

**MULTICLASS CYANOTOXIN ANALYSIS IN RESERVOIR WATERS:  
TANDEM SOLID-PHASE EXTRACTION FOLLOWED BY ZWITTERIONIC HYDROPHILIC  
INTERACTION LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY**

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## Abstract

The presence of cyanobacteria and cyanotoxins in all water bodies, including ocean water and fresh water sources, represents a risk for human health as eutrophication and climate change are enhancing their level of proliferation. For risk assessment and studies on occurrence, the development of reliable and sensitive analytical approaches able to cover a wide range of cyanotoxins is essential. This work describes the development of an HILIC-MS/MS multiclass method for the simultaneous analysis of eight cyanotoxins in reservoir water samples belonging to three different classes according to their chemical structure: cyclic peptides (microcystin-LR, microcystin-RR and nodularin), alkaloids (cylindrospermopsin, anatoxin-a) and three non-protein amino acids isomers such as  $\beta$ -methylamino-L-alanine, 2,4-diaminobutyric acid and N-(2-aminoethyl)glycine). A SeQuant ZIC-HILIC column was employed to achieve the chromatographic separation in less than 12 min. Previously, a novel sample treatment based on a tandem solid-phase extraction (SPE) system using mixed cation exchange (MCX) and Strata-X cartridges was investigated with the aim of extracting and preconcentrating this chemically diverse group of cyanotoxins. The Strata-X cartridge, which was configured first in the line of sample flow, retained the low polar compounds and the MCX cartridge, which was at the bottom of the dual system, retained mainly the non-protein amino acids. The optimization procedure highlighted the importance of sample ion content for the recoveries of some analytes such as the isomers  $\beta$ -Nmethylamino-L-alanine and 2-4-diaminobutyric acid. Method validation was carried out in terms of linearity, limit of detection (LOD) and quantification (LOQ), recoveries, matrix effect and precision in terms of repeatability and intermediate precision. This work represents the first analytical method for the simultaneous analysis of these multiclass cyanotoxins in reservoir water samples, achieving LOQs in the very low range of  $7 \cdot 10^{-3} - 0.1 \mu\text{g} \cdot \text{L}^{-1}$ . Despite high recoveries obtained at the LOQ concentration levels (101.0-70.9%), relative standard deviations lower than 17.5% were achieved.

**Keywords:** Hydrophilic interaction liquid chromatography, Mass spectrometry, Multiclass cyanotoxins, Reservoir water, Tandem solid-phase extraction.

## Introduction

Cyanobacteria (blue-green algae) are microscopic photosynthetic and prokaryotic organisms that form a common and naturally occurring component of most aquatic ecosystems. In low numbers they are important contributors to the aquatic biology of waterways but these species proliferate and present at high concentrations in phenomena known as harmful algal blooms (HABs), which appear to be increasing in frequency, duration, and range due to agriculture runoff, overfishing, and climate change [1].

Most cyanobacteria (CB) are an immense source of several secondary natural products with applications in the pharmaceutical, food, cosmetic, agriculture and energy sectors [2,3]. Different CB species are also well-known cyanotoxin producers and when blooms are formed, the risk of toxin contamination in surface waters increases, posing in some cases serious health problems to animals and humans [4]. Humans may be orally exposed to cyanotoxins by drinking contaminated water, through the consumption of cyanotoxin-containing water fish, crops, and food supplements, or by ingesting water during recreational activities [5]. In addition, the incorporation of cyanobacterial cells and cyanotoxins into spray aerosol of aquatic ecosystems which experience HABs is being under research recently. The inhalation of aerosol poses its own noteworthy health risks beyond the toxicological effects of cyanotoxins [6,7].

Cyanotoxins are usually classified in four groups according to their toxicological target: hepatotoxins (microcystins and nodularins), neurotoxins (anatoxins, saxitoxins and  $\beta$ -methylamino-L-alanine), cytotoxins that produce both hepatotoxic and neurotoxic effects (cylindrospermopsin), and dermatoxins (lypopolysaccharide, lyngbyatoxins and aplysiatoxin). In terms of their chemical structure, cyanotoxins are mainly divided into cyclic peptides, alkaloids, lipopeptides, non-protein amino acids (NPA) and lipoglycans [8,9].

Toxic cyanobacterial blooms in surface water represent a growing public health concern due to the multiple sources of human exposure to toxins. Recently, the United States Environmental Protection Agency (US EPA) has included a number of cyanotoxins produced during HABs in the Drinking Water Contaminant Candidate List 4 (CCL-4) [10]. Therefore, there are relatively few established limits for cyanotoxins. The World Health Organization (WHO) recommends a safe limit for microcystin-LR (MC-LR) of  $1 \mu\text{g}\cdot\text{L}^{-1}$  in drinking water [11]. For

recreational exposure to water, WHO proposes a limit of 20  $\mu\text{g}\cdot\text{L}^{-1}$  MC-LR as a moderate reference value for a health alert [12].

For risk assessment and studies on occurrence, the development of reliable, sensitive and selective analytical approaches able to cover a wide range of cyanotoxins are essential because they normally co-exist in any environment. Each cyanotoxin can be produced by more than one species of cyanobacteria, just as the same species also has the ability to produce more than one toxin and thus greatly contaminate the final products [13]. However, due to their different chemical structures and physicochemical properties, this task is highly challenging [14]. For instance, while biological methods are usually toxin specific, chemical methods, like liquid chromatography coupled with mass spectrometry (LC-MS) also have limitations and quantifying several cyanotoxins might require different sample analysis using diverse chromatographic conditions.

Several LC-MS methods have been developed for the simultaneous analysis of cyanotoxins belonging to different classes. Many of them use a reversed-phase (RP) C18 column for microcystin congeners along with nodularin, anatoxin-a and cylindrospermopsin [15] and recently anabaenopeptins and cyanopeptolin [16,17] have been as well included. When more polar cyanotoxins, like  $\beta$ -methylamino-L-alanine and their isomers, are analyzed together with other toxins, a derivatization step is introduced in order to use a RP C18 column [18,19]. RP-C18 columns (T3) designed to allow a 100% aqueous mobile phases needed for retaining highly polar analytes [20] have been extensively use for cyanotoxin analysis [21,22]. Filatova *et al.* propose the use of two RP columns, a Kinetex C18 for different cyanopeptide classes including microcystins, anabaenopeptins, aeruginosins, cyanopeptolins and microginins and a RP Atlantis T3 C18 column for the more polar compounds, anatoxin-a and cylindrospermopsin, in water from three reservoirs [23].

RP-C18 columns have been used as well in combination with polar columns like amide [24] and about all with hydrophilic interaction liquid chromatography (HILIC) columns [25-31] for multiclass cyanotoxin analysis purposes.

Previously, the use of HILIC columns was restricted to the analysis of the underivatized non-protein amino acid  $\beta$ -methylamino-L-alanine along or with their isomers 2,4-diaminobutyric acid, N-(2-aminoethyl)glycine) and  $\beta$ -amino-N-methyl-alanine, in aquatic organisms [32-35], environmental matrices [36-38] and food supplements [39] because it presents an important alternative to the commonly applied derivatization method.

Regarding sample preparation of the cyanotoxins, it is mainly based on solid phase extraction (SPE) [40]. Usually cation exchange SPE cartridges are used to purify low molecular mass basic compounds such as the NPA isomers in matrices like cyanobacteria extracts [37,41] environmental samples [33,36,42] and food supplements [43]. In contrast, microcystins and nodularin as moderately polar organic compounds are usually retained on reversed phase or hydrophilic-lipophilic balance (HLB) cartridges. On the other hand, porous graphitic carbon (PGC) cartridges are more effective for the retention of cilindrospermopsin and anatoxin-a in some situations due to its high water solubility [25,44-46]. Consequently, achieving a multiclass target analysis is a challenge. In this sense, the use of a dual cartridge assembly of above-mentioned cartridges has been previously investigated for the extraction of cyanotoxins from different families in environmental and food matrices [15,21,22,47,48]. However, the literature often describes one SPE procedure for each family of cyanotoxins or separate elutions of the cartridges [21,49,50] turning the extraction into a time-consuming and tedious process. To the best of our knowledge, the extraction of a mixture consisting of cyclic peptides and alkaloids in combination with the NPA isomers has not been developed previously.

