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Journal of Chromatography A, 1074 (2005) 23–30
JOURNAL OF CHROMATOGRAPHY A
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# Optimization of intracellular microcystin extraction for their subsequent analysis by high-performance liquid chromatography

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Received 10 January 2005; received in revised form 18 March 2005; accepted 22 March 2005
Available online 11 April 2005

**Abstract**

Microcystins are a family of heptapeptide hepatotoxins produced by some genera of cyanobacteria. These toxins have been responsible for the illness and death of both animals and humans. Due to their hazard to human health, extraction of all intracellular microcystin variants is required to characterize and quantify all microcystins present in a sample. To date, there is little work reported comparing results obtained with different extraction methods. Findings reported to date indicate that selection of solvent will vary depending on sample and its microcystin contents. In the present work, a wide range of extraction volumes and solvents were evaluated over a range of pH and extraction times in order to optimize a suitable method for the extraction of a wide range of microcystins. The number of extractions required was also studied. This study was carried out using mainly two laboratory cultures which contain microcystin variants with quite different hydrophobicities. This is the first time that the most commonly used solvents for intracellular microcystin extraction have been studied in detail.


*Keywords:* Cyanobacteria; Microcystins; Nodularins; Intracellular; Extraction; Optimization; HPLC

## 1. Introduction

Cyanobacteria can proliferate rapidly to form blooms and scums under favorable conditions in eutrophic fresh, brackish and marine waters throughout the world. Some bloom-forming cyanobacteria may produce potent toxins, microcystins being the most common hepatotoxins. Microcystins are a family of hepatotoxic peptides produced primarily by freshwater cyanobacteria of *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*) and *Nostoc* genera.

Microcystins have a general structure (Fig. 1) containing three D-amino acids (alanine, β-linked *erythro*-β-methyl-aspartic acid and γ-linked glutamic acid), two variable L-amino acids, $R_1$ and $R_2$, and two unusual amino acids, *N*-methyldehydroalanine (Mdha) and (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-10-phenyl-2, 6, 8-trimethyldeca-4(*E*),6(*E*)-dienoic acid (Adda) [1]. To date, more than 70 microcystin variants have been identified [2].

Microcystins have been proved to cause liver damage as well as tumour promotion [3]. Their toxicity is based on their potent inhibition of protein phosphatases 1 and 2A, two key enzymes in cellular regulation [4]. These toxins have caused illness and death of not only animals but also humans [5–7]. The occurrence of microcystins in water bodies used for aquaculture, recreation and potable water supplies may also constitute a hazard to human health through contact and ingestion of cells or released toxins [8,9].

Considering human health hazards represented by microcystins, the World Health Organization published a provisional guideline level of $1\,\mu g\,l^{-1}$ of total microcystin-LR (intracellular and extracellular) [10]. Therefore, a reliable procedure to extract all microcystins present in a sample is needed in order to evaluate the total microcystin content of a given sample. Nevertheless, there is no agreement about the most suitable method for the extraction of intracellular microcystins, with a wide range of solvents, temperatures, times,

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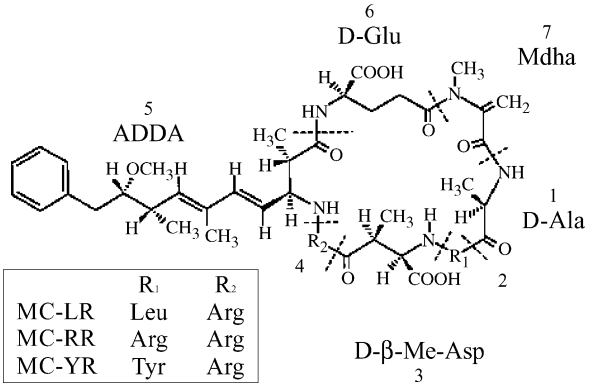
doi:10.1016/j.chroma.2005.03.087




| | $R_1$ | $R_2$ |
|---|---|---|
| MC-LR | Leu | Arg |
| MC-RR | Arg | Arg |
| MC-YR | Tyr | Arg |

Fig. 1. General structure of microcystins.

volumes and extraction techniques described, often with no published report of their efficiency. Furthermore, only few reports comparing different extraction methods are found in the literature [11–13]. Recently, a review on methods for purification of microcystins has been published [14].

