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Development and optimization of a method for the determination of Cylindrospermopsin from strains of *Aphanizomenon* cultures: Intra-laboratory assessment of its accuracy by using validation standards

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ABSTRACT

The occurrence of cyanobacterial blooms in aquatic environments is increasing in many regions of the world due to progressive eutrophication of water bodies. Because of the production of toxins such as Cylindrospermopsin (CYN), contamination of water with cyanobacteria is a serious health problem around the world. Therefore it is necessary to develop and validate analytical methods that allow us to quantify CYN in real samples in order to alert the public of this toxin. In this work, an analytical method has been developed an optimized for the determination of CYN from *Aphanizomenon ovalisporum* cultures. The analytical procedure is based on solvent extraction followed by a purification step with graphitized cartridges and CYN quantification by LC–MS/MS. The extraction and purification steps were optimized using a two-level full factorial design with replications. A suitable and practical procedure for assessing the trueness and precision of the proposed method has been applied by using validation standards. The method has been suitably validated: the regression equation was calculated from standards prepared in extracts from lyophilized *M. aeruginosa* PCC7820 ($r^2 \geq 0.9999$) and the linear range covered is from 5 to 500 μg CYN/L, equivalent to 0.18–18.00 μg CYN/g dry weight lyophilized cells. Limits of detection and quantification were 0.04 and 0.15 μg CYN/g, respectively, the recovery range (%) oscillated between 83 and 94% and intermediate precision (RSD %) values from 5.6 to 11.0%. Moreover, the present method showed to be robust for the three factors considered: the batch of the graphitized carbon cartridges, the flow rate of the sample through the cartridge, and the final re-dissolved water volume after SPE treatment, which permits its validation. The validated method has been applied to different lyophilized cultures of *A. ovalisporum* (LEGE X-001) to evaluate CYN content. This procedure can be used for determining CYN in lyophilized natural blooms samples in environmental studies.

1. Introduction

Harmful cyanobacterial blooms are occurred in eutrophicated freshwater lakes and reservoirs throughout the world and can present a public safety hazard through contamination of drinking water supplies [1–2]. This hazard results from the production of harmful secondary metabolites, otherwise known as cyanotoxins. There are over 40 species representing 20 genera from three cyanobacterial orders known to produce cyanotoxins which include both cyclic peptides and alkaloids [3]. Cylindrospermopsin (CYN) is a hepatotoxic alkaloid consisting of a tricyclic guanidine moiety combined with hydroxymethiluracil which as originally described as being produced by *Cylindrospermopsis raciborskii*. At present, CYN is known to also be produced by *Umezakia natans*, *Aphanizomenon ovalisporum*, *Raphidiopsis curvata*, *Lyngbia wollei*, *Anabaena bergii*, *Aphanizomenos flos-aquae* [4] and more recently by *Oscillatoria* sp. and *Raphidiopsis mediterranea* [3,5]. Two naturally occurring analogs of CYN, 7-epicylindrospermopsin (7-Epi-CYN) and deoxy-cylindrospermopsin (7-deoxy-CYN) have been also identified [6]. 7-Deoxy-CYN was found in *C. raciborskii* as minor metabolite and in *Raphidiopsis curvata* and *Lyngbia wollei* as the major metabolite.

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7-Epi-CYN has so far been detected only in *A. ovalisporum* as minor metabolite. These three closely related molecules were shown previously to be toxic using in vivo assays, and they were shown to inhibit protein synthesis with similar potency [5].

In Palm Island outbreak of hepatoenteritis, Australia 1979, in which 148 people were hospitalized with hepatitis, the organisms in the original bloom of the water supply dam were not identified before treatment with copper sulphate. Retrospectively, the alga *C. raciborskii* was subsequently observed as a seasonally dominant species in the domestic water supply reservoir on Palm Island. Its severe hepatotoxic but also wide-ranging effects in mice make it an organism capable of producing the clinical disease seen at Palm Island, and following the suggestions of Hawkins et al. [7] *C. raciborskii* blooms should be considered as one possible cause. CYN is a highly biologically active molecule that interferes with several metabolic pathways. CYN was shown to be a potent inhibitor of protein synthesis in an in vitro rabbit reticulocyte globin synthesis assay [8] CYN also inhibits glutathione synthesis and induce stress responses using both in vivo and in vitro experimental models [9–11]. CYN is a potent hepatotoxin but it can cause also damage to the kidney, lungs, thymus and heart in several experimental models [8,10,12,13]. Genotoxic effects of CYN include DNA adduction and strand breakage in mouse liver [14] and micronuclei formation in a lymphoblastoid cell line [15]. No dose-response or other available information is available regarding the carcinogenicity of pure CYN [16].

