

Advances in solid phase extraction of the cyanobacterial toxin cylindrospermopsin

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Abstract

Unlike other cyanotoxins, cylindrospermopsin (CYN) is often found in high concentrations in the extracellular fraction, possibly due to a higher release from the producing cells and a limited degradation once liberated. This fact demands effective recovery methods from environmental samples to guarantee a proper quantification. A reliable and simple solid phase extraction (SPE) method was obtained by the sole use of graphitized carbon cartridges and by paying special attention to both the selection of the most suitable solvent and the need of sample preparation prior to SPE. An acidified combination of dichloromethane and methanol not only showed best recoveries, but also allowed drastic reduction to the elution volumes needed with complete recovery being achieved in only 8 mL. Acidification of the sample and addition of sodium chloride are suggested as valuable improvements to the SPE method and turn out to be essential for correct and robust recovery of CYN in environmental samples of great diversity in terms of pH, DOC, and conductivity. Excellent behavior of the proposed method over a wide range of CYN loadings, applied sample volumes, and DOC loadings was also confirmed.

Introduction

Cylindrospermopsin (CYN) is a cyanobacterial alkaloid consisting of a tricyclic guanidine moiety combined with a hydroxymethyluracil initially described as a potent hepatotoxin (Ohtani et al. 1992). Toxic effects have been observed on diverse organisms. For example, and as a result of the studies carried out by Kinnear et al. (2007), potential impacts on amphibian populations and, thus, far-reaching ecological impacts have been predicted. Also bioaccumulation has been observed, for example, in the freshwater mussel *Anodonta cygnea* (Saker et al. 2004). In vertebrates, the main target of CYN seems to be the liver. Still, other organs such as the thymus, kidney, adrenal glands, lungs, intestinal tract, and heart may also be affected. Besides other episodes, CYN has been implicated as cause of hepatoenteritis on Palm Island, Australia, affecting 148 people (Bourke and Hawes 1983; Bourke et al. 1986). Further, assays have shown CYN-induced genotoxicity (Humpage et al. 2000, 2005;

Shen et al. 2002) and evidence for carcinogenicity (Falconer and Humpage 2001).

Several cyanobacterial species are able to produce cylindrospermopsin. Among these species, the most widely distributed is *Cylindrospermopsis raciborskii*, which is now found in many countries (Briand et al. 2004). Other species producing this cyanotoxin are *Umezakia natans* (Harada et al. 1994), *Anabaena bergii* (Schembri et al. 2001), *Raphidiopsis curvata* (Li et al. 2001), *Aphanizomenon ovalisporum* (Banker et al. 1997), *Aphanizomenon flos-aquae* (Preussel et al. 2006), *Anabaena lapponica* (Spooft et al. 2006), or *Lyngbya wollei* (Seifert et al. 2007).

Even though the mechanisms of CYN release from the producing cells are still not completely understood, recently Mihali et al. (2008) suggested CyrK to be a transporter for CYN. In the field, the observation of high extracellular concentrations has been distinctive for this toxin. In this way, Rucker et al. (2007) observed that in 31% of samples collected from twenty-one lakes, dissolved CYN was more than 80% of total CYN. Also in laboratory cultures, a similar behavior has been confirmed by Norris et al. (2001) for a *Cylindrospermopsis raciborskii* culture or Shaw et al. (1999) in the case of *Aphanizomenon ovalisporum*.

The common presence of CYN in the dissolved fraction demands effective methods of extraction and concentration, both for the purpose of recovery of toxin from spent medium as well as for the correct quantification of toxin in field samples.

Norris et al. (2001) performed a complete study with a range of SPE cartridges and observed best results when using

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graphitized carbon. Metcalf et al. (2002) combined graphitized carbon with C18 and obtained good recoveries when working with high concentrations of CYN. In the present study, we focused on further optimization of a graphitized carbon-based SPE method, paying special attention to both solvent selection and sample preparation, aspects that so far had been avoided. Our goal was to obtain a reliable method that not only enables bulk recovery of CYN from spent medium but would allow optimal concentration of even small amounts of CYN from environmental samples.