First reports on the purification of microcystins described the use of 0.1 M ammonium bicarbonate (pH 8.4) [15,16]. Since Siegelman et al. described the use of 1-butanol:methanol:water (5:20:75, v/v) in 1984 [17], many researchers have reported its use to date [18,19]. On the other hand, 5% aqueous acetic acid (v/v, pH 2.7) has been widely used after Harada et al. published its use in 1988 [20]. Nevertheless, it has been demonstrated that acetic acid is less efficient than butanol:methanol:water [11], water, methanol and 70% methanol [12] for microcystin extraction, as microcystin hydrophobicity increases as pH decreases [21]. This finding showed that pH may be an important factor in microcystin extraction. Some authors have also studied sequential extraction where samples were first extracted in a given solvent and cell pellets re-extracted in a different solvent (e.g. 0.05 M acetic acid:ethanol followed by 5% acetic acid; 5% acetic acid–methanol; water–methanol) [22–24].

Another factor affecting the extraction yield is the extraction method. In the literature, extraction methods range from initial mixing and allowing to stand for a given time, continuous stirring or shaking and sonication.

Although volume of extraction to mass of cells ratio may influence extraction efficiency, there are no reports on the evaluation of the optimum ratio. One of the most commonly reported ratios is around 10 g of dry cells per 200 ml of solvent with the highest and the lowest ratios being 10 g per 100 ml [15] and 10 g per 1000 ml [25], respectively.

Extraction times ranging from few minutes to 15 h [26] per extraction have been found in the literature. This parameter will determine total time of sample processing and may affect microcystin stability. Coyle and Lawton [27] compared 5, 30 and 60 min extractions and found that the best yields were obtained when extracting for 60 min, although 85–97% of microcystins were extracted in 30 min [27].

Extraction temperature reported in the literature normally ranges from 4 °C [28] and room temperature. However, Wirsing et al. [29] studied microcystin yields when extracting with 5% acetic acid at 20 and 40 °C, with no significant differences observed. Recently, Metcalf and Codd [30] evaluated microcystin and nodularin extraction using boiling waterbath and microwave extraction (1 and 9 min, respectively) with no degradation reported.

Many authors re-extracted the sample with the same solvent a number of times, generally three times, and, then, pooled the extracts [13]. Since only a few percent of the total microcystin contents was present in the third extract, a total of three extractions seems to extract efficiently intracellular microcystins from cyanobacterial cells [11,27].

In this work, the most common solvents used for intracellular microcystin extraction were evaluated. Furthermore, parameters affecting extraction yield, such as extraction pH, volume, method (discontinuous shaking, no shaking, sonication), time for each extraction method and number of extractions were studied and optimized. Stability at the optimum pH values was also studied.

## 2. Experimental

### 2.1. Chemicals

All chemicals were of analytical-reagent or high-performance liquid chromatographic (HPLC) grade unless stated and obtained from Merck (Darmstadt, Germany). Acetonitrile and methanol were purchased from Rathburn (Walkerburn, UK). Trifluoroacetic acid was from Fisons (Loughborough, UK). High-purity water was obtained from a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Microcystin standards were purified from cultured cyanobacterial cells as previously described [23].

### 2.2. Cyanobacterial material

Cells of *Microcystis aeruginosa* from a water bloom at Rutland Water (Leicestershire, UK) in September 1989 were kindly provided by Anglian Water (Cambridge, UK). Batch cultures of *M. aeruginosa* PCC 7820 (Pasteur Culture Collection, Paris, France), *M. aeruginosa* SCIENTO (Manchester, UK), *M. viridis* NIES-102 (Microbial Culture Collection, National Institute for Environmental Studies, Japan) and *Nodularia spumigena* PCC 7804 (Pasteur Culture Collection, Paris, France) were grown in BG-11 medium [31] plus nitrate (8.8 mM) under continuous illumination and sparging with sterile air. Cells were harvested after approximately 5 weeks growth by tangential flow filtration (Pellicon-2; fitted with three 0.22 μm type GVPP-V filters, Millipore), freeze-dried and stored at −20 °C until required.

### 2.3. Analytical HPLC

Identification and quantification of microcystins and nodularins were performed by HPLC with high-resolution

diode array detection using a Waters 996 detector (Waters Corporation, Milford, MA, USA). Analytes were separated on a Waters Symmetry $C_{18}$ column, 250 mm × 4.6 mm i.d. × 5 μm particle size (Waters Corporation, Milford, MA, USA). Mobile phases were Milli-Q water and acetonitrile, both containing 0.05% (v/v) trifluoroacetic acid (TFA). Chromatographic separation was achieved at a flow rate of 1 ml/min using a linear gradient starting at 30% aqueous acetonitrile increasing to 35% over the next 10 min followed by an increase to 70% over the next 30 min. Detector resolution was set at 1.2 nm and data acquired from 200 to 300 nm [11]. Microcystins were identified on the basis of both their retention time and characteristic UV spectra. Microcystins were quantified using microcystin-LR as standard.