This work describes the development of an advanced analytical method for simultaneous extraction, preconcentration and determination of eight cyanotoxins by HILIC-MS/MS following a tandem SPE sample treatment. In order to cover a wide range of cyanotoxins, a target list of compounds which are representative of the major groups of cyanotoxins present in fresh waters was selected. The toxins included are microcystin-LR, microcystin-RR and nodularin as cyclic peptides, cilindrospermopsin and anatoxin-a as alkaloids and three non-protein amino acids isomers ( $\beta$ -methylamino-L-alanine, 2,4-diaminobutyric acid and N-(2-aminoethyl)glycine)). A Zwitterionic HILIC column is used as unique stationary phase and two cartridges connected in series have been employed for the simultaneous extraction and preconcentration of all cyanotoxins. The method has been thoroughly validated and applied to the analysis of the dissolved cyanotoxins in water samples from several reservoirs located in Granada (Spain).

## 1. Experimental

### 1.1. Reagents and materials

The cyanotoxins studied including microcystin-leucine-arginine (MC-LR  $\geq$  99%), microcystin-arginine-arginine (MC-RR  $\geq$  99%), nodularin (NOD  $\geq$  95%), cylindrospermopsin (CYN  $\geq$  95%) and anatoxin-a (ANA  $\geq$  98%) were supplied by Enzo Life Sciences, Inc. (Lausen, Switzerland). Isomers  $\beta$ -N-methylamino-L-alanine hydrochloride (BMAA  $\geq$  97%), 2-4-diaminobutyric acid dihydrochloride (DAB  $\geq$  95%) and N-  $\beta$ -aminoethylglycine (AEG  $\geq$  98%) were supplied by Sigma Aldrich (Darmstadt, Germany). The structure and physico-chemical properties of the studied cyanotoxins are shown in Table SD1.

Stock standard solutions were prepared by adding 1 mL of the desired solvent directly into the vial of toxin supplied by the manufacturer and gently swirling the vial to dissolve the toxin. The obtained solutions were: 50  $\mu\text{g}\cdot\text{mL}^{-1}$  MC-LR in methanol, 25  $\mu\text{g}\cdot\text{mL}^{-1}$  MC-RR in 80% aqueous MeOH, 50  $\mu\text{g}\cdot\text{mL}^{-1}$  NOD in 50% aqueous MeOH, 25  $\mu\text{g}\cdot\text{mL}^{-1}$  CYN in MeOH, 1000  $\mu\text{g}\cdot\text{mL}^{-1}$  ANA in water. Stock solutions of 1000  $\mu\text{g}\cdot\text{mL}^{-1}$  for the three standard isomer molecules (BMAA, DAB and AEG) were prepared by dissolving the desired amount of analyte in water. All of them were stored in the dark at  $-20$  °C. Intermediate standard solutions of each compound at 2.5  $\mu\text{g}\cdot\text{mL}^{-1}$  were prepared by dilution of the stock solutions with the corresponding solvent for each toxin. These solutions were used to prepare the working solutions that consisted of a mixture of all cyanotoxins in concentration levels according to the experiment in 50% aqueous MeOH. These solutions were stored at 4 °C and equilibrated to room temperature before use.

Unless otherwise specified, analytical grade reagents and HPLC grade solvents were used in this work. Acetonitrile (MeCN) and methanol (MeOH) were purchased from VWR (Radnor, PA, USA). Ammonia solution ( $\text{NH}_3\cdot\text{H}_2\text{O}$ ) (30% assay) was purchased from Merck (Darmstadt, Germany). Formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Oasis WCX cartridges (60 mg), Oasis MCX cartridges of different sorbent mass (60 mg and 150 mg), and Oasis HLB cartridges (60 mg) from Waters (Milford, MA, USA); Strata-X cartridges (200 mg) and Strata WCX cartridges (100 mg) supplied by Phenomenex (Torrance, CA, USA) and WCX cartridges (60 mg) from Supelco Inc. (Bellefonte, PA, USA) were tested for cyanotoxins extraction from water samples. SPE tube adapters from Supelco Inc. (Bellefonte, PA, USA) were employed.

A large variety of syringe filters were evaluated: CLARIFY polytetrafluoroethylene (PTFE) hydrophilic filter (0.2  $\mu\text{m}$   $\times$  13 mm), CLARYFY PTFE hydrophobic (0.2  $\mu\text{m}$   $\times$  13 mm), CLARIFY cellulose acetate (CA) filter (0.2  $\mu\text{m}$   $\times$  13 mm) and CLARIFY glass microfiber (GF) filter (1  $\mu\text{m}$   $\times$  13 mm) from Phenomenex (Torrance, CA, USA); Nylon filter (0.2  $\mu\text{m}$   $\times$  25 mm) and PTFE filter (0.2  $\mu\text{m}$   $\times$  13 mm) from VWR International (West Chester, PA, USA); cellulose acetate (CA) filter (0.2  $\mu\text{m}$   $\times$  13 mm), glass microfiber (GF) filter (0.7  $\mu\text{m}$   $\times$  13 mm) and polyvinylidene fluoride (PVDF) filter (0.2  $\mu\text{m}$   $\times$  13 mm) from Thermo Fisher Scientific (Whatman, Maidstone, UK).

### 1.2. Instrumentation

Chromatographic separation of cyanotoxins was performed on an Agilent 1290 Infinity II System (Agilent Technologies; Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler (with 20  $\mu\text{L}$  injection loop) and a column thermostat. The LC system was coupled to an API 3200 triple quadrupole (QqQ) mass spectrometer (AB Sciex; Darmstadt, Germany) equipped with a Turbo V electrospray ionization source. Instrumental data were collected by the Analyst<sup>®</sup> Software (version 1.5) using the Scheduled MRM<sup>™</sup> Algorithm (AB SCIEX).

Separation was achieved using a SeQuant ZIC-HILIC column (2.1 x 250 mm, 3.5  $\mu\text{m}$  diameter, EMD Millipore; Billerica, MA). A Zorbax RRHD (Rapid Resolution High Definition) Eclipse Plus C18 (2.1 x 50 mm, 1.8  $\mu\text{m}$ ) column from Agilent Technologies (Waldbronn, Germany), a Luna Omega C18 Polar (2.1 x 100 mm, 1.6  $\mu\text{m}$ ) column, a Kinetex Biphenyl (2.1 x 50 mm, 1.7  $\mu\text{m}$ ) column and a Kinetex HILIC (2.1 x 100 mm, 1.7  $\mu\text{m}$ ) column, all supplied by Phenomenex (Torrance, CA, USA), were also tested.

An analytical balance with 0.0001 g resolution (Sartorius; Goettingen, Germany), a multi-tube vortexer (model BV1010 from Benchmark Scientific; Sayreville, USA), a vortex-2 Genie (Scientific Industries; Bohemia, NY, USA), an Universal 320R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany), a pH-meter (Crison model pH 2000; Barcelona, Spain), a nitrogen dryer EVA-EC System (VLM GmbH; Bielefeld, Germany) and a Visiprep solid-phase extraction unit from Supelco (Bellefonte, PA, USA) were used for sample treatment.

### 1.3. Sample preparation

Water samples collected in March 2021 from different freshwater swamps named El Portillo (Castril), Canales (Güéjar Sierra), Bermejales (Arenas del Rey) and Cubillas (Albolote), all located in Granada (Andalucía, Spain), were considered in this study. All samples were collected in amber glass bottles. After sampling procedure, the pH and the conductivity were measured. Conductivity ranged between 115 and 555  $\mu\text{S}\cdot\text{cm}^{-1}$ , while pH was around 8.0 for all of them. The content of sulfates, chlorides, nitrates, fluorides and phosphates determined by ion chromatography is shown in Table SD2. Water samples were filtered through a 0.22  $\mu\text{m}$  cellulose acetate membrane filter to remove suspended particles and they were kept at 4 °C until analysis.