Materials and procedures

Aphanizomenon ovalisporum UAM 290 was grown and harvested when achieving the stationary phase for the obtaining CYN. Cells were separated from the spent media by centrifugation. Both supernatant and pellet were further used as CYN source in the different experiments. Cell-bound CYN in the pellet was extracted with 0.9% saline solution containing 5% formic acid by pulse-pestle ultrasonication on a Branson 450 Sonifier. Ultrasonication was applied in 70% duty cycles during 2 min at maximum energy recommended for micro-tips. CYN in the supernatant was GF/C filtered and stored (-20°C) for further use.

The different recovery experiments were all performed in triplicate and graphitized carbon cartridges (Bond Elut Carbon 500 mg, Varian Inc.) were used. These cartridges were primed with the selected solvent and washed with distilled water. Afterward, the sample was applied at a flow rate of 5 mL min^{-1} , and a further washing step with distilled water and the final elution with the selected solvent were performed. A volume of 10 mL was chosen for each of the priming, washing, and elution steps described above, sample volume was 100 mL unless specified otherwise. Finally, the eluent was vacuum-dried and prepared for HPLC analysis.

Quantification of CYN was performed on a Waters Alliance 2695 HPLC system with a 996 PDA detector equipped with a Waters Spherisorb $5\mu\text{m}$ ODS2 column according to the protocol described by Törökné et al. (2004). The presence of CYN was verified by its UV spectrum and its retention time and quantified with the help of a five-point calibration curve.

All statistics were performed with the XLSTAT software (Addinsoft S.A.R.L.).

Development of the SPE method: selection of solvents and sample preparation—Norris et al. (2001) suggested methanol as the solvent selected for elution of CYN and emphasized the importance of acidification of the solvent. In the present study, we also tested the suitability of acetone and combinations of dichloromethane:methanol (4:1, 1:1, 1:4) as solvents. In all cases, these solvents were acidified with 5% formic acid (v/v). For the selection of the solvent, cartridges were prepared as described above and extracted CYN in saline solution containing 5% formic acid was applied to the cartridges. The eluent was collected in 2 mL fractions and analyzed. Once an appropriate solvent had been selected, importance of sample preparation was studied. Instead of using CYN extracted from the cell-bound fraction into

acidified saline solution, the toxin was obtained from the dissolved fraction naturally present in the spent medium.

To test the possible benefits of acidification, the pH of this dissolved fraction was adjusted respectively to values of 2, 4, 7, and 10, using formic acid and NaOH (0.1 N). Also, the importance of the ionic strength in the sample was tested. Used concentrations were 0%, 0.1%, 0.2%, 0.4%, and 0.9% sodium chloride (w/v).

Suitability of the SPE method for CYN in environmental samples and culture medium—To test the suitability of the sample preparation and extraction method proposed, samples with increasing presence of CYN (0.1 to 30 μg) were applied to the cartridges. The extent of the recovery for the diverse quantities added was used to estimate the linearity of the response. Further, the importance of sample volume and DOC concentration was also studied. To test the suitability of the method for increasing sample volumes, 1 μg CYN was added to 10, 100, 500, and 1000 mL distilled water and applied to the cartridges. In the case of the studies concerning sensitivity to DOC, CYN had to be recovered from 100 mL distilled water to which humic acid (Acros Organics) had been added to final concentrations of 0, 100, 250, 500, and 1000 mg L^{-1} DOC.

Afterward, the fact of working with natural samples of great diversity was considered. GF/C filtered water from five waterbodies was spiked with CYN. The waterbodies selected for this study were four reservoirs: Arcos (Cadiz, Spain), Cogotas (Avila, Spain), Salas (Orense, Spain), and Vega de Jabalón (Ciudad Real, Spain), and the pond in Parque Juan Carlos I (Madrid, Spain). Subsurface water was used in all cases except Cogotas reservoir, for which samples from 18 m depth were used. In both Arcos reservoir and the pond in Parque de Juan Carlos I (JCI), CYN has been detected recently (Quesada et al. 2006; Wormer et al. 2008). Prior to spiking, dissolved organic carbon (DOC), pH, and conductivity were determined in the samples, DOC being quantified on a Shimadzu TOC 5000.

Before applying the spiked water (CYN = 0.89 μg) to the activated cartridges, half the samples were acidified with formic acid 1% (v/v), and sodium chloride was added (0.1% w/v). The other half were applied without further preparation. A combination of dichloromethane:methanol (1:4) with the addition of 5% formic acid was selected as solvent. In parallel, these environmental samples were also analyzed without external addition of CYN.