### 2.4. Optimization of intracellular microcystin extraction conditions

#### 2.4.1. Optimization of the extraction solvent

Freeze-dried cells (25 mg) from each culture, *M. aeruginosa* PCC 7820 and SCIENTO, were extracted three times with 1 ml of solvent for an hour with shaking every 10 min. Experiments were performed in triplicate. The solvents evaluated were water, methanol, butanol:methanol:water 1:4:15 (v/v), 5% acetic acid, 0.1 M ammonium hydrogen carbonate, acidified methanol (0.1% TFA, v/v) and 10–90% aqueous methanol. Different methanol–water extraction sequences were also studied: methanol–water–methanol–water, water–methanol–water–methanol, water–water–methanol–methanol and methanol–methanol–water–water. Finally, water with two different kinds of surfactants (0.1% Triton X-100 and 0.1% Tween-20) was evaluated. Extracts corresponding to each extraction step were analyzed separately by HPLC/DAD as described in Section 2.3.

#### 2.4.2. Optimization of the extraction pH

Methanolic extraction at different pH values, ranging from pH ∼ 1 (0.5% TFA) to pH ∼ 6 (0.000005% TFA), were studied following the experimental procedure detailed above in order to optimize the extraction pH.

#### 2.4.3. Stability at pH ∼ 1 and ∼2 at room temperature over 48 h

Freeze-dried cells (50 mg) from each culture were extracted with 1 ml of acidified methanol (pH ∼ 1 and ∼2) for an hour with shaking every 10 min. Extracts corresponding to a specific culture were analyzed by HPLC/DAD every 2 h.

#### 2.4.4. Optimization of the extraction volume

Freeze-dried cells of *M. aeruginosa* PCC 7820 and SCIENTO, *M. viridis* NIES-102 and *N. spumigena* PCC 7804 cultures were used. Freeze-dried cells from a water bloom sample collected in Rutland water were also analyzed. Each sample (25 mg) was extracted with 250, 500 and 1000 μl of acidified methanol (pH ∼ 2). Furthermore, 10 and 5 mg of each sample were extracted with 1 ml of acidified methanol (pH ∼ 2). Extraction was carried out as explained above and extracts from each extraction step were analyzed separately.

#### 2.4.5. Optimization of the extraction time for different types of extraction

Freeze-dried cells (25 mg) of *M. aeruginosa* PCC 7820 and SCIENTO were extracted as detailed above for different periods of time: 5, 15, 30, 45, 60, 90 and 120 min for extraction with shaking every 10 min; 5, 15, 30, 45, 60, 90, 120, 150 and 180 min for extraction with no agitation; and 5, 10, 15, 20, 25, 30, 40, 50 and 60 min for extraction by sonication. Extracts corresponding to each extraction step were analyzed separately.

#### 2.4.6. Number of extractions

Freeze-dried cells from each culture (25 mg), *M. aeruginosa* PCC 7820 and SCIENTO, were extracted three times by sonicating for 15 min with 1 ml acidified methanol (pH ∼ 2). Each experiment was carried out in triplicate. Extracts from each extraction step were analyzed separately.

## 3. Results and discussion

### 3.1. Optimization of the extraction solvent

Extraction of microcystins with various solvents revealed that methanol and water were better at extracting hydrophobic and hydrophilic microcystins, respectively (Fig. 2). Nevertheless, additional contaminants, which may interfere in HPLC/DAD analysis, were present in the water extract (Fig. 3). Butanol–methanol–water extracted all microcystins although its efficiency was lower than that of water or methanol. As expected, acetic acid was shown to be the least efficient extraction solvent, recovering only trace amounts of the more hydrophobic microcystins (Fig. 2), since microcystin solubility changes due to protonation of carboxylic groups. These results are consistent with previous results reported by Lawton et al. [11]. However, great differences were not observed when extracting with 0.1 M ammonium hydrogen carbonate (pH 8.4). Finally, addition of surfactants did not seem to improve extraction of microcystins, apart from that of hydrophilic microcystins (Fig. 2).

Mixtures of methanol and water extracted more efficiently hydrophilic and hydrophobic microcystins compared to extraction with methanol and water, respectively. The best extraction seemed to be obtained at 60–70% for hydrophilic microcystins and 80–90% for hydrophobic microcystins, with 80% aqueous methanol selected as the most suitable mixture for the extraction of both hydrophobic and hydrophilic microcystins (Fig. 2). These results were consistent with those previously reported in the literature [11,12,32].