#### 1.4. SPE procedure

A sample treatment was developed for cyanotoxin extraction and preconcentration using an assembly of two cartridges, a Strata-X (200 mg, 6 mL) and Oasis® MCX (150 mg, 6 mL), connected in series, which enabled the simultaneous extraction and preconcentration despite variations in their physico-chemical properties. The SPE was carried out using a 12-port SPE vacuum manifold. The optimized SPE procedure was as follows: an aliquot of 25 mL of freshwater sample previously acidified to pH 3 with 37% commercial concentrated hydrochloric acid was placed in a volumetric flask and spiked at the desired analyte concentration levels. The two cartridges were conditioned and activated separately with 3 mL of MeOH followed by 3 mL of deionized water at pH 3. After that, the cartridges were connected in series: Strata-X cartridge (retaining low polar and moderately polar compounds such as microcystins (MCs), NOD, CYN and ANA) was configured first in the line of sample flow, followed by the MCX cartridge (retaining the highly polar and water-soluble non-protein amino acids BMAA, DAB and AEG, as well as the rest of positive charged cyanotoxins that might pass through Strata-X cartridge). Samples were loaded onto the dual-cartridge set-up. This step was vacuum-assisted in order to maintain a uniform and constant sample flow rate of 1 mL $\cdot\text{min}^{-1}$ . Then, the cartridges were dried under vacuum for 5 min by passing low-pressure nitrogen. Before the elution step, the order of the cartridges was reversed, being MCX at the top and Strata-X at the bottom of the dual system. Elution of the analytes was carried out using 5 mL of 10%  $\text{NH}_3\cdot\text{H}_2\text{O}$  in MeOH. The eluate was evaporated to dryness in a heating block (30 °C) under a gentle stream of nitrogen and then the residue was re-dissolved with 250  $\mu\text{L}$  of 60% MeCN with 0.1% of FA. The final extract was filtered through a CLARIFY-



PTFE hydrophilic filter (0.2  $\mu\text{m}$   $\times$  13 mm), transferred to a glass insert and analyzed by the proposed HILIC-MS/MS method.

### 1.5. HILIC separation

Chromatographic separations were carried out on a SeQuant ZIC-HILIC column at 55 °C. Mobile phase flow was set at 0.2 mL $\cdot$ min<sup>-1</sup>. It consisted of water as eluent A and MeCN as eluent B, both containing 0.3% (v/v) of FA as volatile acid. Separation was accomplished under gradient elution conditions. The composition (expressed as a percentage of eluent B in mobile phase) was established as follows: 0 min, 60% (v/v); 3 min, 60% (v/v); 6 min, 40% (v/v); 12 min, 40% (v/v); and 13 min, 60% B (v/v). Finally, initial conditions were maintained for 22 min in order to guarantee column equilibration between runs (35 min of total analysis time). Injection volume was set to 20  $\mu\text{L}$  (full loop injection).

### 1.6. MS/MS parameters

The detection of the target cyanotoxins was carried out using tandem mass spectrometry with electrospray ionization operated under positive mode (ESI (+)-MS/MS) in multiple reaction monitoring (MRM) conditions. Source parameters were set as follows: source temperature (TEM), 550 °C; ion spray (IS) voltage, 5500 V; nebulizing and drying gases (GS1 and GS2, nitrogen), 50 psi (344.7 kPa), curtain gas (CUR, nitrogen), 25 psi (172.4 kPa) and collision gas (CAD, nitrogen) was set to 10 psi (69 kPa). The precursor and product ions of individual analytes were identified by tuning after direct infusion of individual standard solutions into ESI-MS/MS system. One precursor ion and two product ions were monitored for all analytes. The protonated form of cyanotoxins (*i.e.*,  $[\text{M}+\text{H}]^+$ ) was selected as precursor ion for all analytes except for MC-RR, which tends to ionize as the diprotonated molecular ion (*i.e.*,  $[\text{M}+2\text{H}]^{2+}$ ). The fragment ion with the highest intensity was used for quantification ( $Q_{\text{ion}}$ ) whereas the second one was acquired for identification ( $I_{\text{ion}}$ ). Main MRM parameters, including declustering potential (DP), entrance potential (EP), collision entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP), are given in Table 1.

**Table 1.** MRM parameters for the analysis of cyanotoxins by HILIC-ESI-MS/MS.

Analyte	Retention time (min)	Molecular ion	Precursor ion (m/z)	DP (V)	EP (V)	CEP (V)	Product ions	CE (V)	CXP (V)
MC-LR	3.1	[M+H] <sup>+</sup>	995.6	136	10.5	32	Q <sub>ion</sub> 135.2	93	4
				136	10.5	32	I <sub>ion</sub> 105.0	129	4
NOD	3.3	[M+H] <sup>+</sup>	825.4	96	6.5	24	Q <sub>ion</sub> 135.1	89	2
				96	6.5	100	I <sub>ion</sub> 103.2	129	4
CYN	4.2	[M+H] <sup>+</sup>	416.0	51	11.0	18	Q <sub>ion</sub> 194.2	49	6
				51	11.0	18	I <sub>ion</sub> 175.9	49	6
MC-RR	4.3	[M+2H] <sup>2+</sup>	519.8	41	6.0	18	Q <sub>ion</sub> 135.2	39	4
				41	6.0	18	I <sub>ion</sub> 103.2	91	6
ANA	4.9	[M+H] <sup>+</sup>	166.2	36	4.5	12	Q <sub>ion</sub> 149.2	19	4
				36	4.5	12	I <sub>ion</sub> 131.2	19	4
BMAA	9.6	[M+H] <sup>+</sup>	119.2	26	4.0	6	Q <sub>ion</sub> 44.1	27	2
				26	4.0	6	I <sub>ion</sub> 102.1	15	4
DAB	10.6	[M+H] <sup>+</sup>	119.1	21	3.0	14	Q <sub>ion</sub> 101.0	11	4
				21	3.0	14	I <sub>ion</sub> 56.0	31	2
AEG	11.8	[M+H] <sup>+</sup>	119.1	26	3.5	10	Q <sub>ion</sub> 102.0	11	4
				26	3.5	10	I <sub>ion</sub> 56.0	25	4

Abbreviations: declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; collision energy, CE; collision cell exit potential, CXP; quantification ion, Q<sub>ion</sub>; identification ion, I<sub>ion</sub>.

## 2. Results and discussion

### 2.1. Optimization of the chromatographic conditions and MS/MS detection

The development of suitable chromatographic separation conditions for the eight target cyanotoxins is challenging due to the large differences in analyte structure and polarity (log P ranging from 2.4 to -4.0, calculated by using Advanced Chemistry Development software V11.02 (ACD/Labs, Toronto, Ontario, Canada) [51]). Method optimization was carried out for a mixture of eight cyanotoxins at concentrations between 30 and 400  $\mu\text{g}\cdot\text{L}^{-1}$ , depending on the sensitivity of each analyte. BMAA, DAB and AEG are isomers, hence they have the same molecular ion  $[\text{M}+\text{H}]^+$  at  $m/z$  119. Product ion spectra show that they have very similar fragmentation patterns, with some minor differences being that BMAA has a more intense fragment at  $m/z$  102.1, DAB at  $m/z$  101.0 and AEG at  $m/z$  102.0. However, although they were the most intense fragments obtained by direct infusion of individual standard solutions, for BMAA the fragment  $m/z$  44.1 was finally selected as  $Q_{\text{ion}}$  because higher signal-to-noise ratio was obtained in real samples. As described above, the challenge of developing a chromatographic separation method for the target cyanotoxins is to ensure that the baseline separation of isomers is successfully achieved to prevent misidentifications, while retaining the rest of the cyanotoxins which are less polar than the non-protein amino acids.