The observation that CYN is generated by a range of cyanobacteria species has initiated efforts to define the spatial distribution and sources of CYN [17]. This toxin is widely distributed in tropical and subtropical freshwaters, e.g., Australia [18], and Florida [19], but is also found in temperate regions such as Europe. CYN was detected in Europe for the first time in Germany in 2002 [20], then in Italy [21,22], Spain [23], Finland [24], Poland [25] and Czech Republic [26]. Due to the toxicity of CYN, a derivation of a guideline value for CYN is in progress by the WHO [27], and a guideline safety value (GV) of 1 μg/L in drinking water has been proposed by Humpage and Falconer [28].

The monitoring of drinking water supplies for the presence of this toxin is of critical importance for the assessment of environmental and health risks. Compared to microcystins and saxitoxins, relatively little work has been done on methods for the detection of CYN. Common methods for quantitative determination of this toxin are liquid chromatography coupled with photo diode array detection (LC-PDA) with a C18 reverse phase column, and both, isocratic and gradient mobile phase methods have been developed. Harada et al. [29] developed the first screening method for CYN using reverse phase high performance (LC/PDA), and CYN has an easily identifiable peak and maximum UV absorbance at 262 nm. Welker et al. [30] developed an alternative method to analyze environmental samples for their content of CYN based on LC/DAD; the application of the protocol to natural samples proved to be hampered by the fact that the extraction with pure water, though very efficient for CYN, gave a considerable matrix background and occasionally covered CYN completely in chromatograms. In an interlaboratory comparison trial on CYN measurement in lyophilized cyanobacterial cells that involved six laboratories of five countries, it was shown that the most effective extraction method employed 5% aqueous formic acid, providing efficient extraction and fewer contaminants peaks than the extraction method using water only, when analyzed by LC-PDA employing an isocratic mobile phase of 5% (v/v) methanol plus 0.1% (v/v) TFA [31]. Due to its hydrophilic nature, some authors indicated that CYN cannot be extracted and concentrated from water samples with SPE cartridges such as C18, but SPE with graphitized carbon, has been used successfully [32,33]. Wormer et al. [34] obtained a reliable method by the sole use of graphitized carbon cartridges for the concentration of CYN from culture medium (*A. ovalisporum*) or from diverse environmental samples, using a previous sample preparation, a combination of dichloromethane:-methanol (DCM:MeOH, 1:4) with 5% formic acid as solvent, and quantification of CYN by LC-PDA.

Whilst the use of LC/DAD is less expensive alternative to MS/MS, it not adequately detect trace quantities of CYN [30]. Liquid chromatography–mass spectrometry (LC–MS) protocols, including triple quadrupole LC–MS/MS, have been established as a standard method for the identification and quantification of CYN in freshwaters and fish muscle [35–38]. The combination of hydrophilic interaction liquid chromatography with electrospray mass spectrometry (HILIC–MS) was applied to the analysis of field and cultured samples of *A. circinalis* and *C. raciborskii* [1]. Kubo et al. [2]proposed a fractionation method for CYN analysis from cells of *C. raciborskii* using a double-cartridges column (styrene polymer+anion exhange) with 0.1 M carbonate buffer at pH 10.5 followed by LC-PDA or LC–MS analysis of the extracts. In order to determine CYN at traces levels from cultures of cyanobaterial cells, the development of highly sensitive and quantitative validated method is essential. Classical approaches to analytical method validation consisted of checking the conformity of a performance measure to a reference value, but this does not reflect the end–user of the data or the consumer's needs [39]. Assessment of accuracy of analytical methods is a fundamental stage in method validation, and some procedures of intra-laboratory testing of method accuracy have been discussed by González et al. [40].

The aim of this work was to develop an analytical procedure based on solvent extraction followed by a purification step with graphitized cartridges and LC–MS/MS technique for the CYN determination from lyophilized cultures. The extraction and purification steps were optimized using a two-level full factorial design with replications. A suitable and practical procedure for assessing the trueness and precision of the proposed method has been applied by using validation standards, according to González et al. [40]. The present procedure has been intended for routine determination of aquatic samples (water, blooms, cultures) in order to detect CYN at trace levels.

2. Materials and methods

2.1. Reagents and materials

Cylindrospermposin (CYN) standards (purity > 95%) were supplied by Alexis Corporation (Lausen, Switzerland). The chemical structures of CYN and 7-deoxi-CYN are shown in Fig. 1. Standard solutions of CYN were prepared in water milli Q (100 μg/mL) and diluted as required for their use as working solutions (5–500 μg/L).