Sequential extraction using methanol and water also increases extraction yields of those microcystins not well-extracted by the first solvent, either methanol or water, depending on the sequence (Fig. 2, treatments 15–18). In

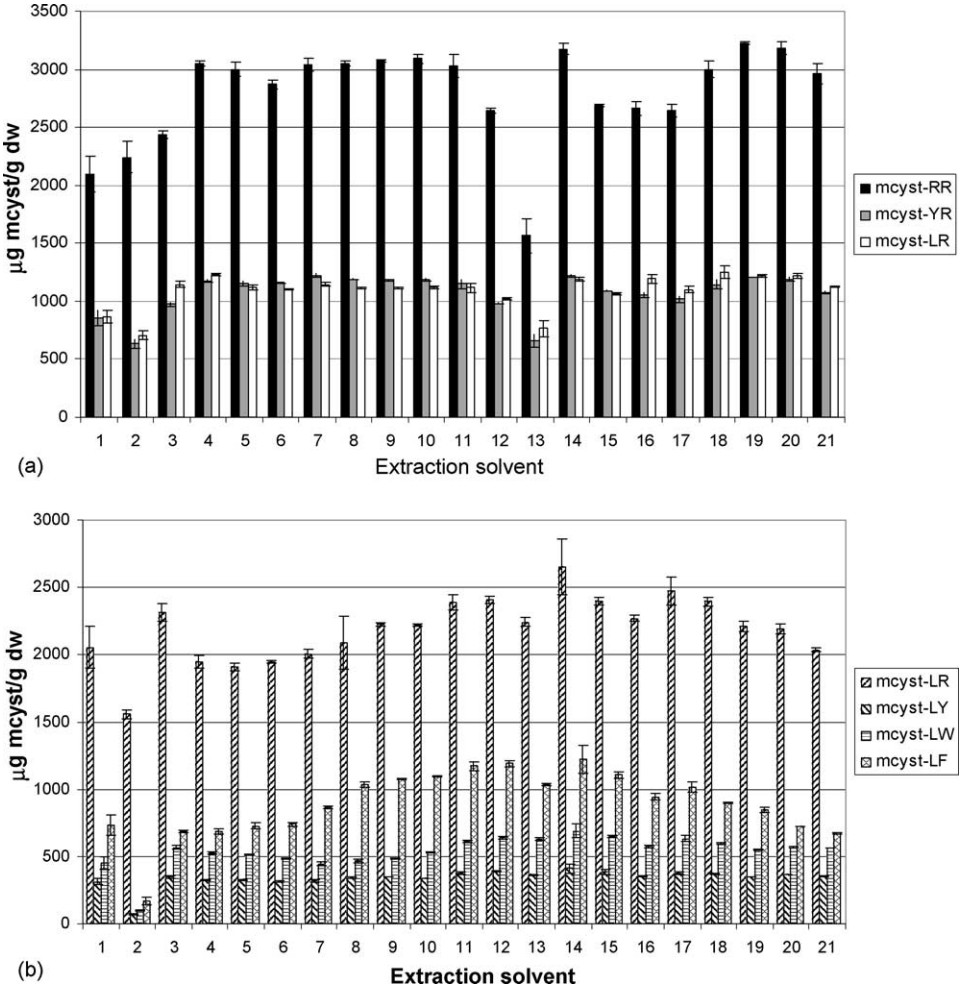



Fig. 2. Extraction of 25 mg of freeze-dried cells of *M. aeruginosa* (a) SCIENTO and (b) PCC 7820 in (1) butanol–methanol–water (1:4:15); (2) 5% acetic acid; (3) water; (4–12) 10–90% aqueous methanol; (13) methanol; (14) acidified methanol (0.1% TFA); sequencial extraction with (15) methanol–water–methanol–water, (16) water–methanol–water–methanol, (17) methanol–methanol–water–water and (18) water–water–methanol–methanol; (19) 0.1% Triton X-100; (20) 0.1% Tween-20 and (21) 0.1 M ammonium hydrogen carbonate. Mean values of triplicate analysis, error bars indicate standard deviation.

previous work, Fastner et al. [13] obtained the best yields with sequential extraction (three extractions with methanol followed by three extractions with water) when comparing microcystin extraction of *M. aeruginosa* PCC 7820 with sequential extraction, water and methanol [13]. The sequential extraction with methanol–water–methanol–water did not increase significantly hydrophobic microcystin yields obtained with methanol. However, substantial differences were observed for hydrophilic microcystins, with some extraction yields around two-folds higher than those obtained with methanol. The sequential extraction with water–methanol–water–methanol improved extraction yields of hydrophobic microcystins achieved with water. Results obtained with methanol–methanol–water–water were similar to those obtained with methanol–water–methanol–water. Despite extraction yields for hydrophobic microcystins being similar to those with methanol, they were higher for hydrophilic microcystins. When extracting with water–water–methanol–methanol, extraction yields for hydrophilic microcystins were similar to those obtained with water. However, they were improved for hydrophobic microcystins. These results were similar to those obtained with water–methanol–water–methanol.