Initially, several stationary phases were tested, including both reversed phase (RP) columns (Zorbax RRHD Eclipse Plus  $\text{C}_{18}$ , Luna Omega  $\text{C}_{18}$  Polar and Kinetex Biphenyl) and two HILIC columns (Kinetex HILIC and SeQuant ZIC-HILIC). For RP columns MeCN (eluent B) and  $\text{H}_2\text{O}$  (eluent A) were used as mobile phases employing isocratic mode at different percentages of eluent B (25%, 15% or 10%). Flow rate was  $0.3 \text{ mL}\cdot\text{min}^{-1}$ , temperature was set at  $40 \text{ }^\circ\text{C}$  and an injection volume of  $20 \mu\text{L}$  (full loop) was used. RP columns gave either no significant or slight retention on the columns resulting in broad peaks. HILIC employs a polar stationary phase becoming an alternative approach to effectively separate small polar compounds such as NPA. With the aim of testing HILIC columns, the same chromatographic conditions were employed in isocratic mode at 70% of eluent B. Among the two tested HILIC columns SeQuant ZIC-HILIC, which has a sulfobetaine type zwitterionic stationary phase covalently attached to silica particles, showed the best results in terms of resolution, although the isomers were not eluted under the isocratic mode. Thus, a gradient elution program was investigated to provide

the elution of BMAA, DAB and AEG. The final gradient program started at 60% B (held for 3 min), decreasing to 40% B in 3 min (held for 6 min), and back to 60% B in 1 min. An equilibration time of 22 min with the initial gradient conditions was needed after each sample run (35 min total analysis time) to ensure the reproducibility of the analysis. With these conditions, all cyanotoxins eluted as separate peaks. Commonly, the use of a volatile acid in the mobile phase leads to an improvement in the ionization step under ESI+ conditions. Therefore, the addition of FA to both eluent A and B was tested between 0% and 0.5% (v/v). It was observed that the presence of FA in the mobile phase improved the sensitivity and peak shape although signal suppression was observed at the highest concentrations of acid. In this sense, the best signal-to-noise ratio was achieved when 0.3% of FA (v/v) was added in both, eluent A and B. The effects of the mobile phase flow rate were investigated in the range between 0.2 and 0.35 mL·min<sup>-1</sup>. Although retention time decreased with high flow rates, system pressure increased above 400 bar. This was a constraint to the applicability of this method because it exceeded the maximum working pressure that the employed SeQuant HILIC column can withstand, so 0.2 mL·min<sup>-1</sup> was selected with a pressure of 315 bar at the initial part of the gradient. Column temperature was evaluated in the range of 25 to 60 °C while 55 °C was found to be the optimum value as narrower peaks and slightly better sensitivity were obtained. Finally, injection solvent and injection volume were studied. Sample solvents with higher elution strength than the initial mobile phase composition cause a less effective retention on the stationary phase with undesirable peak distortion, peak broadening and earlier elution. This is even more relevant in HILIC separation as it is generally more sensitive to the solvent strength mismatch between the sample solvent and the mobile phase in comparison to RP chromatography. HILIC requires the injection of low water content solution to maintain acceptable chromatographic peak shapes [52]. However, this can cause solubility issues with highly polar analytes, such as BMAA, DAB or AEG. Different percentages of aqueous MeCN were tested as sample solvent. The increment of MeCN up to percentages above the 60% improved the analyte peak shapes. Finally, it was observed that the addition of FA to the injection solvent increased peak areas and provided significant narrower and better peak shapes for the isomers, especially for DAB, thus a 60% of aqueous MeCN with 0.1% of FA was selected as the optimum sample solvent.

The ionization source parameters as well as the pressure of the CAD were optimized to enhance signal sensitivity for each cyanotoxin. Source temperature was evaluated between 350 and 650 °C. The signal for the majority of analytes increased with the temperature up to 550 °C, so this was selected as optimum. CAD was studied between 5 and 15 psi and 10 psi was selected. IS voltage was studied in the range of 4500 to 6000 V and it was observed that an increment in IS voltage slightly decreased peak area for most of the analytes, while in the case of MC-LR, peak area dramatically increased. Thus, in order to have a better sensitivity for MC-LR, 5500 V was selected as optimum. Pressure of CUR was studied between 10 and 40 psi. High values of CUR increased peak area for the majority of the analytes except for MC-LR and NOD which disappeared when a pressure of 40 psi was applied. A pressure of 25 psi (172.4 kPa) was selected as optimum CUR as it was the highest value that did not impair the sensitivity of any analyte. GAS 1 and GAS 2 (both of them nitrogen) were studied between 40 and 60 psi and both were set to 50 psi (344.7 kPa).

## *2.2. Optimization of SPE procedure using standard solutions*

Simultaneous SPE of the target cyanotoxins is a challenge due to their diverse physico-chemical properties. MC-LR and MC-RR have a non-protein amino acid named (all-S,all-E)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) with a lipophilic side chain, two negatively charged carboxylic groups at neutral pH and a positive charge in the guanidine group ( $pK_a > 12$ , calculated by using Advanced Chemistry Development software V11.02 (ACD/Labs, Toronto, Ontario, Canada) [51]) as both are arginine-containing MCs. NOD is structurally and toxicologically related to MCs and it also presents amino groups positively charged at neutral pH. CYN is highly hydrophilic and carries both a positive and a negative charge at neutral pH. ANA is also hydrophilic and has a positive charge at neutral pH. Isomers BMAA, DAB and AEG are small molecules and polar NPA, so they can be in positive, negative or zwitterionic form depending on the pH. At a pH below their isoelectric point, they carry a net positive charge, which facilitates their interaction with cation exchangers (Table SD1).

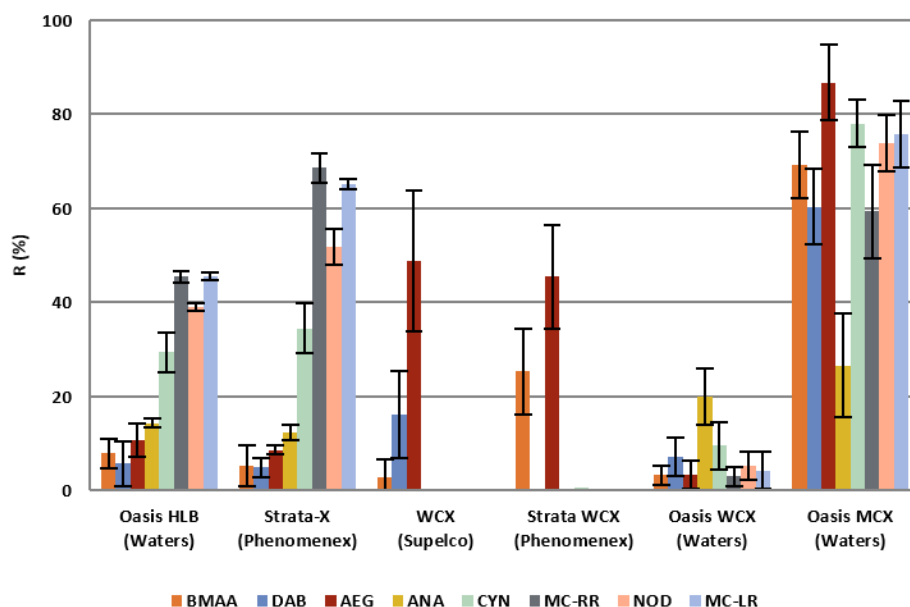
Our study was focused on developing a SPE method and optimizing parameters that could affect the simultaneous extraction of the cyanotoxins from reservoir water samples. Thus, extraction parameters such as sorbent type and size, sample pH, composition and volume of the elution solvent and breakthrough volume were evaluated to select the optimum

conditions by assessing the recovery of each step. For SPE optimization, samples were aqueous standard solutions containing  $30 \mu\text{g}\cdot\text{L}^{-1}$  for MC-LR, NOD, CYN and MC-RR,  $300 \mu\text{g}\cdot\text{L}^{-1}$  for ANA and AEG and  $400 \mu\text{g}\cdot\text{L}^{-1}$  for BMAA and DAB. Samples were loaded into the cartridge without subsequent washing steps. After that, the cartridge was dried prior the elution step. Eluate was collected, evaporated to dryness, reconstituted in  $250 \mu\text{L}$  of 60% MeCN with 0.1% FA (v/v), filtered with hydrophilic PTFE filters and injected into the HILIC-MS/MS system.

