-ups ($n=2$)

Clean-up solvent	Microcystin-RR	Microcystin-YR	Microcystin-LR
5 ml, 20% methanol–water	73±8	82±4	89±4
5 ml, 30% methanol–water	76±4	78±3	78±3
5 ml, 40% methanol–water	74±7	108±8	76±6

Table 4
Analytical data for microcystins in SPE–LC

	Microcystin-RR	Microcystin-YR	Microcystin-LR
Relative standard deviation at 0.5 μg/l ($n=3$)	8.2%	5.3%	4.7%
Calibration curve equation[a]	$y=0.4+14.1x$	$y=0.7+13.7x$	$y=0.3+14.1x$
Correlation coefficient	0.997	0.997	0.995
Detection limits (μg/l) ($S/N=3$)			
Drinking water	0.03	0.03	0.03
Surface water	0.1–0.2	0.1–0.2	0.1–0.2

[a] Equation of the calibration curve: y = peak height, in arbitrary units, x = concentration, in μg/l.

ratio equal to three, were measured on the peak heights at 238 nm. Using this method, it proves possible to detect 30 ng/l of each microcystin in drinking water and 0.1–0.2 μg/l in surface water, depending on the matrix. As an example, Fig. 4 shows the analysis after SPE of 500 ml of drinking water (Fig. 4A) and surface water (Fig. 4B), both spiked with microcystins at 0.1 μg/l. This concentration corresponds to the detection limit in surface water, but is still above that value in drinking water. The detection limit can be reduced by increasing the injection volume to at least 50 μl. The detection limits reported here correspond to the SPE–classical LC system. They are similar to those obtained using the SPE–micro-LC system, as expected from respective detection limits (in pg) and

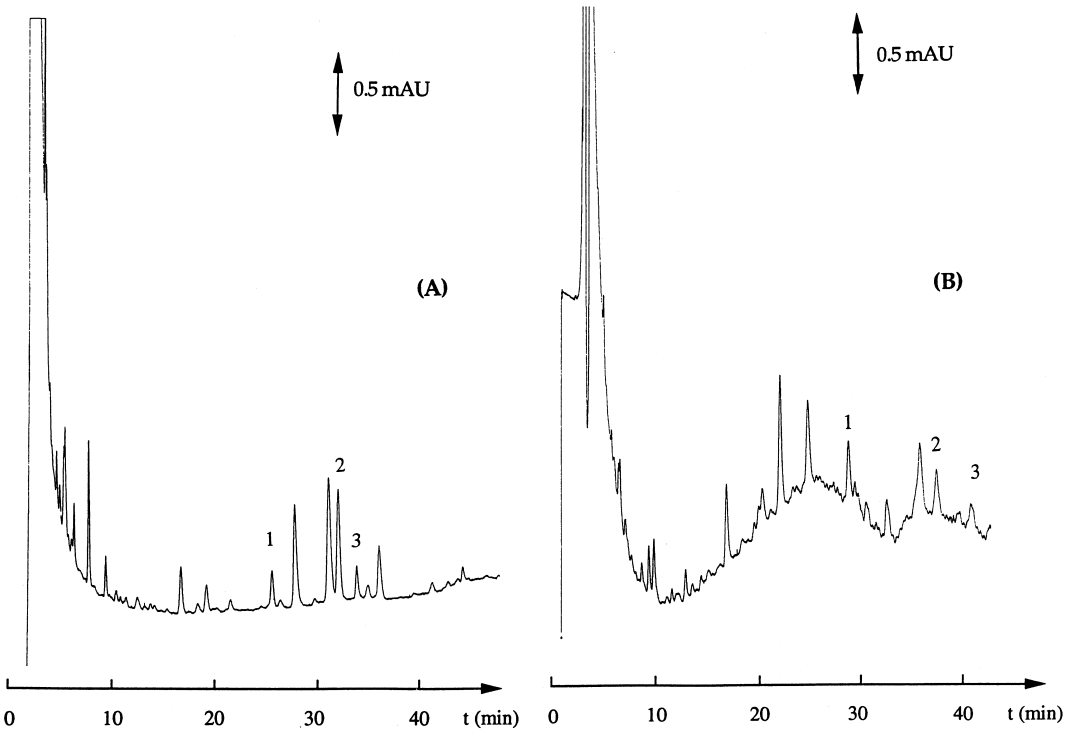

Fig. 4. Analysis of 500 ml of (A) drinking water and (B) surface water spiked with 0.1 μg/l of each toxin using SPE–HPLC [microcystin-RR (1), microcystin-YR (2) and microcystin-LR (3)]. Experimental conditions as in Fig. 3C, except the elution gradients (see Section 2.5).

the enrichment ratio. These values meet the suggested limits of 0.1 μg/l in drinking water for a long term exposure [11].