Finally, the capacity of the proposed method to guarantee good recovery of CYN in a wide range of concentrations was tested. Therefore, a declining culture of *Aphanizomenon ovalisporum* UAM 290 was sonicated by pulse-pestle ultrasonication on a Branson 450 Sonifier in the spent medium and centrifuged (3000g, 15 min). The supernatant was GF/F filtered and applied to the cartridges as described above. 1784 μg were applied in a sample volume of 200 mL, effluents containing nonretained CYN were collected at 10 mL intervals. Afterward, CYN retained in the cartridge was recovered according to the proposed method.

Assessment

Development of the SPE method: selection of solvents and sample preparation—Percentual recoveries achieved by the use of different solvents are shown in Fig. 1. The use of acetone showed the worst recovery values of all solvents tested; in 20 mL, only 43% of the initially added CYN was eluted. The use of methanol clearly improved the recoveries observed, achieving a value of 88%. Finally, the addition of dichloromethane to the methanol further improved the obtained results. Not only higher recoveries were achieved (98%), but also the needed elution volume was drastically reduced.

Once the benefits of adding dichloromethane to methanol were established, different combinations of both solvents were tested, always in presence of 5% formic acid. The three combinations tested (dichloromethane:methanol, 4:1, 1:1, and 1:4) showed similar recoveries and elution profiles. Beside this, an improvement in the chromatographical resolution of the peaks was observed as the percentage of dichloromethane decreased (data not shown).

The importance of acidifying the sample to be passed through the cartridge is shown in Table 1. Recoveries are not significantly different (Student's *t*-test, $\alpha = 0.05$), although slight improvements may be observed at lower pH, up to 96.4% of the initial CYN added was retrieved. Also, it should be noted that an increased tailing of the chromatographical peak was observed when the samples were not acidified (Fig. 2), in some cases the importance of this tailing was as high as 15% of total peak area (Fig. 2B).

Also, the importance of concentration of sodium chloride was evaluated (Table 1). Although only small differences in the recovery values were observed, the presence of sodium chloride seemed to be related with more robust measurements as expressed by decreasing standard deviations, the differences observed being statistically significant (Student's *t*-test, $P = 0.002$). For concentrations above 0.1%, no additional effects nor further improvement could be described.

Suitability of the SPE method for CYN in environmental samples and culture medium—Excellent linearity between CYN applied and recovered was achieved for quantities between 0.1 and 30 μg CYN (Fig. 3). Also, recovery of 1 μg CYN from samples with volumes increasing from 10 to 1000 mL was very stable and remained above 97% for all volumes tested (data not shown). Concerning the importance of DOC (Fig. 4), loadings as high as 25 mg DOC did not affect the recovery of CYN by our method. Loadings of 50 mg seem to have slight negative impact, as recovery drops to 85.3%. Extremely high DOC loadings (100 mg) result in a more evident negative impact, as recovery was only 73.9%.

Before spiking the natural waters with CYN, the environmental samples selected were characterized (Table 2). Salas reservoir shows lower conductivity and slightly acid waters while the other waterbodies are characterized by higher conductivity and pH. In the case of Cogotas reservoir, the sample

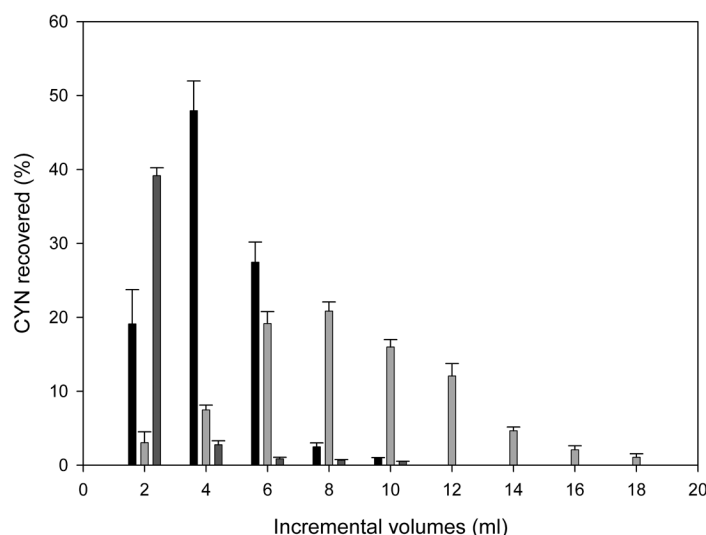


Fig. 1. Elution profile of CYN applied to graphitized carbon cartridges. Different solvents were tested: dichloromethane:methanol (4:1), black bar; methanol, light gray bar; acetone, dark gray bar. All solvents were acidified (5% formic acid). Mean and standard deviations are represented ($n = 3$)