As expected, acidified methanol led to the highest yields of most microcystins, since hydrophobic character of microcystins improves as the pH value decreases [21]. As an example, Fig. 2 shows that microcystin-LR yield in acidified methanol was twice that obtained in methanol alone. All these results suggest that acidified methanol was the most suitable solvent for the extraction of both hydrophilic and hydrophobic microcystins.

### 3.2. Optimization of the extraction pH

As can be observed in Fig. 4, the best extraction efficiencies for all microcystins studied were obtained when extracting with acidified methanol at pH $\sim$ 1. The greatest increase in extraction efficiency was observed for the more

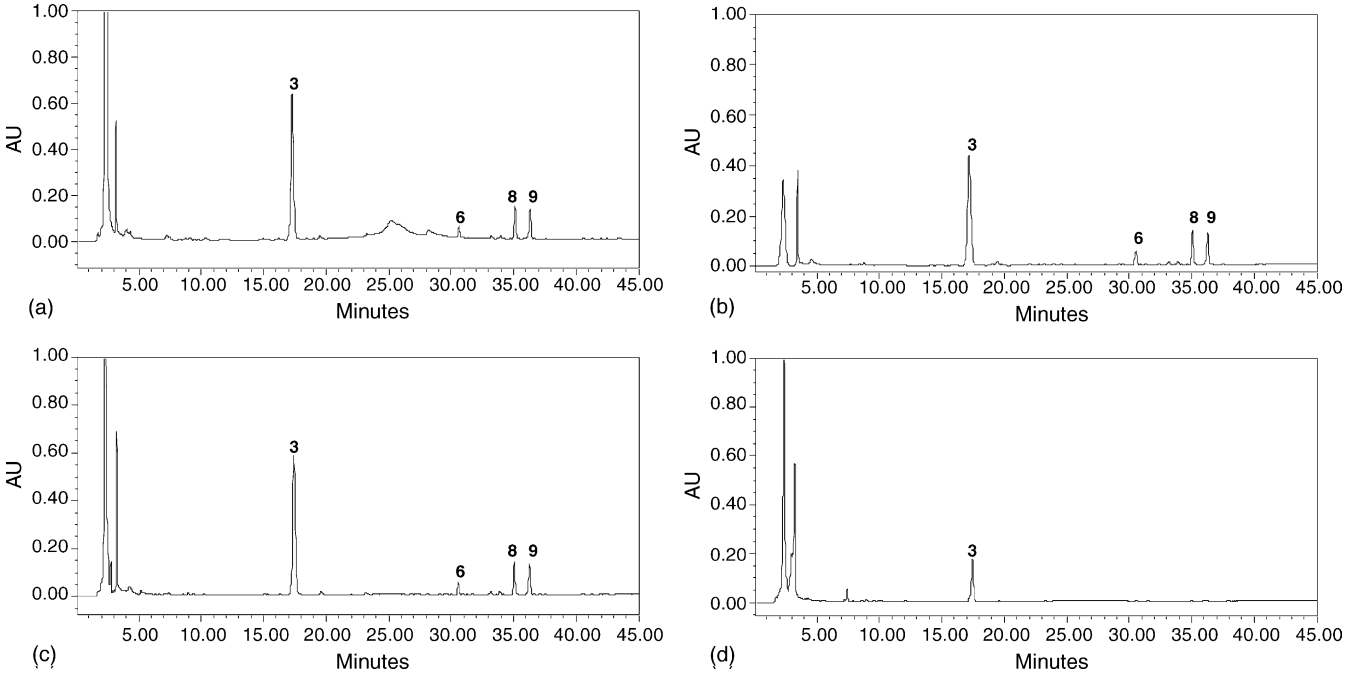



Fig. 3. HPLC/UV chromatogram at 238 nm of a 25 mg extract of freeze-dried cells of *M. aeruginosa* PCC 7820 in (a) water, (b) methanol, (c) butanol–methanol–water (1:4:15) and (d) 5% acetic acid: microcystin-LR (3), microcystin-LY (6), microcystin-LW (8), microcystin-LF (9).

hydrophilic microcystins found in *M. aeruginosa* SCIENTO. In particular, at pH ∼ 1 around 100% more microcystin-RR was observed compared to pH ∼ 3 and above. However, after 48 h, some peaks corresponding to microcystin-like compounds were observed by HPLC/DAD in the extract obtained at pH ∼ 1 (data not shown). These peaks may be related to microcystin breakdown products, as they were not present in the initial sample and microcystins were under very high acidic conditions (pH ∼ 1). In order to avoid potential sample degradation, pH ∼ 2 was chosen as the optimum extraction pH. At this pH value, no degradation was observed after 48 h (data not shown) and good recoveries were still achieved.