### 2.2.1. Type of sorbent

Taking into account that all the target cyanotoxins contain amino groups, which are positively charged at pH below their pKa, the use of cation exchangers were thoroughly investigated. Non-ionic cartridges were also evaluated. The selection of the appropriate cartridge was based on the evaluation and comparison of a wide variety of stationary phases from several suppliers. Preliminary studies were performed with 1 mL of deionized water spiked at the desired concentrations of each cyanotoxins and following the general SPE procedure recommended by the manufacturer for each sorbent. Non-ionic stationary phases such as Oasis HLB (60 mg, Waters) and Strata-X (60 mg, Phenomenex) were tested, eluting with MeOH. Weak cation exchange cartridges such as WCX (60 mg, Supelco) and Strata WCX (100 mg, Phenomenex) and a mixed-mode weak cation exchange Oasis WCX (60 mg, Waters) were tested, eluting with 2% HCl or  $\text{NH}_3\cdot\text{H}_2\text{O}$  in MeOH. Finally, the mixed-mode strong cation exchange cartridge Oasis MCX (60 mg, Waters) was tested eluting with 5%  $\text{NH}_3\cdot\text{H}_2\text{O}$  in MeOH. In all cases, 5 mL of eluent was employed. The non-ionic cartridges (HLB and Strata-X) showed an acceptable performance with non-polar compounds, although NPA isomers and ANA were scarcely retained. The weak cation exchange cartridges showed very low reproducibility and recoveries for most of the compounds, except for AEG, which was retained in both Supelco and Strata WCX cartridges. However, the MCX cartridge provided satisfactory recoveries for the three isomers as well as for the rest of cyanotoxins, with values above 55% for all of them except for ANA (**¡Error! No se encuentra el origen de la referencia.**). In light of the results obtained from the overall assessment of individual SPE cartridges and in accordance with other authors suggestions [44], a more specific optimization was carried out using the strong cation exchange adsorbent Oasis MCX. These cartridges contain a mixed-mode polymeric sorbent with reversed-phase and strong cation functionalities (by sulfonated groups) that are able to interact selectively with neutral and basic compounds, therefore the retention

mechanism of compounds by MCX includes both ion exchange and electrostatic interaction. Once the cartridge was selected, the sorbent mass of the cartridge (60 and 150 mg) was evaluated showing that an increase in the amount of sorbent also increases the recoveries, especially for the NPA isomers, so a 150 mg MCX cartridge was selected.



**Figure 1.** Performance of diverse SPE cartridges in standard solution. Error bars represent the standard error.

### 2.2.2. Elution solvent

When using a MCX cartridge, a neutral organic eluate is not enough to desorb the analytes. Thus an alkaline methanolic solution that helps to neutralize the charged cyanotoxins leading to their elution is necessary. Moreover, elution volume showed a crucial role especially for MC-RR elution [53]. In order to assess this influence, 1 mL of spiked deionized water was loaded onto the MCX cartridge and 5%  $\text{NH}_3 \cdot \text{H}_2\text{O}$  in MeOH was selected as the elution solvent. Volumes from 1 to 10 mL were studied at a flow rate of  $1 \text{ mL} \cdot \text{min}^{-1}$ . It is noteworthy that when 1 mL of eluent was employed MC-LR, MC-RR, NOD and ANA were not eluted from the cartridge, showing higher interaction than the NPA isomers and CYN. However, increasing the elution volume up to 10 mL enhanced the recoveries for all analytes, so it was selected as the optimum value. Once the influence of elution volume was checked, the percentage of ammonia solution in MeOH was evaluated between 5-15% (v/v). Slightly better recoveries were obtained when increasing the percentage of aqueous ammonia up to 10%, so it was

selected as the optimum composition. Then, the temperature of elution solvent was tested in order to improve the overall recoveries, however no significant differences were obtained between elution at room temperature or at 40 °C, so heating was not required.

### 2.2.3. Sample volume and sample pH

Sample volume was evaluated by spiking different volumes of deionized water (1, 10, 50, 75 and 100 mL) while keeping the cyanotoxin masses constant in all cases, loading them onto the MCX cartridge at a flow rate of 1 mL/min and eluting with 10 mL of 10% NH<sub>3</sub>·H<sub>2</sub>O (v/v) in MeOH. Sample volume was not significant for most of the analytes except for CYN, the recoveries of which highly decreased when the loading volume increased, which was in accordance with a previous publication [44]. This suggests that CYN is not strongly adsorbed in the stationary phase, probably due to its secondary amino group, which does not interact with the sulfonic groups as strongly as a primary amino group does, and also probably due to its high water solubility, which facilitates the early elution from the sorbent during the sample loading of relatively large volumes of water. Consequently, 10 mL was selected as sample loading volume.

The target cyanotoxins present amino groups positively charged at pH below their pKa (Table SD1); therefore, acidification of water samples might be preferable prior to SPE to achieve satisfactory retention. We checked two pH values: deionized water without pH adjustment and pH 3 (adjusted with 37% HCl). Recoveries of NPA isomers slightly improved at pH 3 so it was selected as the optimum sample pH.

### 2.2.4. Evaporation and filtration

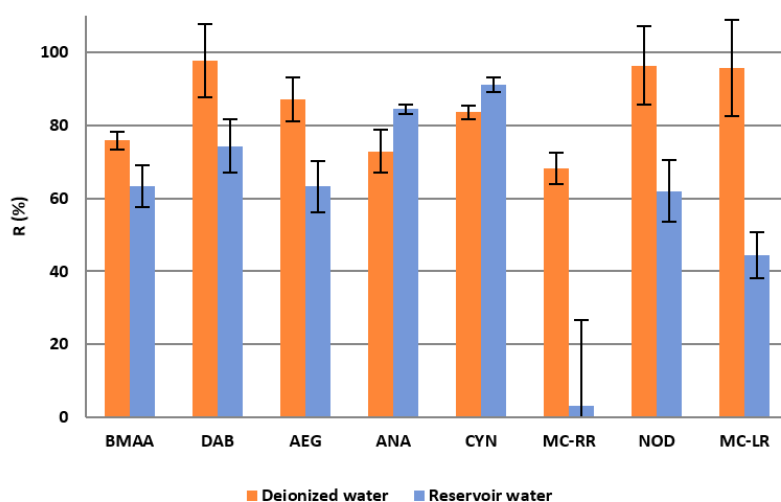
Evaporation after elution from the SPE cartridge permits preconcentration of the analytes and reconstitution of the sample with the most suitable solvent for injection in the HILIC column. Evaporation was carried out in a heating block under a gentle stream of N<sub>2</sub> at 30 °C. Filtration of the final extract is useful to extend column lifespan. However, this step may cause the loss of the target cyanotoxins. In order to reduce undesirable interactions between analytes and the filter membrane, different materials such as CA, GF, PTFE, Nylon or PVDF from different suppliers were tested by comparing filtrated standard solutions with unfiltered standard solutions. Hydrophilic PTFE filter membrane was the most reliable filter material for



the elimination of suspended material as no significant differences were observed when comparing with unfiltered standard solution.