Table 1. Effect of sample preparation by modification of pH and/or addition of sodium chloride on recovery of CYN from graphitized carbon cartridges ($n = 3$)*

pH [†]	NaCl concentration (% w/v)	CYN recovery (%)
10	0	94.6 ± 1.87
7	0	94.6 ± 2.81
4	0	96.4 ± 1.66
2	0	96.1 ± 2.23
	0.1	96.2 ± 0.68
	0.2	94.8 ± 0.94
	0.4	95.8 ± 0.75
	0.9	96.3 ± 0.74

*Mean and standard deviation are shown.

[†]pH values of 4 and 2 are achieved by the addition of 0.01% and 1% formic acid (v/v) respectively.

shows a higher pH, but conductivity remains low. DOC concentrations were quite high and ranged from 12.8 mg L⁻¹ in Cogotas reservoir to up to 34.5 mg L⁻¹ in Salas reservoir.

Concerning the recovery of CYN in these spiked samples (Table 2), on the one hand, results were always above 90% when the samples were acidified and sodium chloride was added. Best recoveries were achieved for samples from Parque Juan Carlos I (JCI) and Cogotas reservoir, while in Arcos reservoir the worst recoveries (91.8%) were obtained. Naturally occurring CYN was only detected in the pond in Parque Juan Carlos I, concentration after SPE was determined to be 8.41 μg L⁻¹. The recovery values for this waterbody when working with spiked samples were obtained by subtracting the naturally