Table 5 shows that recoveries are similar in drinking and surface water samples.

In Fig. 4A, a coeluted compound interfered with the microcystin-YR peak. Its presence was detected by the modification of the elution conditions (the gradient used for the detection limit in surface water was then employed), which enabled the separation of its peak from the microcystin-YR peak. Interferences are an inherent problem in any method that is employed alone to assess unknown samples. Here the elution gradient was modified to separate the interferent from the toxin. When both compounds have the same retention time, the use of DAD enables the comparison of the UV spectrum of the unknown compound to that of the toxin. Moreover, confirmation of the presence or the absence of toxins can be obtained using other methods such as an enzyme-linked immunosorbent assay (ELISA) or an enzyme inhibition test, which will be described in detail in another paper.

3.3. Determination of the microcystin-LR content in cultures of cyanobacteria

The methodology developed above was employed to follow through the toxin content in cultures of cyanobacteria. The conditions required for a cyanobaterial strain to synthesize toxins are not well known. Natural blooms of the same species do not show the same toxicity and the toxicity of a given species can vary during time. Strains of *Microcystis aeruginosa* known to produce toxins in Europe were chosen as to study the variation in the toxic content according to the growth stage. After filtration to separate cell material from culture media, cellular components were extracted in methanol and the culture media underwent the same treatment as water samples. Both methanolic extracts and culture media were then assessed for the microcystin standards using micro-LC followed by DAD. Micro-LC was employed rather than classical LC to minimize the injected amount. Toxins were identified by spiked samples and comparison of their retention time and their UV spectrum to that of the standards.

Microcystin-LR was present in each sample. On the other hand, no trace of microcystin-YR nor -RR was detected. The variations in the microcystin-LR concentration in the cells and in the medium according to the growth curve are reported in Fig. 5 (A, B and C, respectively). At the five day stage, a small amount of toxin is present in both cells and medium (2.3 ± 0.5 μg/g and 11 ± 1 μg/l, respectively). A sharp increase in the toxin production can be observed from the 15th day to the 22nd day (and maybe later) when the cellular content reaches 43 ± 5 μg/g. The start of the toxin mass production corresponds to the middle of the exponential growth phase. This suggests that there is no correlation between the toxin content and the biomass. During this time, the toxin essentially accumulates inside the cyanobacteria. Only 4% of the total toxin amount is found outside the cells at the 22nd day stage, as shown in Fig. 5D. The leakage of microcystin-LR does not evolve a lot since its content in the liquid medium only increased to 17 ± 2 μg/l at the 18th day. During

Table 5
Recoveries (%) (±relative standard deviation) of microcystins in 500 ml of drinking and surface water samples, spiked with different concentrations (μg/l) of each toxin ($n=3$)

Sample	Concentration (μg/l)	Microcystin-RR	Microcystin-YR	Microcystin-LR
Drinking water	1	76±4	78±3	78±3
	0.8	78±5	83±3	79±3
	0.5	74±8	82±5	70±5
	0.3	74±8	93±7	75±5
	0.1	106±15	101±10	98±9
Surface water	1	104±8	95±5	91±6
	0.5	126±12	92±5	100±7
	0.1	111±17	100±14	61±20