### 2.3. Optimization of SPE procedure using reservoir water samples

After SPE optimization studies using spiked deionized water, reservoir water samples from El Portillo (Granada, Spain) were submitted to the final SPE protocol. Although the recoveries for all cyanotoxins with MCX cartridges were above 60% in deionized water, a decrease was observed for the NPA isomers, NOD and MCs when reservoir water samples were analyzed (**¡Error! No se encuentra el origen de la referencia.**). The main difference was related to MC-RR, the recoveries of which were negligible



**Figure 2.** Performance of the optimized SPE using standard solution comparing with reservoir water samples. Error bars represent the standard error.

when spiked reservoir water was analyzed. This result could be explained by two main reasons: a practically non-existent retention of the target cyanotoxin in the sorbent, or an ineffective elution from the cartridge. The analysis of the collected reservoir water sample after the loading step showed that MC-RR was retained entirely on Oasis MCX cartridge therefore elution was not occurring, probably due to some interaction caused by compounds present in natural water samples. In order to verify the negative influence of reservoir water matrix in the elution of MC-RR from the cartridge, mixtures of different proportions of reservoir water and deionized water (from 0% to 100% of reservoir water) were spiked and

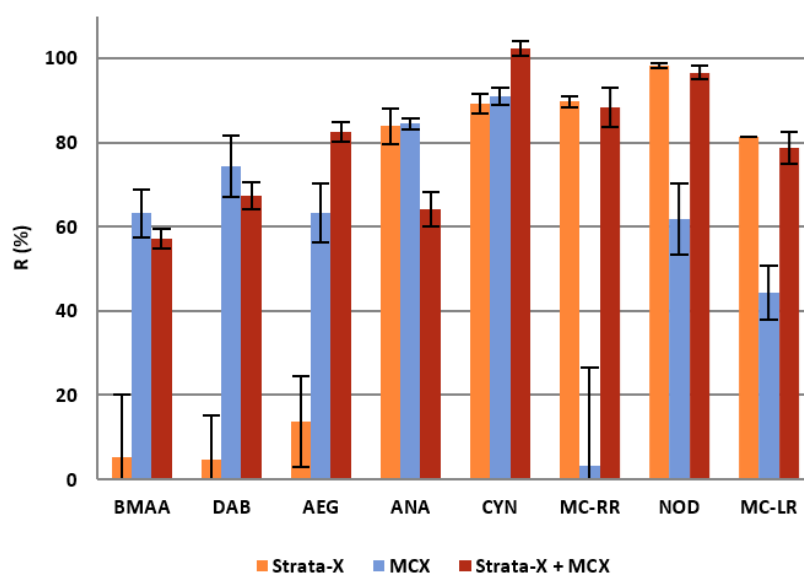
submitted to the whole SPE procedure. In accordance with the aforementioned, the elution of MC-RR decreased when the proportion of reservoir water in the sample increased, being negligible when 100% of reservoir water sample was analyzed. The obtained results indicated that a modified sample treatment approach was necessary. Alternatively, the use of a tandem SPE assembly with an extra cartridge could enhance the overall efficiency of the method, enabling the simultaneous extraction and elution of all cyanotoxins despite their different characteristics.

### 2.3.1. Evaluation of tandem SPE assembly

Two types of non-ionic cartridges (Oasis HLB and Strata-X, 200 mg) were evaluated under MCX optimized conditions. The combination of the two sorbents could allow a successful recovery of MC-RR, since it could be retained on the non-ionic cartridge, which would be configured first in the line of sample flow, followed by the MCX cartridge.

To that end, 10 mL of reservoir water sample spiked with  $30 \mu\text{g}\cdot\text{L}^{-1}$  for MC-LR, NOD, CYN and MC-RR,  $300 \mu\text{g}\cdot\text{L}^{-1}$  for ANA and AEG and  $400 \mu\text{g}\cdot\text{L}^{-1}$  for BMAA and DAB were loaded onto the cartridges which were previously conditioned with 3 mL of MeOH and 3 mL of deionized water at pH 3. After drying the cartridges, analytes were eluted with 10 mL of 10%  $\text{NH}_3\cdot\text{H}_2\text{O}$  (v/v) in MeOH. The extract was dried under a gentle stream of  $\text{N}_2$  and reconstituted in 250  $\mu\text{L}$  of 60% MeCN 0.1% FA prior to the injection into the HILIC-MS/MS system. Both sorbents showed a similar trend, however, the reversed phase functionalized polymeric sorbent Strata-X cartridge provided better overall recoveries for MCs, NOD, CYN and ANA while NPA isomers were scarcely retained in both Strata-X and HLB cartridges. Then, Strata-X and MCX cartridges were connected in series, being Strata-X cartridge configured at the top and MCX cartridge at the bottom of the tandem SPE system. After the conditioning step, spiked reservoir water sample was loaded at  $1 \text{ mL}\cdot\text{min}^{-1}$  onto the dual-cartridge set-up and then they were completely dried. The non-polar compounds were highly retained in the Strata-X cartridge, at the top of the tandem-SPE assembly, while the more polar compounds leached through the first cartridge and were retained on the subsequent MCX cartridge. It is noticeable that compounds partially retained in the first cartridge like ANA and CYN, would be retained in the MCX cartridge, minimizing analyte losses. For analyte elution the order of the cartridges was reversed, MCX being at the top and Strata-X at the bottom of the dual SPE system. This

inversion is required to avoid MC-RR retention on the MCX sorbent. The elution step was performed using 10 mL of 10% NH<sub>3</sub>·H<sub>2</sub>O in MeOH. Although usually the Strata-X cartridges are eluted with acidified methanol, an alkaline methanolic solution is necessary to neutralize and desorb the positively charged compounds from MCX cartridge. In spite of this, no negative effects were observed in the recoveries of non-polar compounds retained in Strata-X. In fact, overall recoveries highlighting that the proposed dual SPE strategy avoided the drawbacks resulting from the retention of MC-RR on MCX cartridge, achieving recoveries above 85% (¡Error! No se encuentra el origen de la referencia.). At this point, some parameters affecting the dual SPE performance were re-optimized in natural reservoir water samples.

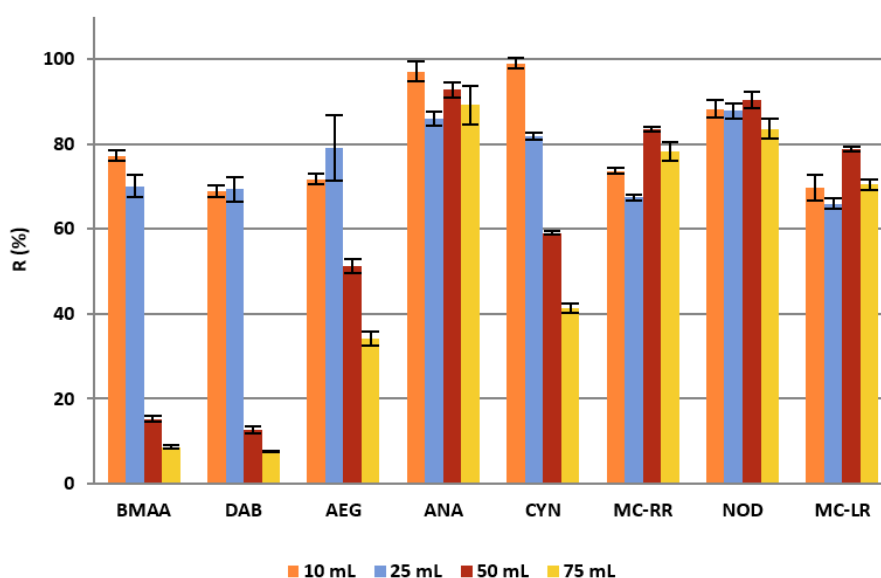


**Figure 3.** Performance of tandem SPE compared with single cartridge SPE in reservoir water samples. Error bars represent the standard error.

### 2.3.2. Breakthrough volume

During the SPE procedure, a sudden drop in the recovery values can occur due to sorbent saturation when large volumes of sample are loaded. The breakthrough volume is important because it is related to the preconcentration factor, thus affecting the sensitivity of the method. To evaluate the breakthrough volume, a series of experiments with increasing volumes of spiked reservoir water samples (10, 25, 50 and 75 mL) maintaining the cyanotoxin masses constant in all cases were carried out using the previously explained procedure. It was observed that an increase in the sample volume barely affected the retention of the non-

polar MCs and NOD; however, volumes above 25 mL highly decrease the recoveries of the most polar compounds, especially BMAA and DAB, although AEG and CYN were also negatively affected (*Error! No se encuentra el origen de la referencia.*). The obtained results were in line with a previous work that highlighted that the competition with natural organic matter greatly reduced the adsorption capacity of cartridges and accelerated the leakage of BMAA and DAB [36]. Thus, the breakthrough volume of the entire method was set at 25 mL. Taking into account that 250  $\mu$ L was the volume of injection solvent employed to re-dissolve the dried extracts, a preconcentration factor of 100 was achieved. It is noteworthy that no washing step was needed.