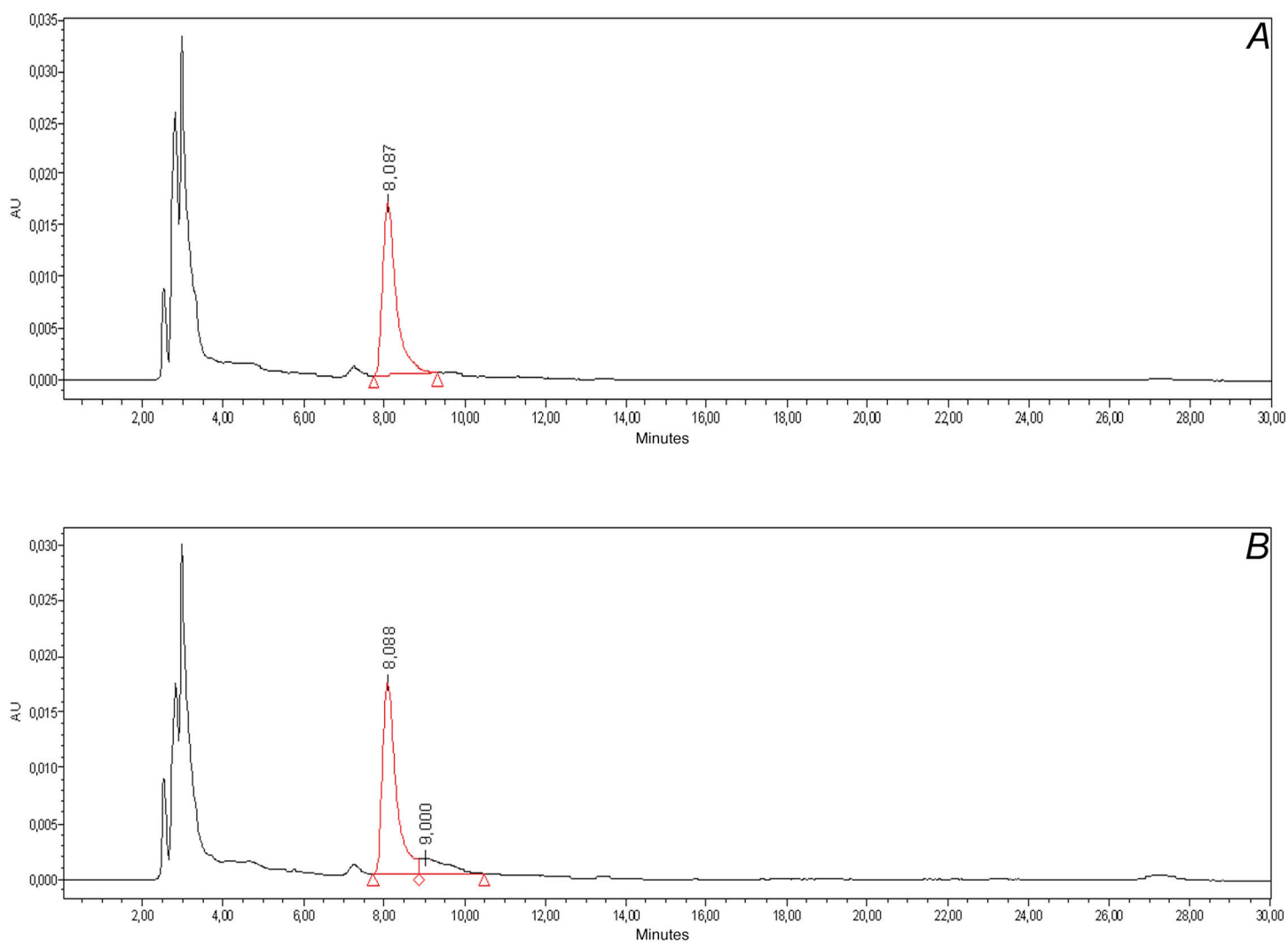


Fig. 2. HPLC chromatograms of CYN recovered by SPE. Extracellular CYN (2.07 μg) in spent medium was used and pH was adjusted to values of 2 (Fig. A) and 7 (Fig. B) before applying to the cartridge. AU, absorbance units.

occurring amount of CYN in the 100 mL sample from the total amount recovered.

On the other hand, when the sample was applied to the cartridges untreated, recovery values clearly decreased (Table 2). In Arcos reservoir for example, recovery dropped to 80.2%, which is 11.6% less than with sample preparation. Only in Salas reservoir, where the natural pH is lowest (pH = 6.04), no differences in recovery were observed (Student's *t*-test, $P = 0.813$). Differences in the recovery values were statistically significant for all samples (Student's *t*-test, $\alpha = 0.05$) except for the above-mentioned Salas reservoir and for Vega de Jabalón reservoir (Student's *t*-test, $P = 0.094$).

When establishing the natural concentration of dissolved CYN without sample preparation, the toxin could also only be detected in the pond in Parque de JCI. Also, important differences were observed between the two methods studied. Without

sample preparation, a concentration of 7.6 $\mu\text{g L}^{-1}$ was calculated, which is 10% less than what was obtained when the sample was previously treated.

Finally, the capacity of our method to extract high amounts of CYN from culture medium was tested (Table 3). For loadings as high as 1517 μg , no CYN could be detected in the effluent. Further loading of the cartridges results in nonretained toxin being observed in the last three effluent fractions. Still, the sum of CYN in these fractions accounts for only 1.00 μg (0.056% of overall CYN loaded). Afterward, CYN retained by the cartridge was eluted following the proposed method, 1515 μg CYN were regained in only 10 mL solvent.

Discussion

Field studies carried out all over the world in different types of bodies of water have shown that very high proportion of

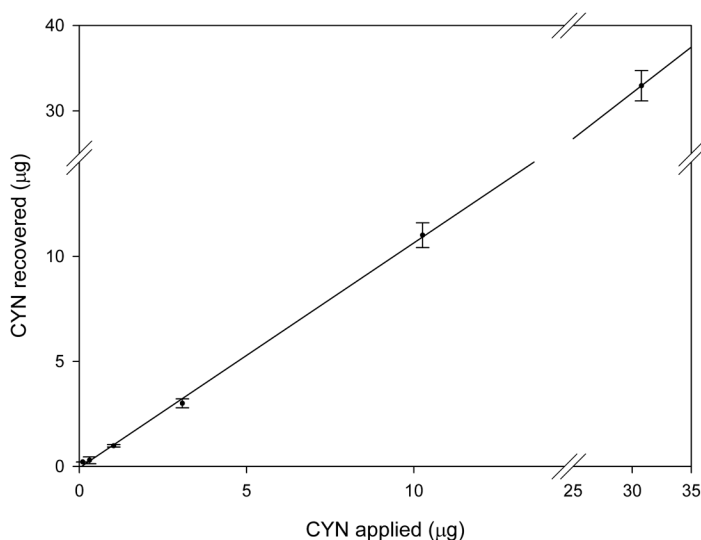


Fig. 3. Recovery of increasing quantities of CYN applied to SPE cartridges. Mean and standard deviations are represented ($n = 3$). Linear regression ($R^2 = 0.999$) is described by the equation $y = 10691x$

