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Capillary electrophoresis tandem mass spectrometry to determine multiclass cyanotoxins in reservoir water and spinach samples

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A R T I C L E I N F O

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A B S T R A C T

Cyanotoxins constitute a group of toxic secondary metabolites, the presence of which in any water body poses a major health risk. Moreover, advanced organisms such as edible plants exposed to these toxins, are a possible pathway for human exposure. Green analytical chemistry is demanding environmentally friendly analytical techniques. In this sense, we propose the use of capillary electrophoresis coupled to tandem mass spectrometry (CE-MS/MS) to determine a mixture of eight cyanotoxins belonging to three different classes: cyclic peptides (microcystin-LR, microcystin-RR and nodularin), alkaloids (cylindrospermopsin and anatoxin-a) and three isomeric non-protein amino acids (β-methylamino-l-alanine, 2,4-diaminobutyric acid and N-(2-aminoethyl) glycine). Separation was achieved by using an acidic background electrolyte consisting of 2 M formic acid and 20% acetonitrile in water. Parameters affecting MS/MS detection and the sheath-liquid interface were also studied. Finally, a combination of pH-junction, field-amplified sample stacking (FASS) and acid barrage as online preconcentration strategies, was employed to improve sensitivity and efficiency. The online preconcentration applied, in combination with a dual cartridge solid-phase extraction (SPE) system, allows to obtain limits of detection in the very low range of $\mu g \cdot L^{-1}$ for these multiclass cyanotoxins in reservoir water samples (from 0.005 to 0.10 $\mu g \cdot L^{-1}$). Furthermore, for the first time cyanotoxins are analysed in spinach samples through CE-MS/MS using the same SPE procedure, following lyophilisation and solid-liquid extraction with 6 mL 80 % aqueous MeOH.

1. Introduction

Cyanobacteria are a group of primitive microorganisms present in any aquatic and terrestrial ecosystems, from tropical forests to deserts, oceans, and lakes [1,2]. The health of aquatic ecosystems are being endangered globally by the proliferation of harmful cyanobacterial blooms (cyanoHABs) that have increased because of the nutrient oversupply and temperature changes caused by humans. Some cyanobacteria species produce toxins, which is why this increase may have a negative impact on water quality, ecosystem integrity and human health through the production of cyanotoxins in drinkable, fishable and recreational water, or the ingestion of vegetables irrigated with this contaminated water [3–5].

Most analytical methods to determine cyanotoxins in water target microcystins (MCs) and nodularin (NOD), both being large and hydrophobic cyclic peptides with hepatotoxic activity [6–8]. MCs are normally inside cells until cyanobacterial lysis, whereas cyanotoxins with a higher hydrophilic nature are predominantly dissolved in the surrounding water. Cylindrospermopsin (CYN) has emerged as one of the most important toxins in freshwater worldwide [9,10]. This polar alkaloid with hepatotoxic, cytotoxic, dermatotoxic and possibly carcinogenic activity is the first cyanobacteria toxin strongly implicated in causing human illness after ingestion of conventionally-treated municipal drinking water. Other well-known polar alkaloids with neurotoxin activity are anatoxins, with anatoxin-a (ANA-a) being the most studied. ANA-a is produced by different species that are distributed worldwide and grow in brackish, marine and fresh water [11,12]. Small, polar and water-soluble diamino-acids β-N-methylamino-l-alanine (BMAA) and its structural isomers, 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl)glycine (AEG) [13], are as well in cyanotoxins research spotlight. It has been reported that about 95 % of free-living cyanobacteria genera tested produced BMAA [14] and other microorganisms such as diatoms

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and dinoflagellates [15]. Large exposure to these neurotoxins is correlated with the risk of developing amyotrophic lateral sclerosis (ALS), demonstrating that accurate detection and quantification of BMAA in the environment may help to prevent the disease [16].