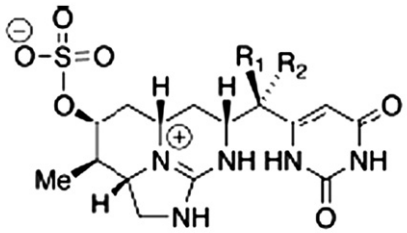
All chemicals and reagents used in this study were analytical grade materials. HPLC-grade methanol, dichloromethane, formic acid, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (> 18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

BOND ELUT® Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe).

2.2. Cyanobacterial cultures samples

2.2.1. Microcystis aeruginosa

PCC7820, a non-CYN-producing strain (CYN-), was obtained from the Pasteur Culture Collection (Paris, France). The culture was maintained in sterilized 250 mL Erlenmeyer flask containing 100 mL of BG11 medium (+1 M $K_2HPO_4 \cdot 3 \cdot H_2O$ + 5 mM $NaNO_3$ + 12 mM $NaHCO_3$) at 30 °C under continuous illumination with an



Compound name	R_1	R_2	Mw
Cylindrospermopsin (7-*R*)	OH	H	415.42
7-Epi-cylindrospermopsin (7-*S*)	H	OH	415.42
7-Deoxy-cylindrospermopsin	H	H	399.42

Fig. 1. Structures of Cylindrospermopsin (CYN) and its analogs ([5]).

intensity of 28 μmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent tubes, and later transferred to bottles containing 20 L of BG11 medium. After the period of 21 days, cultures were harvested and concentrated by centrifugation in continuous (14000 rpm).

2.2.2. Aphanizomenon ovalisporum

(LEGE X-001) cyanobacterial CYN-producing strain (CYN+) was supplied by the Marine Research Center (Porto, Portugal). Being isolated from Lake Kinneret, Israel [41]. Two cultures of this strain were maintained in Z8 medium at 25 °C under continuous illumination with an intensity of 28 μmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. After 33 days, cultures were harvested by decantation with a plankton net (20 μm diameter).

Both concentrated biomass were preserved at −80 °C until lyophilization (Telstar Cryodos, Madrid).

2.3. Solvent extraction and purification procedures

Both the extraction (SPE) and the purification (Clean-up) steps were optimized, according to the methods from Welker et al. [30], intended to modify final volume of milliQ water for extracting CYN, as well as the procedure of Wormer et al. [34], regarding to the proportion of solvents employed and its detection and quantification (LC–MS/MS instead of LC-DAD used in this work). After optimization of several variables through a full factorial design 2^3, the following extraction procedure was adopted: CYN content was extracted from the lyophilized cells of *A. ovalisporum* culture (14 mg) with 3 mL of MilliQ water, sonicated for 15 min, stirred for 1 h and sonicated again for 15 min. The resulting mixture was centrifuged at 4500 rpm. for 10 min, after which the supernatant was collected and 6 μL of 0.1% trifluoroacetic acid (TFA) were added. Then, it was stirred for 1 h and allowed to stand for 3 h. The supernatant was taken for further purification/concentration.

For the clean-up procedure, graphitized carbon cartridges are packed Bond Elut® which were activated with 10 mL of a solvent mixture of DCM/MeOH (10/90) and rinsed with 10 mL of MilliQ water. Subsequently, the sample is passed through the cartridges, washed with 10 mL of MilliQ water and eluted with 10 mL DCM/MeOH (10/90). For concentration of the sample, extract is evaporated in a rotary evaporator and resuspended in 500 μL MilliQ water, prior to its LC–MS/MS analysis.

Extraction efficiencies were performed in triplicate by spiking the matrix, lyophilized cells of *M. aeruginosa* PCC7820 strain (CYN-) with CYN standard solutions at three concentration levels: 20, 200 and 500 μg/L. Besides, a robustness study was carried out by spiking the matrix with a standard solution of 200 μg CYN/L.

2.4. Chromatographic conditions

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of an hybrid triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a 150 × 2.1 mm Zorbax Sb-Aq column. The flow rate was 0.2 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) methanol. Both components contained 0.05% TFA (v/v). The injection volume was 20 μL. The elution profile was: 0% B (1 min), linear gradient to 90% B (10 min), 90%B (5 min) and finally 0% B (5 min).

Multiple Reaction Monitoring (MRM) experiment was applied where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The transitions for the detection of CYN are: 416.2/194.0, 416.2/ 274.0, 416.2/336.0 and 416.2/175.9. The transition 416.2/194.0 was chosen for quantification of CYN. For the detection of 7-deoxy-CYN the following transitions are: 400.0/194.0, 400.0/320.0, and 400.0/274.0. For LC-ESI–MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, source gas 60 psi, ion spray voltage 5500 V, and the Turboprobe temperature was maintained at 350 °C.