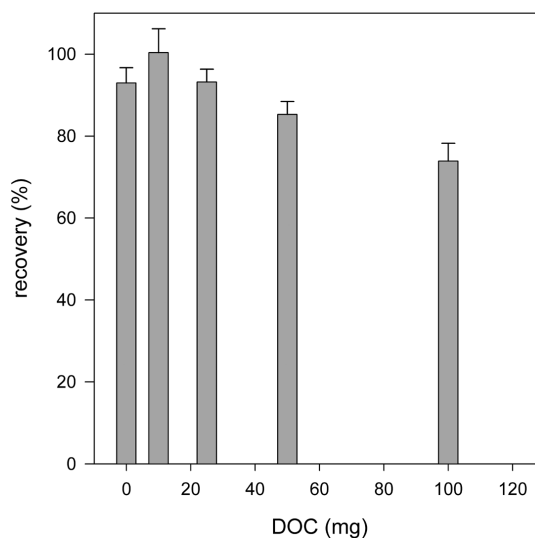


Fig. 4. Recovery of CYN applied to SPE cartridges in samples with increasing presence of DOC. Mean and standard deviations are represented ($n = 3$).

Table 2. Characterization of the water samples used to test suitability of the method proposed and recovery of CYN ($n = 3$)^{*}

	Date of sampling	Depth of sampling	Conductivity ($\mu\text{S cm}^{-1}\text{s}^{-1}$)	pH	DOC (mg L^{-1})	CYN recovery (1% formic acid, 0.1% NaCl added)	CYN recovery (untreated sample)
Cogotas	28/06/2007	18 m	170	8.37	12.8 ± 0.05	0.95 ± 0.05 (106.6%)	0.83 ± 0.04 (93.5%)
Salas	18/02/2007	subsurface	65	6.04	34.5 ± 0.08	0.87 ± 0.09 (97.9%)	0.89 ± 0.02 (99.8%)
Arcos	26/09/2007	subsurface	1320	7.94	24.6 ± 0.6	0.82 ± 0.06 (91.8%)	0.71 ± 0.03 (80.2%)
JC I	05/09/2005	subsurface	608	9.12	30.9 ± 0.7	$0.87 \pm 0.05^*$ (98.3%)	$0.77 \pm 0.04^*$ (86.7%)
Vega de Jabalón	03/07/2007	subsurface	1394	7.89	18.6 ± 0.3	0.86 ± 0.06 (97.4%)	0.78 ± 0.03 (87.8%)
JC I unspiked	05/09/2005	subsurface	608	9.12	30.9 ± 0.7	$8.41 \pm 0.34^\dagger$	$7.60 \pm 0.27^\dagger$

^{*}Recovery is expressed as recovered μg CYN and as percentage of initial CYN spiked ($0.89 \mu\text{g}$)

[†]Recovery obtained by subtracting the naturally occurring CYN amount from total recovery CYN concentration in $\mu\text{g L}^{-1}$ is shown.

Table 3. Retention capacity of SPE method for CYN^{*}

Fraction	CYN fed (μg)	CYN in effluent (μg)	Ratio effluent:feed [†] (%)
1–17	1517	nd	nd
18	1606	0.120	0.13
19	1695	0.313	0.35
20	1784	0.568	0.64

^{*}Effluent is collected in 20 fractions and analyzed.

[†]Effluent:feed ratio is calculated considering CYN fed and CYN in effluent in each fraction (nd: not detected).

total CYN can be found in the dissolved fraction (Table 4). This accumulation in the extracellular fraction has not been completely explained yet, but could be related to an increased CYN release from the cells and a long persistence of the toxin in the dissolved state due to limited degradation (Chiswell et al. 1999; Smith et al. 2008; Wormer et al. 2008).