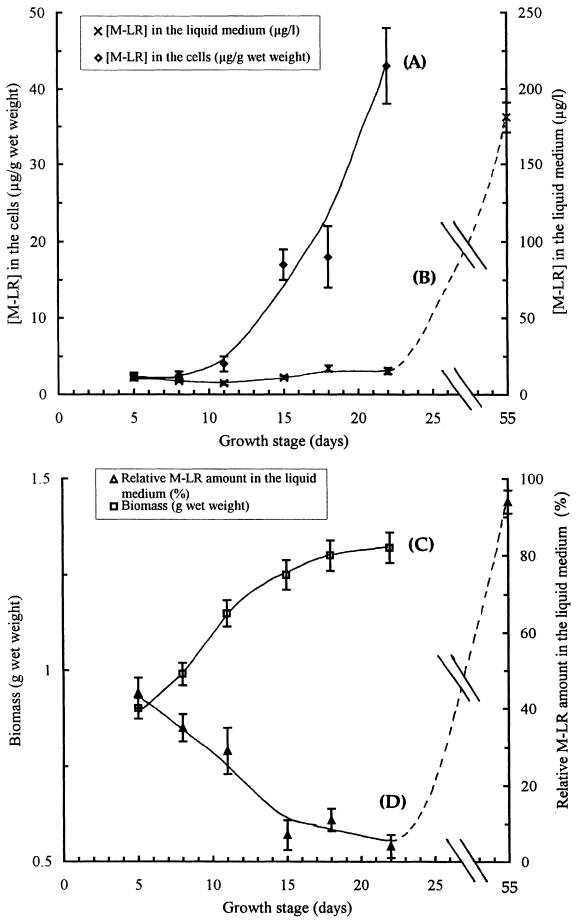

Fig. 5. Variations in microcystin-LR content in cellular extracts (µg/g wet mass) (A) and in culture media (µg/l) (B), in biomass (g wet mass) (C) and in the relative amount released in water to the total microcystin-LR amount (D), during the growth of the cyanobacterium *Microcystis aeruginosa* PCC7806.

the three week study, the toxin concentration in the liquid is then not proportional to the cellular content. When the cyanobacteria get older however, cell lysis enables the release of the toxin outside the cells. In cultures of *Microcystis* harvested two weeks after the beginning of the decline phase, microcystin-LR was identified in the media at concentrations of 170±10 and 280±15 µg/l in the PCC7806 and the PCC7813 strains, respectively. Fig. 6 shows the analysis of the culture medium of the *Microcystis aeruginosa* PCC7806 strain harvested at this stage. The toxin was also quantified in the cells [15], and the concentrations in the media corresponded to 94% and 99% of the total toxin amount found in the PCC7806 and the PCC7813 strains, respectively. For old cultures, the main part of microcystin-LR synthesized by *Microcystis* strains is thus found in water, clearly indicating a toxin release in the medium when the cells die.

It must be underlined that both strains of *Microcystis* probably synthesize other microcystins than microcystin-LR. Two reasons support this assessment. On one hand, some compounds have UV spectra similar to that of microcystins standards, with a maximal absorbance at 238 nm, as shown in Fig. 6. We can actually expect that other microcystins will present UV spectra very close to the ones of the standards, because we noticed that the spectra of the three standards were similar. On the other hand, a rapid ELISA test seems to confirm the presence of other microcystins than microcystin-LR in the media. One of these probable microcystins (compound 6), more hydrophobic than microcystin-LR, is detected from the 18th growth day at 0.9±0.2 µg/g (wet mass) in the cells and is present in the culture medium of old cultures at 10±2 µg/l. This shows that this compound undergoes the same process of production and release in the medium as microcystin-LR. Identification using LC–mass spectrometry (MS) is required to confirm this result. This shows the real need of having other microcystin standards.

4. Conclusions

The physicochemical parameters of microcystins have been measured using micro- and classical LC in order to have a better understanding of their environmental behaviour and to optimize their trace enrichment in water. However, the main problem encountered in microcystin analysis is the lack of standards for confirmation. Microcystin determination cannot be performed using only the three standards available. Our results indicate that other microcystins and/or transformation products are produced by cyanobacterial strains commonly found in Europe. Besides the necessary HPLC–MS coupling for identification, isolation and commercialization of the microcystins identified in strains of