2.5. Evaluation of CYN in different lyophilized cultures of Aphanizomenon ovalisporum (LEGE X-001)

Two different cultures of *A. ovalisporum* were used for our study. They were cultured under conditions referred to in paragraph 2.2.2. Levels of CYN from those samples of lyophilized *A. ovalisporum* cultures were analyzed according to the proposed and validated method. The total time for carrying out the complete procedure (including the freezing and lyophilization steps) oscillated between 2 and 3 days.

3. Results and discussion

3.1. General aspects

In order to develop the LC–MS/MS for the detection of CYN, commercially available standards solutions of CYN were assayed to acquire mass spectra and adjust mobile phase strength. Fig. 2 shows the MS/MS product ion spectrum and fragmentation scheme of CYN. The spectrum was obtained on collision of m/z 416, corresponding to the pseudomolecular ion $[M+H]^+$. The signals at m/z 336 and 318 were assigned to the loss of SO_3 and H_2O from the pseudomolecular ion, respectively. Another fragment ion, at m/z 274 corresponds to the loss of the [6-(2-hydroxy-4-oxo-3-hydropyrimidyl)]hydroxymethinyl moiety of the molecule. Finally, the ions at m/z 194 and 176 correspond to the loss of SO_3 and H_2O from the fragment ion at m/z 274 [1]. Quantitation of CYN was achieved using the 416/194 transition with other transitions monitored as confirmation ions.

3.2. Results from the optimization of the extraction procedure

The extraction procedure was optimized by a full factorial 2^3 design with replications. The considered factors were: amount of lyophilized cells of *A. ovalisporum* (X_1), volume of TFA (X_2) to precipitate dissolved cell components, and dichloromethane

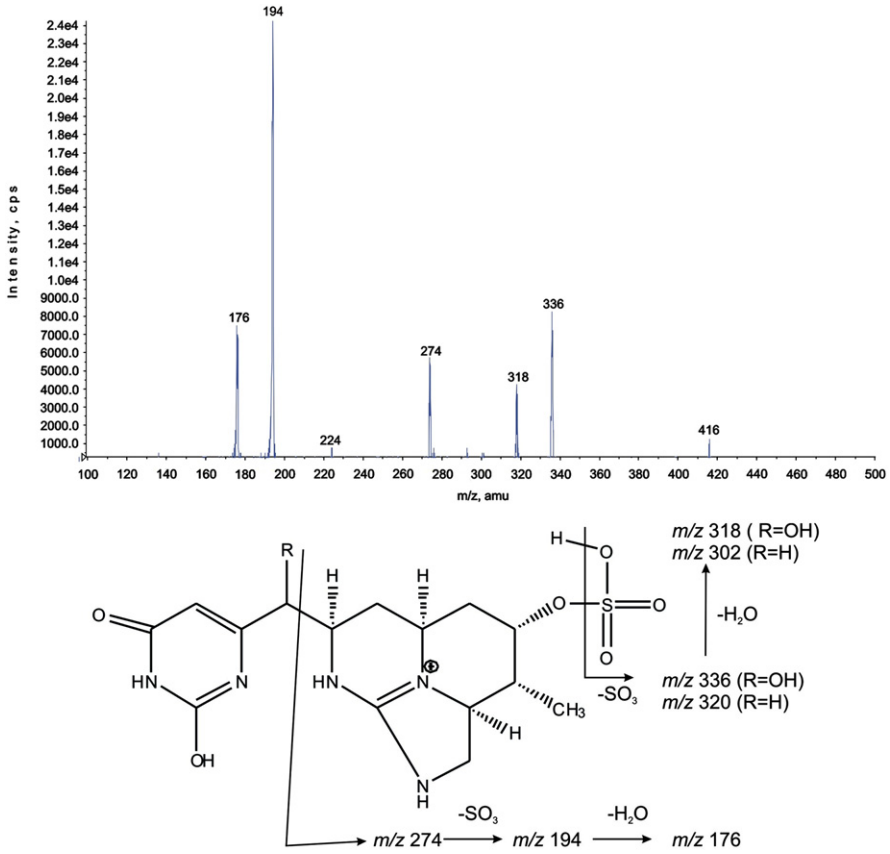


Fig. 2. MS/MS spectrum of the product ions of Cylindrospermopsin $[M+H]^+$ ion at m/z 416. The ion at m/z 194 was monitored for quantitation using the multiple reaction monitoring mode (a). Assignments of labeled fragment ions of CYN and its deoxy-derivative (7-deoxy-CYN) are shown (b).

Table 1
Tested values in the full factorial design for the variables related to the extraction and purification step in CYN determination lyophilized culture.