### 3.3. Optimization of the extraction volume

No significant differences were observed in microcystin yields when extracting with different biomass–solvent volume ratios, except for microcystin-RR (the only microcystin present with two basic arginine units in the molecule) which showed a maximum at 25 mg/1 ml (Fig. 5). To confirm this finding, *M. viridis* NIES-102, *N. spumigena* PCC 7804 and *M. aeruginosa* from the water bloom collected in Rutland water were also studied. Results obtained for these two cultures and the water bloom sample were consistent with the previous results explained above, observing a maximum yield when extracting 25 mg of freeze-dried cells in 1 ml of sol-

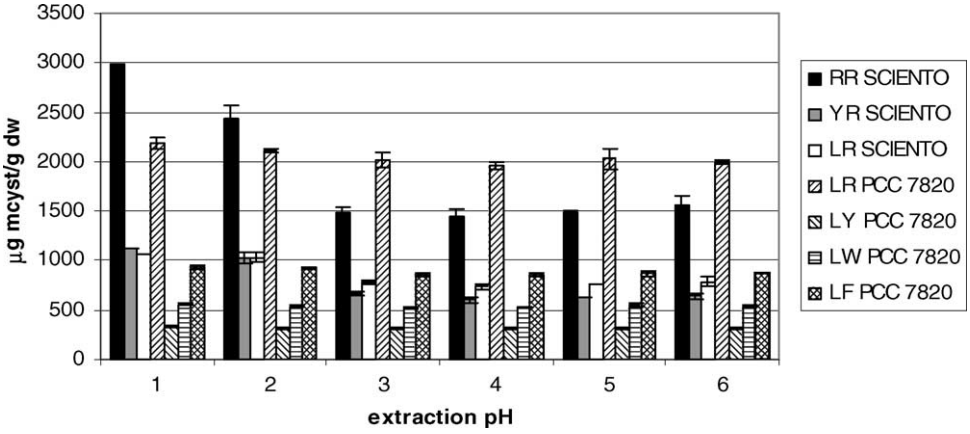



Fig. 4. Extraction of 50 mg of freeze-dried cells of *M. aeruginosa* SCIENTO and PCC 7820 with acidified methanol at different pH values. Mean values of triplicate analysis, error bars indicate standard deviation.

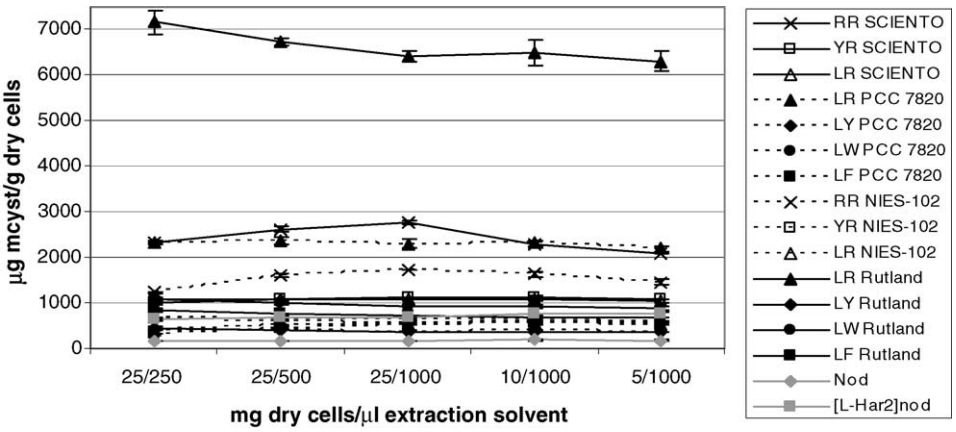



Fig. 5. Extraction of 25 mg of freeze-dried cells of *M. aeruginosa* SCIENTO and PCC 7820, *M. viridis* NIES-102, *N. spumigena* PCC 7804 and Rutland water bloom with different extraction volumes of acidified methanol (pH ~ 2). Mean values of triplicate analysis, error bars indicate standard deviation.