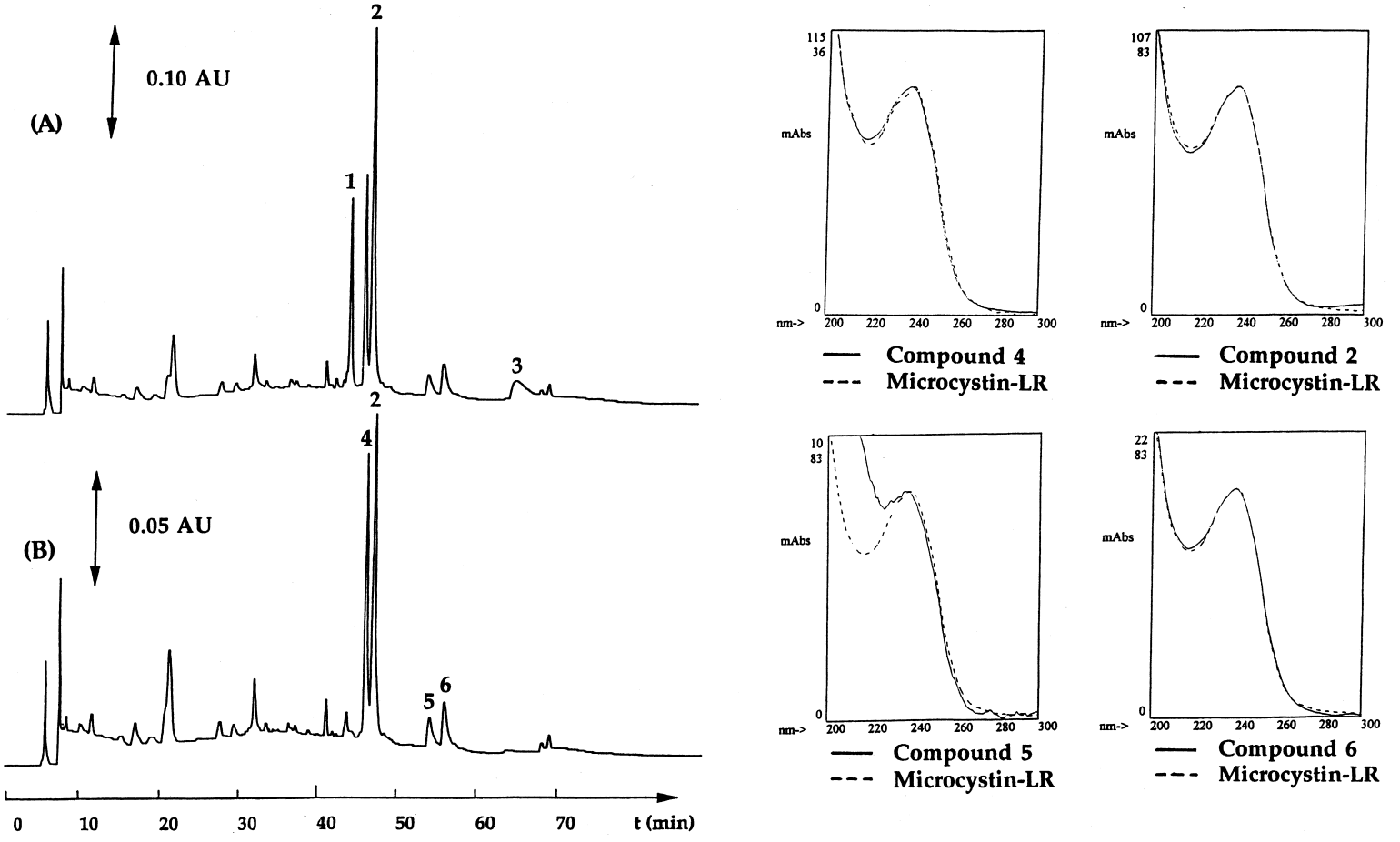

Fig. 6. Analysis by SPE–micro-LC of the culture medium of *Microcystis aeruginosa* PCC7806: (A) non-spiked, (B) spiked with 20 ng of each toxin [microcystin-YR (1), microcystin-LR (2), microcystin-RR (3)]. Experimental conditions as in Fig. 3C except sample volume (130 ml), injection volume: 200 nl; column: 20 cm×0.32 mm I.D. packed with 5 μm BioSil ODS; mobile phase: gradient (see Section 2.5) of acetonitrile–phosphate buffer $5 \cdot 10^{-3}$ *M* acidified to pH 2; flow-rate: 3.5 μl/min.

cyanobacteria commonly found in the environment is required for water quality monitoring.