Variable	Tested value	Coded	Tested value	Coded	Tested value	Coded
Amount of lyophilized (mg) (X_1)	14	−1	28	0	42	+1
TFA volume (mL) (X_2)	2	−1	6	0	10	+1
Solvent proportion (mL) (X_3) (DCM/MeOH)	10/90	−1	20/80	0	30/70	+1

/methanol (DCM/MeOH) proportion used for the clean up procedure (X_3) to elute the toxin from the SPE cartridges. The levels are coded according to the rule: high level = +1, central level = 0 and low level = −1 as it is depicted in Table 1.

The results of the three factors, two-level full factorial design with replications of the extraction procedure of CYN from the lyophilized culture (explained in Section 2.3), are shown in Table 2. The significant factors were: amount of lyophilized material (X_1) and solvent proportion (X_3), as well as the interactions X_{23} and X_{13}, because their corresponding coefficients are significant. According to this, the best results were obtained taking 14 mg of lyophilized cells of *A. ovalisporum*, 6 μL of TFA, and using the proportion 10/90 of the solvent mixture DCM/MeOH. Thus, the experiment should be performed at −1 level for the factors X_1 and X_3, the other variable (X_2) is not affecting the results. This would lead to the best CYN recovery.

3.3. Calibration study

The response as a function of concentration was measured by a 6-point calibration curve with a linear range within 5–500 μg/L, equivalent to 0.18–18.00 μg/g. The regression equation was calculated from standards prepared in extracts from lyophilized *M. aeruginosa* PCC7820 ($r^2 \geq 0.9999$) Fig. 3.

3.3.1. Linear range

Response linearity was established according to Huber, 1998 [44] by plotting the called response factors (signal response/analyte concentration) against their respective concentrations, obtained from six lyophilized *M. aeruginosa* PCC7820 (CYN-) extracts spiked with standards ranging in concentrations from 5 to 500 μg CYN/L (equivalent to 0.18–18.00 μg CYN/g dry weight) and submitted to the proposed method (by triplicate). Fig. 4 shows the Huber plot. The target line has zero slopes and the intercept is just the median of the response factors obtained. Two parallel horizontal lines are drawn in the graph at 0.95 and 1.05 times the median value of the response factors in a fashion similar to the action limits of control charts. As no intersections with the lines were found, the linear range of the method applies to the full range studied.

3.3.2. Goodness of the fit

Linear calibration function was obtained by preparing six calibration standards in extracts of lyophilized *M. aeruginosa*

Table 2
Results from the three factors, two-level full factorial design with replications.

	Estimate	Standard error	*t*-Value df=3	Significance
b0	19.12273	0.309066	61.8726	YES
b1	−4.27250	0.362412	−11.7891	YES
b2	−1.04500	0.362412	−2.8835	NO
b3	−2.02750	0.362412	−5.5945	YES
b12	1.08750	0.362412	3.0007	NO
b13	−1.51000	0.362412	−4.1665	YES
b23	2.84250	0.362412	7.8433	YES
b123	−0.02000	0.362413	−0.0552	NO

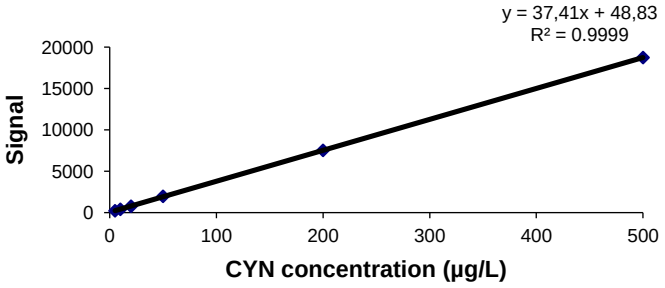


Fig. 3. Linear calibration function for the proposed procedure.

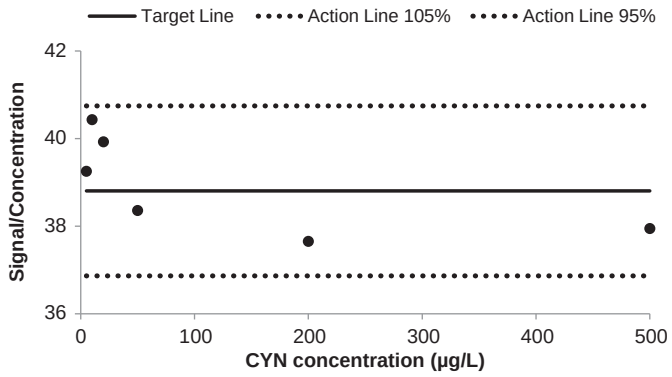


Fig. 4. Huber plot for assessing linear range.