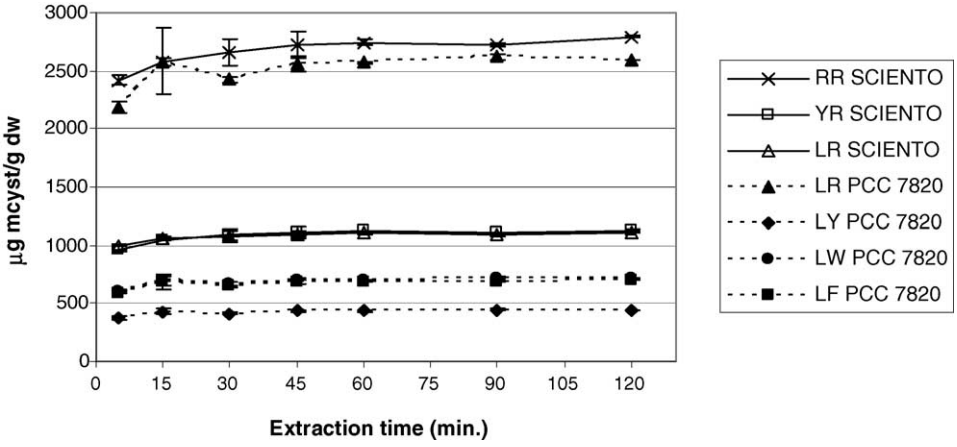



Fig. 6. Extraction of 25 mg of freeze-dried cells of *M. aeruginosa* SCIENTO and PCC 7820 by shaking every 10 min with 1 ml of acidified methanol (pH ~ 2) for different extraction times. Mean values of triplicate analysis, error bars indicate standard deviation.

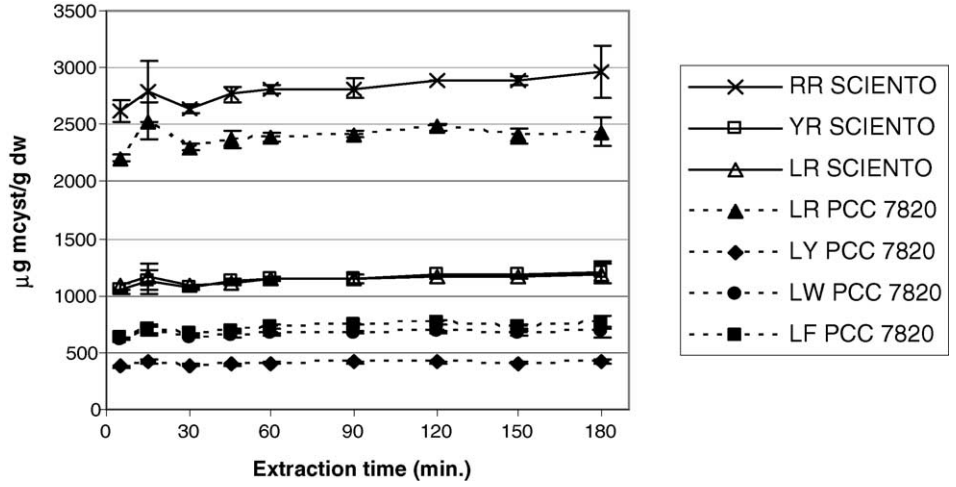



Fig. 7. Extraction of 25 mg of freeze-dried cells of *M. aeruginosa* SCIENTO and PCC 7820 with no shaking and 1 ml of acidified methanol (pH ~ 2) for different extraction times. Mean values of triplicate analysis, error bars indicate standard deviation.

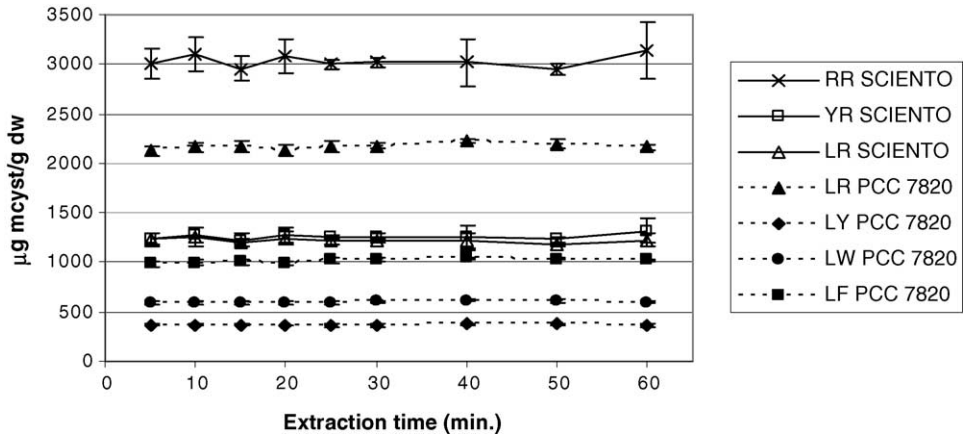



Fig. 8. Extraction of 25 mg of freeze-dried cells of *M. aeruginosa* SCIENTO and PCC 7820 by sonicating with 1 ml of acidified methanol (pH ~ 2) for different extraction times. Mean values of triplicate analysis, error bars indicate standard deviation.

vent only for microcystin-RR (Fig. 5). On the other hand, an important amount of microcystins, 20–35% of total microcystins, was present in the third extract when extracting 25 mg of freeze-dried cells in 250 or 500 μl. For these reasons, 25 mg of freeze-dried cells in 1 ml of acidified methanol was considered to be the optimum biomass-extraction volume ratio.