This high presence in the dissolved state demands efficient and robust methods for quantification. While quantification methods are well developed (Eaglesham et al. 1999; Törökne et al. 2004), concentration and extraction of dissolved CYN from natural samples has not been widely developed or published. Norris et al. (2001) completed a first approach toward an effective SPE method by selecting graphitized carbon as the most suitable compound when compared with a wide range of the most usual SPE sorbents. Also, in their work, the need for acidification of the solvents was established. Finally, when applying culture medium used to grow *Cylindrospermopsis raciborskii* to graphitized carbon cartridges with bed weights of 300 mg, up to 861 μg CYN was retained, thus showing the great capacity of these cartridges for CYN. In the present study, we assumed these results but studied whether an improvement of recoveries and in the elution profile could be obtained by the use of other solvents and conditions. While acetone was clearly not

Table 4. Data on the occurrence of particulate and dissolved cylindrospermopsin

Sampling site	Number of samples	Dissolved CYN ($\mu\text{g L}^{-1}$) [% of total CYN]	Particulate CYN ($\mu\text{g L}^{-1}$)	Source
Hervey Bay water storage facility, 1997-1998 (Australia)	8	7–63 [19%–95%]	0.7–29	Chiswell et al. 1999
Palm Lakes, 1997 (Australia)	2	22–120 [85% to 100%]	0–4	Shaw et al. 1999
Ocean Park, pond 1, 1997 (Australia)	2	14 – 16 [87.5%–100%]	0–2	Shaw et al. 1999
Ocean Park, pond 2, 1997 (Australia)	1	4 [100%]	0	Shaw et al. 1999
Aquaculture pond, 1997 (Australia)	1	39 [6.6%]	550	Saker and Eaglesham, 1999
NE German lowlands, 2005 (Germany)	115 (21 lakes)	0–11.8	0–0.5	Rücker et al. 2007
Parque de Juan Carlos I, 2005 (Spain)	3	2.93–7.83 [52%–75%]	2.63–3.7	Wormer et al. 2008
Arcos reservoir, 2004-2005 (Spain)	4	0.18–3.17 [25%–63%]	0.54–9.4	Quesada et al. (2006) and unpublished data

useful, the addition of dichloromethane maximizes recoveries and reduces elution volumes.

At this stage, attention should be paid to the differences in the elution volume for methanol obtained in our work when compared with those from Norris et al. (2001) and Metcalf et al. (2002), where total recovery was obtained in about 8 mL, half the volume observed in the present study. An explanation to this different behavior may be found in the bed weight of the cartridges. We used cartridges of 500 mg graphitized carbon, while Norris et al (2001) used 300 mg. In Metcalf et al. (2002), no indication about the quantity used could be found. According to diverse manufacturers, the 500 mg cartridges will allow loadings of more than 1 g analyte and are recommended for large sample volumes (100 mL–1 L). Low concentration of the toxin in field samples usually demands higher volumes to be passed through the SPE cartridges. Thus, in our opinion, the larger cartridges are more suitable for natural samples and should be considered as standard method for routine analysis. We consider the modification of solvents proposed here essential to allow correct concentration and quantification of the toxin in environmental samples.

Also, Norris et al. (2001) and the later work from Metcalf et al. (2002) did not explore the possible need of some previous sample preparation. But in this work, we have been able to demonstrate the improvement achieved by acidification and addition of sodium chloride, when working with natural samples of diverse characteristics. Even though acidification and addition of sodium chloride did not result in great improvements when applied to CYN present in spent medium—possibly due to peculiar characteristics of growth media concerning, for example, high concentration of salts—they were essential for accurate recoveries in spiked environmental samples and in the unspiked sample from the pond in Parque de Juan Carlos I. When sample preparation was avoided, up to 11.6% less CYN was recovered from spiked samples, and even more importantly, without sample preparation, CYN concentration in an environmental sample was estimated to be 10% lower than if sample was treated. These losses were apparently related to samples that showed pH values above 7, as shown

by the fact that only in the case of Salas reservoir (pH = 6.04), recoveries were similar. As the presence of phytoplankton in water, and thus the consumption of CO_2 via photosynthesis, will increase pH values, toxic blooms can be expected to occur mainly in waters with high pH. It should be noted that—at least in Spain—CYN has been observed only in basic bodies of water, namely Arcos reservoir and the pond in Parque de Juan Carlos I (Quesada et al. 2006; Wormer et al. 2008). Also the appearance of CYN-producing organisms in Shaw et al. (1999) or Seifert et al. (2007) is related to sites presenting pH values above 7. Sample preparation thus seems to be most needed in the types of bodies of water that have been described to house CYN-producing organisms.