PCC7820 (CYN-) cells (in triplicate) from 5 to 500 µg/L (equivalent to 0.18–18.00 µg CYN/g dry weight), and recording the signal response according to the proposed procedure. Here, drinking or recreational processed waters are taken as "placebo" and the analyte is spiked in the natural environment required for future samples. So, these calibration standards can be also considered as validation standards (VS). The calibration line has a correlation coefficient of 0.9999 and the corresponding ANOVA of the regression line indicates a lack-of-fit F ratio of 0.82 (Fig. 3). Consequently, there is not lack-of fit and the calibration function can be considered as linear.

3.3.3. Detection limit and quantification limit

The limits of detection (LOD) and quantification (LOQ) were calculated from the expression $Y_{LOD/LOQ}=Y_{blank}+nS_{blank}$, where Y_{blank} and S_{blank} are the average value of 10 independent blank samples and its corresponding standard deviation. In these expressions, $n=3$ in the case of LOD and $n=10$ in the case of LOQ. Afterwards, Y_{LOD} and Y_{LOQ} values are converted in concentration units by using the calibration function. The LOD and LOQ obtained were 0.04 µg CYN/g and 0.15 µg/ g, respectively. These values are similar to those found by Fastner et al. [42] (around 0.1 µg/g dry weight) when they analyzed lyophilized crude extracts using the MRM for CYN analysis by LC–MS/MS. Higher values of LOD and LOQ, 16 and 52 µg CYN/g, respectively, were reported by Liu and Scott (2011) [43] in algal food supplements extracts.

3.4. Accuracy study

3.4.1. Intermediate precision and recovery studies

According to ICH guidelines, 2005 [45], precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed as intra-assay or within-run. Intermediate precision applies to within-laboratory variations: different days, different analysts or equipments, and is sometimes called between-run or inter-assay precision [40]. The third level, reproducibility, expresses the between-laboratories precision like in collaborative studies, and it will not be considered in this work.

On the other hand, the trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted, either a conventional value or an accepted reference value like validation standard (VS) [40].

Repeatability and intermediate precision were calculated analyzing five replicates of *M. aeruginosa* PCC7820 strain (CYN-) extracts spiked with different concentrations of standard CYN (20, 200 and 500 µg/L) on the same day and in two different days, respectively.

Considering two different days, as the main source of variation, an analysis of variance (ANOVA) was performed for each concentration, obtaining estimations of within-condition variance (S_w^2), also known as repeatability (S_r^2), and between-condition variance (S_B^2). Also, the intra laboratory reproducibility or intermediate precision, is obtained as $S^2_{IP}=S_r^2+S_B^2$ [39,40]. All these parameters are shown in Table 3.

From these data, the corresponding relative standard deviations, RSD_R are calculated, and were compared with the acceptable RSD percentages obtained from the AOAC Peer Verified Methods (PVM) program [40,44]. As a quick rule [40], the RSD_{IP} results should be compared with one-half the corresponding RSD values tabulated. Our results, at the three concentration levels considered, were lower or the same order than the one-half $\%RSD_{AOAC}$ tabulated (11–15%) (Table 3).

Trueness can be expressed as the bias or recovery obtained for each validation VS assayed [46]. The total recovery for VS is defined as the ratio between the observed estimation of the VS concentration, and the "true" value T, expressed as percentage or as fraction. The recoveries (%) computed for the three VS considered are show in Table 3. We checked them for suitability by comparison with the published acceptable recovery range as a

Table 3
Estimations of within-condition (repeatability), between-condition, intermediate precision (intra laboratory reproducibility) and recoveries of CYN assayed in lyophilized cells of *M. aeruginosa* PCC7820, at three concentrations levels, in two different days.

	CYN concentration level		
	20 µg/L	200 µg/L	500 µg/L
S_w	1.73	16.62	10.16
S_B	2.49	24.98	37.31
S_{IP}	2.01	19.80	23.09
RSD_{IP} (%)	10.70	11.00	5.60
Recovery (%)	94	89	83

function of the analyte concentration [40,44]. In our method, as the CYN concentration of the three VS ranged between 20 and 500 μg/L, the recovery range (%) could oscillate between 80 and 110% for the three of them. The recoveries obtained oscillated between 94% for 20 μg/L and 83% for 500 μg/L. Thus, the method can be considered as acceptable in terms of recoveries.

These recoveries are higher than those obtained by Kubo et al. [2] ($75 \pm 1\%$) in extracts of *C. raciborskii* analyzed by LC/MS. Authors suggested that some compounds in the algal extract could interfere with the isolation of CYN. Recently, Liu and Scott (2011) [43] have determined CYN in algal food supplements extracts by LC–UV and the recoveries ranged between 70 and 90%.