**Figure 4.** Study of sample loading volume on the tandem-SPE procedure.

### 2.3.3. Volume of elution solvent

With the aim of shortening the time of the sample treatment while maintaining the extraction efficiency, the volume of the elution solvent was evaluated using natural reservoir water; therefore 5, 7.5 and 10 mL were tested. It was observed that the elution volume could be reduced to only 5 mL of 10%  $\text{NH}_3 \cdot \text{H}_2\text{O}$  (v/v) in MeOH maintaining satisfactory recoveries.

## 3. Validation of the method

The proposed tandem-SPE-HILIC-MS/MS method was validated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), extraction efficiency, matrix effect and

precision (i.e., repeatability and intermediate precision), using reservoir water samples from El Portillo (Granada, Spain). To assess specificity, blank samples were analyzed and no interfering peaks were observed comigrating at the retention time of the analytes.

### 3.1. Calibration curves and analytical performance characteristics

The calibration curves were established for the studied analytes in reservoir water samples according to the optimized tandem SPE-HILIC-MS/MS procedure described above. Procedural calibration curves were established using reservoir water samples from El Portillo spiked at different concentrations of the eight studied cyanotoxins and submitted to the whole analytical procedure (from  $7 \cdot 10^{-3}$  to  $0.15 \mu\text{g} \cdot \text{L}^{-1}$  for MC-LR and NOD, from  $4 \cdot 10^{-3}$  to  $0.10 \mu\text{g} \cdot \text{L}^{-1}$  for CYN and MC-RR, from 0.1 to  $2 \mu\text{g} \cdot \text{L}^{-1}$  for ANA, from 0.05 to  $1 \mu\text{g} \cdot \text{L}^{-1}$  for BMAA, from 0.03 to  $1 \mu\text{g} \cdot \text{L}^{-1}$  for DAB and from 0.02 to  $0.4 \mu\text{g} \cdot \text{L}^{-1}$  for AEG). Procedural calibration curves compensate both the matrix effect and the sample treatment losses. Six concentration levels were evaluated, with the lowest ones corresponding to the LOQ. Two samples per each concentration level were processed following the SPE method and injected in triplicate ( $n=6$ ). Sample concentration levels were selected based on the preconcentration factor achieved by the sample preparation method. Peak area was considered as the response signal, being linearly dependent on the concentration of analyte in the sample. In all cases, acceptable linearity ( $R^2 > 0.991$ ) was achieved. Statistical parameters, calculated by least-square regression, the performance characteristics of the tandem-SPE-HILIC-MS/MS method for reservoir water samples and LOD and LOQ values are shown in Table 2. LODs of each analyte were calculated as the lowest concentration for which the peak height was, at least, three times the signal-to-noise ratio while LOQs were calculated as the lowest concentrations, which fulfilled method performance acceptability criteria of a signal-to-noise ratio of at least 10. Very low LOQ values (between  $7 \cdot 10^{-3}$  and  $0.1 \mu\text{g} \cdot \text{L}^{-1}$ ) were reached as a consequence of the high analyte preconcentration obtained during the sample treatment.

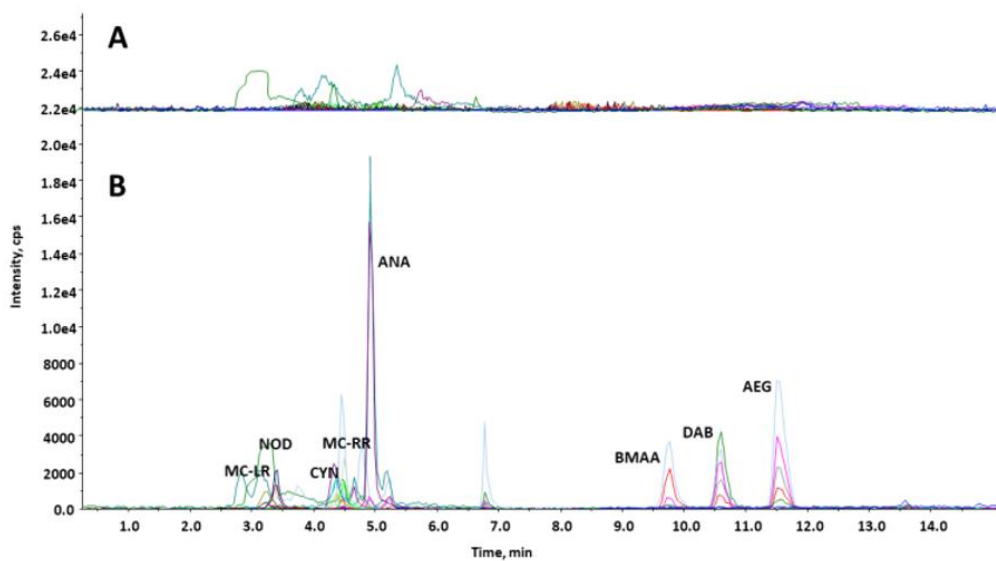
### 3.2. Matrix effect

The matrix effect (ME) is the result produced on the analytical response by the components of the sample other than the analytes. ME was assessed by comparing the analyte response from blank samples spiked after the tandem-SPE procedure with the analyte response of standard solutions at the same concentration levels. Two concentration levels, L1 and L2,

corresponding with the LOQ values and 7.5 x LOQ values, respectively, were studied for each analyte (Table 2). A ME higher than 100% means that the analytical response is enhanced while a ME lower than 100% means that the analytical response is suppressed. A significant signal suppression can be observed for ANA while a signal enhancement occurred for AEG at the lowest level of concentration. However, procedural calibration curves take into account these matrix effects.

### 3.3. Precision

The precision of the method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the whole tandem-SPE-HILIC-MS/MS method to reservoir water samples spiked at L1 and L2 concentration levels. Repeatability was assessed over three samples for each concentration level and analyzed in triplicate (n=9) on the same day under the same conditions. Intermediate precision was evaluated with a similar procedure, with five samples analyzed in triplicate for five consecutive days (n=15). The results, expressed as RSD of peak areas, are shown in Table 2. Satisfactory precision was obtained, as in all cases relative standard deviation (RSD) was lower than 14.1%. Chromatograms of a reservoir water sample spiked at L2 concentration for each cyanotoxin and its corresponding blank are shown in **¡Error! No se encuentra el origen de la referencia..**



**Figure 5.** Chromatogram of a blank of reservoir water sample (A) and a reservoir water sample spiked at L2 for each analyte (B).

**Table 2.** Performance characteristics, precision and matrix effect for the proposed tandem SPE-HILIC-MS/MS method.

Analyte	Linear range ( $\mu\text{g}\cdot\text{L}^{-1}$ )	LOD ( $\mu\text{g}\cdot\text{L}^{-1}$ )	LOQ ( $\mu\text{g}\cdot\text{L}^{-1}$ )	R <sup>2</sup>	Intra-day precision (RSD %) n=9		Inter-day precision (RSD %) n=15		Matrix effect (%) n=9	
					L1	L2	L1	L2	L1	L2
MC-LR	7·10 <sup>-3</sup> - 0.15	2.1·10 <sup>-3</sup>	7·10 <sup>-3</sup>	0.997	10.8	6.9	10.6	7.5	130.7	123.1
NOD	7·10 <sup>-3</sup> - 0.15	2.1·10 <sup>-3</sup>	7·10 <sup>-3</sup>	0.995	5.7	5.6	9.3	7.3	92.0	90.8
CYN	4·10 <sup>-3</sup> - 0.10	1.2·10 <sup>-3</sup>	4·10 <sup>-3</sup>	0.997	5.5	3.3	6.4	5.1	69.8	62.0
MC-RR	4·10 <sup>-3</sup> - 0.10	1.2·10 <sup>-3</sup>	4·10 <sup>-3</sup>	0.999	8.2	8.0	6.1	9.0	78.9	67.6
ANA	0.10 - 2.00	0.03	0.10	0.997	4.5	3.6	6.4	14.0	11.4	16.8
BMAA	0.05 - 1.00	0.015	0.05	0.991	7.9	14.1	7.7	12.2	75.9	52.1
DAB	0.03 - 1.00	9·10 <sup>-3</sup>	0.03	0.998	7.3	4.6	9.7	7.7	132.2	120.0
AEG	0.02 - 0.40	6·10 <sup>-3</sup>	0.02	0.994	3.7	2.8	12.7	5.7	253.2	134.1

Concentration levels were established for each cyanotoxin as the LOQ (L1) and 7.5 x LOQ (L2). L1 and L2 were set to 7·10<sup>-3</sup> and 0.05  $\mu\text{g}\cdot\text{L}^{-1}$  for MC-LR and NOD; 4·10<sup>-3</sup> and 0.03  $\mu\text{g}\cdot\text{L}^{-1}$  for CYN and MC-RR; 0.1 and 0.75  $\mu\text{g}\cdot\text{L}^{-1}$  for ANA; 0.05 and 0.375  $\mu\text{g}\cdot\text{L}^{-1}$  for BMAA; 0.03 and 0.225  $\mu\text{g}\cdot\text{L}^{-1}$  for DAB and 0.02 and 0.15  $\mu\text{g}\cdot\text{L}^{-1}$  for AEG.