Also, it should be noted that the high concentrations of DOC present in the spiked environmental samples did not affect recovery of CYN, the proposed SPE method remained reliable at DOC values of up to 34.5 mg L^{-1} . This is consistent with our experimental results (Fig. 4) that show that loadings of up to 25 mg DOC do not affect the SPE of CYN and that loadings of even 50 mg DOC have only very small negative effect on recovery of CYN. This is important as graphitized carbon shows extraordinary adsorption of organics to its surface, and, consequently, problems with the extraction of compounds from samples with high DOC can be expected. These saturation effects have not been found in our experiments, possibly due to the larger bed weight in the cartridges, which guarantees an increased adsorption surface.

Another aspect that should be noted is that the proposed method shows very good linearity when comparing applied to recovered CYN for quantities ranging from 0.1 to 30 μg . This indicates that, on the one hand, small amounts of toxin can be correctly concentrated and recovered, and on the other hand, risk of breakthrough of the cartridges should be very low for environmental samples. Small deviations when applying 0.1 μg CYN can be explained due to chromatographic limitations. As recovered samples are concentrated in 1 mL saline solution, but only 100 μL are injected, quantification is being made at the limit of our system (10 ng CYN injected to the chromatography column). The sample volume applied to the

cartridges has shown to have no impact at all on the recovery of CYN. Therefore, when working with HPLC systems similar to those used in the present study, we suggest sample volumes to be at least 500 mL. This allows correct detection of CYN concentrations close to the provisional standard for drinking waters of 1 µg L⁻¹ and in samples with DOC concentrations ranging as high as 50 or even 100 mg L⁻¹.

Finally, the excellent capacity of the proposed method to recover high amounts of toxin is remarkable. In our study (Table 3), 1784 µg CYN were loaded, and the sum of nonretained CYN in effluents accounts for only 0.056% of this amount. In their experiments, Norris et al. (2001) were able to retain up to 861 µg CYN on SPE, calculated as the difference in feed and effluent. In our case, retention increased to more than 2-fold that amount. Note that in the case of Norris et al. (2001), such recoveries are achieved with high breakthrough already occurring. In our case, breakthrough is only starting, because concentration in the final effluent fraction is only 0.64% of feed concentration. Besides studying the retention of CYN, we were also interested in the ability to efficiently regain this high amount of toxin. In only 10 mL, up to 1515 µg were recovered (84.9% of CYN in feed). The use of cartridges with larger bed weights, together with the selection of the most appropriate solvents seem to be responsible for this high retention capacity and massive recovery. Our data thus clearly show that very substantial amounts of CYN can be easily extracted from culture medium and regained in small volumes. It should be considered that all results are obtained by the sole use of graphitized carbon cartridges. Avoiding the use of an additional C 18 cartridge, as proposed by Metcalf et al. (2002), is a further advantage in terms of optimal time and resource management.

Therefore, for analysis of dissolved CYN in environmental samples and recovery from growth medium, we suggest a SPE method consisting of a sample preparation with 1% formic acid and 0.1% sodium chloride and the use of a combination of dichloromethane:methanol (1:4) acidified with 5% formic acid as solvent for the graphitized carbon cartridges used.

Comments and recommendations

Solid phase extraction by the sole use of graphitized carbon cartridges is confirmed as a suitable method for the concentration of CYN from culture medium or from very diverse environmental samples. The use of a solution of methanol:dichloromethane as eluent is strongly recommended, as it allows the use of increased bed weights while guaranteeing optimum recovery in small elution volumes. Eluents used so far, namely methanol, result in an excessive broadening of the elution profile, thus complicating total recovery.

Previous sample preparation, namely acidification and addition of sodium chloride, has been avoided so far, but turns out to be essential for correct and steady recovery of CYN from very diverse environmental samples, and thus for correct analysis of water quality. Excellent response over a wide range

of CYN concentrations, sample volumes, and DOC loadings further backs the method proposed.

Considering analysis of CYN in other environmental samples, and especially in drinking water, influence of treatment processes on the method should be studied separately.

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