3.4.2. Robustness study

The strategy for carrying out our robustness study is based on a landmark procedure suggested by Youden [47] and García et al. [48], according to the practical guide of González and Herrador (2007) [39]. These factors, relative to the SPE procedure,

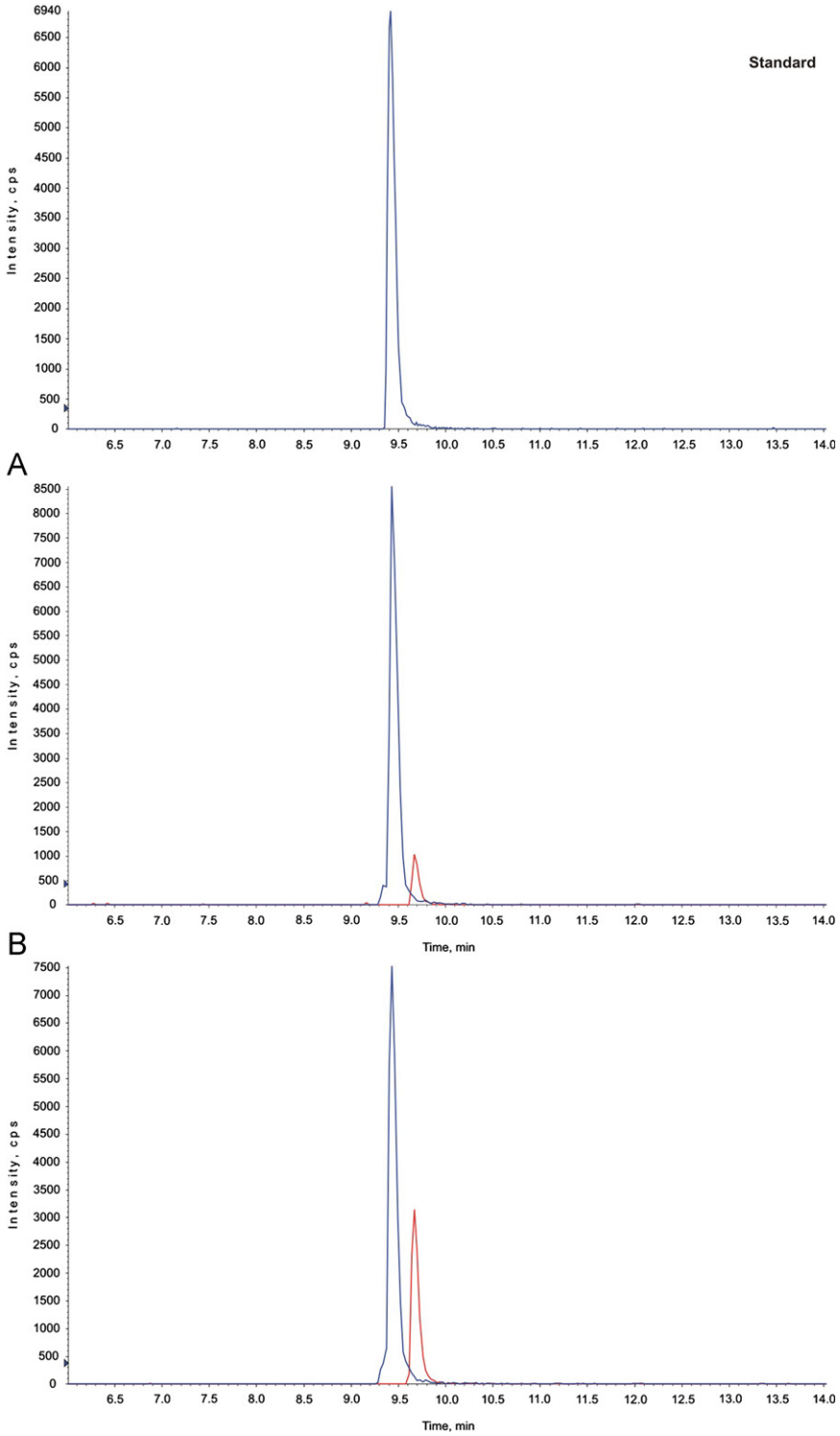


Fig. 5. LC–MS/MS Chromatograms of CYN standard and CYN and 7-deoxy-CYN from diluted extracts from two different samples of *Aphanizomenon ovalisporum* cultures (sample A and sample B, respectively).