### 3.4. Recovery assays

Recovery experiments were carried out in reservoir water samples from different locations (see section 1.3) spiked at two concentration levels (L1 and L2). Three water samples from each reservoir were spiked at each concentration level, processed according to the tandem SPE procedure and subsequently analyzed by HILIC-MS/MS in triplicate (n=9). In all cases a blank sample was analyzed in order to check the absence of matrix compounds co-eluting with cyanotoxin peaks. Recoveries were estimated as the relation between the peak area of samples spiked before the sample treatment and the peak area of samples spiked after sample treatment. Results showed recoveries ranging between 70.9 and 94.7% with associated RSD < 15.3% for El Portillo reservoir water samples, and ranging between 75.4 and 93.6% with associated RSD < 17.3% for Canales reservoir water samples (Table 3). However, when water samples from Bermejales and Cubillas reservoirs were submitted to the recovery study, low recoveries were obtained for the isomers BMAA and DAB (16.2-33.3%). Similarly to our results, in a previous work where BMAA and DAB were extracted by an SPE protocol using Oasis MCX cartridges, an important reduction of the SPE performance occurred when

natural water samples were analyzed instead of pure water [36]. This issue was attributed to the presence of cations, which compete for the active ion-exchange sites, and to the presence of natural organic matter, which causes pore blocking. In fact, the recoveries obtained from the diverse reservoir water samples in the present work are aligned with their content of total ions. Thus, Bermejales and Cubillas reservoir waters, which have the highest conductivity values (367 and 555  $\mu\text{S}\cdot\text{cm}^{-1}$ , respectively) as a consequence of their highest amount of ions, especially sulfates (153 and 124  $\mu\text{g}\cdot\text{kg}^{-1}$ , respectively), present the lowest recovery values for BMAA and DAB. On the contrary, Canales reservoir water samples, which has a much lower conductivity, similar to that of El Portillo (115 and 191  $\mu\text{S}\cdot\text{cm}^{-1}$ , respectively), provided satisfactory recovery values with acceptable associated precision, showing that the present method could satisfactorily be applied to determine BMAA and DAB on waters with conductivities below 200  $\mu\text{S}\cdot\text{cm}^{-1}$ .

**Table 3.** Recovery assessment for reservoir water samples of different origins in Granada province (Spain).

	El Portillo				Canales				Bermejales				Cubillas			
	L1		L2		L1		L2		L1		L2		L1		L2	
	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %
<b>MC-LR</b>	88.5	9.6	86.8	8.0	93.2	16.5	84.0	7.0	74.6	4.6	80.6	4.7	76.7	9.4	75.1	4.8
<b>NOD</b>	92.8	6.8	94.7	5.7	87.9	10.8	88.9	6.5	101.0	10.5	91.8	1.7	86.2	13.6	91.2	9.9
<b>CYN</b>	88.8	6.2	90.6	2.2	80.7	17.1	75.4	7.7	81.8	15.4	85.2	9.1	90.6	8.2	89.1	9.3
<b>MC-RR</b>	87.8	6.7	89.2	9.3	84.7	5.8	88.4	6.6	92.1	10.2	89.5	6.2	91.9	17.5	88.3	9.2
<b>ANA</b>	88.1	5.8	82.9	6.2	83.7	10.3	82.3	3.2	88.2	3.8	82.3	3.5	92.8	9.7	84.3	7.1
<b>BMAA</b>	74.6	15.3	70.6	7.8	87.1	17.3	80.4	7.1	16.2	34.0	26.1	11.9	27.2	36.6	26.7	10.5
<b>DAB</b>	70.9	10.9	74.5	5.6	93.6	8.4	80.9	7.5	26.2	35.9	26.9	11.3	33.3	15.8	25.5	10.7
<b>AEG</b>	71.6	4.8	80.7	4.1	87.6	15.9	76.6	14.0	91.4	13.7	75.6	5.3	76.0	12.4	94.7	15.0

Concentration levels were established for each cyanotoxin as the LOQ (L1) and 7.5 x LOQ (L2). L1 and L2 were set to  $7\cdot 10^{-3}$  and  $0.05\ \mu\text{g}\cdot\text{L}^{-1}$  for MC-LR and NOD;  $4\cdot 10^{-3}$  and  $0.03\ \mu\text{g}\cdot\text{L}^{-1}$  for CYN and MC-RR; 0.1 and  $0.75\ \mu\text{g}\cdot\text{L}^{-1}$  for ANA; 0.05 and  $0.375\ \mu\text{g}\cdot\text{L}^{-1}$  for BMAA; 0.03 and  $0.225\ \mu\text{g}\cdot\text{L}^{-1}$  for DAB and 0.02 and  $0.15\ \mu\text{g}\cdot\text{L}^{-1}$  for AEG. BMAA and DAB for reservoir water samples from Bermejales and Cubillas (in red color) cannot be quantified.

#### 4. Conclusions

The presence of cyanobacteria and their toxins in drinking or recreational waters may present a risk for human health. In this context, our study was focused on the development of a new



method for the determination of 8 multiclass cyanotoxins (MC-LR, MC-RR, NOD, ANA, CYN, BMAA, DAB and AEG) in reservoir water samples. Separation based on HILIC coupled with tandem MS detection was carried out in less than 12 minutes. Prior the chromatographic separation, a tandem-SPE procedure was developed for the simultaneous extraction of all cyanotoxins, despite their different physical-chemical properties. For this purpose, a Strata-X and a MCX cartridges were connected in series, being the Strata-X cartridge at the top and the MCX cartridge at the bottom of the dual system. Before the elution step, the order of the cartridges was reversed in order to fully elute MC-RR. In this manner, it was possible to extract the target cyanotoxins from reservoir water samples by means of a single sample loading step and the use of a single eluent in the elution step. This makes the process much more efficient, in contrast with previous studies where different procedures are employed for each family of cyanotoxins [21,49,50]. Furthermore, this is a novel work as it includes the joint extraction of NPA isomers with other cyanotoxins from different families such as microcystins, nodularin, anatoxin and cylindrospermopsin. The method was validated reporting adequate linearity, repeatability, reproducibility, recoveries and matrix effect yields for the cyanotoxins considered. The resulting LOQs, ranging from  $7 \cdot 10^{-3}$  to  $0.1 \mu\text{g} \cdot \text{L}^{-1}$ , were significantly lower than the guideline value proposed by WHO for MC-LR in drinking water. In addition, LOQs were lower than those obtained previously with other dual SPE systems prior to the application of LC-MS/MS for the quantification of cyanotoxins [15,22,47,48]. This method was satisfactorily applied to reservoir water samples from different locations, suggesting its possible application in the monitoring of the presence of target analytes in these matrices. Only BMAA and DAB showed a reduction of their recoveries when reservoir water samples with high content of ions were submitted to the tandem-SPE procedure. In conclusion, this work will advance our knowledge of cyanotoxins in natural waters allowing, for the first time, the simultaneous extraction and separation of the target multiclass cyanotoxins using HILIC-MS/MS.

## 5. Acknowledgements

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