### 3.4. Optimization of the extraction time for different types of extraction

#### 3.4.1. Discontinuous agitation

When extraction was performed with discontinuous shaking, microcystin extraction yields increase as the extraction time increases for extraction times between 5 and 45 min (Fig. 6). Extraction time longer than 45 min did not improve significantly the extraction yield, so the suitable extraction time was chosen to be 45 min.

#### 3.4.2. No agitation

Intracellular microcystin levels when extracting with no agitation (Fig. 7) were unexpectedly similar to those obtained with discontinuous agitation. This observation may be due to the destruction of the cell wall during freezing and freeze-drying of cells prior to extraction. For extraction times lower than 90 min, a slight increase in microcystin yield was observed as the extraction time increases. However, longer extraction time did not increase significantly the extraction yield. So, 90 min seems to be the optimum extraction time.

#### 3.4.3. Sonication

In contrast to the other two extraction methods, when sonicating, no significant increase was observed in microcystin extraction yield over time (Fig. 8). From these observations, 15 min was chosen as the extraction time. Furthermore, sonicating was chosen as the extraction method due to the short time of analysis involved.

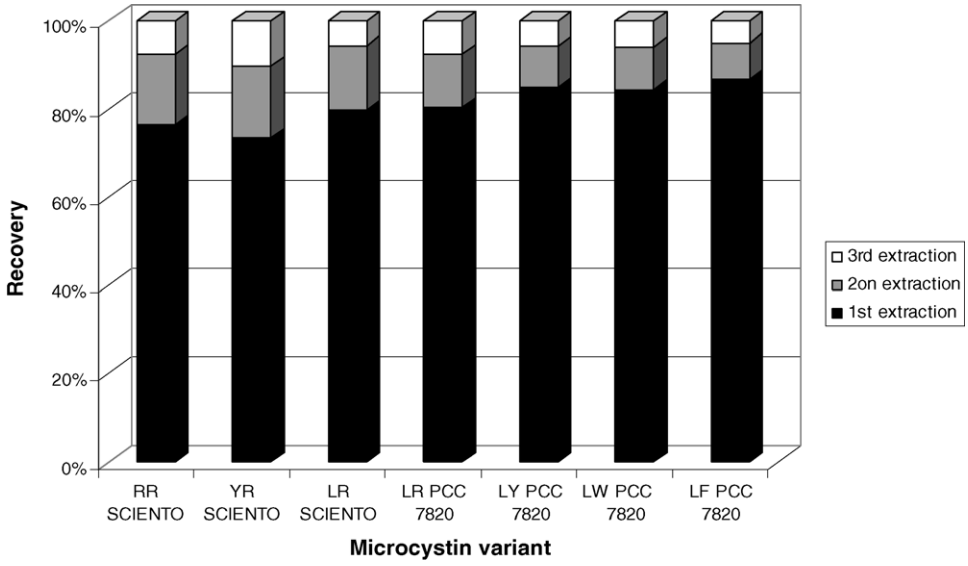



Fig. 9. Microcystin yield of each extraction step. Extraction of 25 mg of freeze-dried cells of *M. aeruginosa* SCIENTO and PCC 7820 by sonicating with 1 ml of acidified methanol (pH ~ 2) for 15 min.

### 3.5. Number of extractions

It could be concluded from results obtained (Fig. 9) that three extractions will extract most microcystin contents from cyanobacterial cells, as the third extract only contains about 5–9% of total microcystin.

## 4. Summary

This paper represents the first wide and detailed evaluation of most reported solvents used for extraction of intracellular microcystins. Efficiency of each extraction solvent as well as other parameters affecting extraction efficiency including extraction pH, volume, method (discontinuous shaking, no shaking and sonication), time for each extraction method and number of extractions were evaluated and optimized. Stability at the optimum pH values was also studied. This compilation of results will be of great value for most scientists already or just working in this field. It may also help to achieve a world-wide consensus on the best extraction method for the routine analysis of intracellular microcystins. Moreover, it may be valuable for purification scale. In the present work, sonicating with acidified methanol (pH $\sim$ 2) has been shown to be a rapid and efficient method for the routine analysis of a wide range of intracellular microcystins as well as nodularins with no recorded degradation. Analysis of triplicate samples demonstrated good reproducibility of results. This method can be also adapted and applied to the analysis of intracellular microcystins in water bloom samples, contained in cells usually trapped on GF/C filters.

## Acknowledgements

This work has been supported by a project from the Spanish Government (CICYT AMB1999-0718). M. Barco would like to thank CIRIT for the predoctoral fellowship (1998FI 00035 APMARN CSIC) and the financial support for her two stays at The Robert Gordon University.

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