were: (Z_1) the batch of the graphitized carbon cartridges employed; (Z_2) flow rate of the water sample through the cartridge, and (Z_3) final redissolved water volume after SPE treatment. The levels are coded according to the rule: high value$=+1$ ($Z_1=1$ (batch no. 1); $Z_2=1$ min; $Z_3=500$ μL), and low level$=-1$ ($Z_1=2$(batch no. 2); $Z_2=2$ min; $Z_3=520$ μL). The effect of each considered factor is estimated as the difference of the mean result obtained at the level$+1$ from that obtained at the level -1. Once effects have been estimated, to determine whether variations have a significant effect on the results, a significance *t*-test is used [49], and the *t*-values (Z_k) are compared with the 95% confidence level two-tailed tabulated value with the degrees of freedom coming the precision study for each concentration. In our study, the experiments were carried using culture extracts of cyanobacterial cells (CYN-) solutions spiked with 200 μg/L, and each factor was analyzed by quintuplicate in two different days. So, for 9 degrees of freedom, the *t*-values obtained were 0.768, 6.869, and 0.409, for Z_1, Z_2 and Z_3 factors, respectively. For the Z_1 and Z_3 $t(X_k)<t_{tab}$ (2.262), and then the procedure can be considered as robust against these two factors (at the levels fixed in the study). Accordingly, the flow rate in this case is an important factor to take into account when carrying out this method. Thus, a new assay was performed by selecting new levels of this factor: high value$=+1$($Z_2=60$ s) and low level$=-1$($Z_2=75$ s). Now, the *t*-values obtained were 0.910, 0.803, and 0.589 for Z_1, Z_2 and Z_3 factors, respectively, and $t(Z_k)<t_{tab}$ (2.262) always. Hence, the procedure can be considered as robust against the three factors (at the levels fixed in the study).

3.5. Evaluation of CYN in different lyophilized cultures of Aphanizomenon ovalisporum (LEGE X-001)

Levels of CYN from two different samples of *A. ovalisporum* cultures were analyzed according to the proposed and validated method, previously diluting the extracts 1/100 in milli-Q water. CYN was detected (retention time of 7.55 min) and quantified, and the results were 3675 (sample A) and 3979 μg CYN/g (sample B) (Fig. 5). Moreover, its deoxy-derivative (7-deoxy-CYN) has been also detected in both samples, with a retention time of 7.76 min. In this case, the full scan and tandem mass spectra of 7-deoxy-CYN were very similar, the only difference being the shift of ions at m/z 336 and 318 in CYN down 16 mass units in 7-deoxy-CYN (Fig. 2). As no pure standard of this isomer was available, the quantification of 7-deoxy-CYN was made using the CYN calibration curve (equivalent of CYN).The values obtained were 1405 μg 7-deoxy-CYN/g and 427 μg 7-deoxy-CYN/g, for samples A and B, respectively. The ratio between both toxins (CYN/7-deoxy-CYN) were 3/1 and 9/1 in both samples, respectively. The predominance of CYN agrees with previous results found by Li et al. [50] in a *C. raciborskii* strain (CY-Thai); they detected CYN and Deoxy-CYN in a ratio of 10/1 (CYN/7-deoxy-CYN) when they analyzed both toxins by HPLC–MS/MS. Nevertheless, other authors analyzed the production of CYN and Deoxy-CYN by HPLC–MS/MS from *Raphidiopsis mediterranea* showing a production of 917 and 1065 μg/g of CYN and Deoxy-CYN, respectively, reporting a ratio 1/1 [3]. Therefore, the ratio CYN/7-deoxy-CYN may depend on the cyanobaterial producing strain and/or the culture conditions.

4. Conclusion

This report presents a sensitive, reproducible, accurate, and robust method for extraction and determination of CYN in lyophilized cells, using SPE with graphitized carbon cartridges and quantification by LC–MS/MS. The recoveries (83–94%) and intermediate precision values obtained (5.6–11.0%), as well as the robustness of the method for the three factors considered, permit its validation. This method provides detection and quantification limits acceptable for environmental studies and proves its utility for determining CYN in lyophilized natural blooms samples. The total time for carrying out the complete procedure (including the freezing and lyophilization steps) oscillated between 2 and 3 days. Consequently, this LC–MS/MS method is appropriate to confirm and quantify CYN in natural samples previously assayed using screening methods (e.g. ELISA). Therefore, its usefulness would be as a confirmatory method to those employed in monitoring water for public health protection.

Novelty statement

We have developed and validated a sensitive, reproducible, accurate, and robust method for extraction and determination of CYN from *A. ovalisporum* lyophilized cells. This method is based on SPE with graphitized carbon cartridges for the extraction and quantification by LC–MS/MS. The recoveries (83–94%) and intermediate precision values obtained (5.6–11.0%), as well as the robustness of the method for the three factors considered relative to the SPE procedure (the batch of the graphitized carbon cartridges employed; flow rate of the water sample through the cartridge, and final redissolved water volume), permit its validation. This method provides detection and quantification limits acceptable for environmental studies.

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