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References

[1] G.A. Codd, S.G. Bell, W.P. Brooks, Water Sci. Technol. 21 (1989) 1.
[2] W.W. Carmichael, in C.L. Owny and G.V. Odell (Editors), Proceedings of the 9th World Congress on Animal, Plant and Microbial Toxins, Stillwater, OK, 1988, Pergamon Press, Oxford 1989, p. 3.
[3] W.W. Carmichael, Scientific Am. January (1994) 78.
[4] W.W. Carmichael, J. Appl. Bacteriol. 72 (1992) 445.
[5] F.D. Galey, V.R. Beasley, W.W. Carmichael, G. Kleppe, S.B. Hooser, W.M. Haschek, Am. J. Vet. Res. 48 (1987) 1415.
[6] L.A. Lawton, G.A. Codd, J. Institute Water Environ. Manag. 5 (1991) 460.
[7] R.E. Honkanan, B.A. Codispoti, K. Tse, A.L. Boynton, Toxicon 32 (1994) 339.
[8] R. Nishiwaki-Matsushima, T. Otha, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W.W. Carmichael, H. Fujiki, J. Cancer Res. Clin. Oncol. 118 (1992) 420.
[9] R. Matsushima, S. Yoshizawa, M.F. Watanabe, K.-I. Harada, M. Furusawa, W.W. Carmichael, H. Fujiki, Biochem. Biophys. Res. Commun. 171 (1990) 867.
[10] I.R. Falconer, M.D. Burch, A. Steffensen, M. Choice, O.R. Coverdale, Environ. Toxicol. Water Qual.: Int. J. 9 (1994) 131.
[11] I.R. Falconer, Proceedings of the Symposium Eutrophication, Causes, Consequences and Remediation, Porto, 21–23 May 1995, p. 8.
[12] L.A. Lawton, C. Edwards, G.A. Codd, Analyst 119 (1994) 1525.
[13] K. Tsuji, S. Naito, F. Kondo, M.F. Watanabe, S. Suzuki, H. Nakazawa, M. Suzuki, T. Shimada, K.-I. Harada, Toxicon 32 (1994) 1251.
[14] M.-C. Hennion, V. Pichon, Environ. Sci. Technol. 28 (1994) 576.
[15] C. Rivasseau, M.-C. Hennion, P. Sandra, J. Microcol. Sep. 8 (1996) 541.
[16] A.J. van der Westhuizen, J.N. Eloff, Planta 163 (1985) 55.
[17] K. Sivonen, Appl. Environ. Microbiol. 56 (1990) 2658.
[18] P. Gorham, in D.F. Jackson (Editor), Algae and Man, Plenum Press, New York, 1964, p. 307.
[19] M.F. Watanabe, K. Harada, K. Matsuura, M. Watanabe, M. Suzuki, J. Appl. Phycol. 1 (1989) 161.
[20] R.Y. Stanier, R. Kuniswa, M. Mandel, G. Cohen-Bazire, Bacteriol. Rev. 35 (1971) 171.
[21] E.P. Serjeant and B. Dempsey, Ionisation Constants of Organic Acids in Aqueous Solution, IUPAC Chemical Data Series, No. 23, Pergamon Press, 1979, p. 177.
[22] T. Brauman, J. Chromatogr. 373 (1986) 191.
[23] R. Kaliszan, J. Chromatogr. A 656 (1993) 417.
[24] OECD Guidelines for Testing of Chemicals, Partition Coefficients (*n*-octanol–water), High-Performance Liquid Chromatography Method, No. 117, Paris, 1989.
[25] Y. Ueno, S. Nagata, T. Tsutsumi, A. Hasegawa, M.F. Watanabe, H.-D. Park, G.-C. Chen, G. Chen, S.-Z. Yu, Carcinogenesis 17 (1996) 1317.
[26] C.M. McDermott, R. Feola, J. Plude, Toxicon 33 (1995) 1433.
[27] M.-C. Hennion and P. Scribe, in D. Barcelo (Editor), Environmental Analysis – Techniques, Applications and Quality Assurance, Vol. 13, Elsevier, Amsterdam, 1993, p. 24.