Due to the dangerous biological activity of cyanotoxins, several methods have been developed to evaluate their presence and amount in the environment and drinking water [17–23]. In addition, the use of water containing cyanobacteria and their toxins in agriculture produce the consequent contamination of agricultural crops that can increase human exposure to cyanotoxins via consumption of contaminated plant food. The accumulation of cyanotoxins, especially MCs and CYN in tissues has been studied in a wide range of agricultural crops [24–27]. Fresh leafy vegetables such as lettuce, cabbage or spinach are minimally processed and could bioconcentrate 3-times more toxins in their edible parts than other agricultural plants [28]. Although post-harvesting processes for cyanotoxins attenuation are being studied [29], there are still knowledge gaps about cyanotoxins bioaccumulation in plants and utilization of water resources to minimize crop contamination.

Reversed-phase Liquid Chromatography coupled to Mass Spectrometry (LC-MS) has been the technique of choice for cyanotoxin determination, mainly for analytes belonging to the same family [20,21]. However, to achieve multiclass cyanotoxin separation, hydrophilic interaction liquid chromatography (HILIC) coupled to MS has recently been proposed, due to its efficiency to separate analytes showing large differences in the polarity [30]. A possible alternative to avoid the use of this kind of columns, which are expensive and need long washing and reequilibration steps, is capillary electrophoresis (CE). CE can be considered a green and sustainable analytical technique, which is why some authors state the feasibility of replacing LC with greener CE methods [31]. In the recent years, the development of CE-MS methods has become an attractive alternative in food, environmental and pharmaceutical or metabolomic analysis, providing not only high-resolution separations but also mass information, enabling molecular characterization based on MS/MS fragmentation [32–34].

The main limitation of capillary electrophoresis is the poor sensitivity, which is why on-line preconcentration strategies have been extensively proposed [35]. These strategies, commonly known as stacking techniques, can be classified based on the mechanism that causes the variation in the electrophoretic velocity of the analytes which promotes their preconcentration. Among them, field-amplified sample stacking (FASS) is a commonly used and simple technique based on a higher conductivity of the sample matrix compared to the background electrolyte (BGE), so that analytes move faster in the sample zone and get stacked when they reach the BGE. Another well-known strategy which bases the analyte velocity variation on chemical phenomena is the pH-junction, which modifies analyte ionization [36].

Up to now, few studies based on CE-MS for cyanotoxin determination have been published. All of them analyse toxins belonging to the same class. Most of them are focused on MCs analysis [37–39], but also BMAA [40] and saxitoxin (STX) [41].

Solid phase extraction (SPE) is the main sample preparation method used for cyanotoxin extraction and preconcentration. Cation exchange SPE cartridges are used to retain basic compounds such as BMAA and its isomers [42]; but MCs and NODs are usually purified on hydrophilic-lipophilic balance (HLB) cartridges due to their less polar nature [22]. CYN and ANA-a, as more water-soluble compounds, can be extracted in graphitized non-porous carbon cartridges [23]. Therefore, achieving an adequate sample treatment for the analysis of multiclass cyanotixins is a challenge. In our group, Aparicio-Muriana et al. [30] developed a tandem dual cartridge SPE sample treatment for the satisfactory extraction and cleanup of water samples containing the multiclass cyanotoxins proposed in this study.

In this study, we propose for the first time a capillary zone electrophoresis coupled to tandem mass spectrometry (CZE-MS/MS) method as an alternative to the HILIC-MS/MS method to determine a mixture of eight cyanotoxins belonging to three different classes: large and hydrophobic cyclic peptides (microcystin-LR, microcystin-RR and nodularin), relatively polar alkaloids (cylindrospermopsin, anatoxin-a) and three small and polar isomeric non-protein amino acids such as β-methylamino-l-alanine (BMAA), 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl)glycine (AEG). The method has been validated for its application to reservoir water and spinach samples.

2. Materials and methods

2.1. Reagent and materials

The cyanotoxins studied including microcystin-leucine-arginine (MC-LR ≥ 99 %), microcystin-arginine-arginine (MC-RR ≥ 99 %), nodularin (NOD ≥ 95 %), cylindrospermopsin (CYN ≥ 95 %) and anatoxin-a (ANA-*a* ≥ 98 %) were supplied by Enzo Life Sciences, Inc. (Lausen, Switzerland). Isomers β-N-methylamino-l-alanine hydrochloride (BMAA ≥ 97 %), 2–4-diaminobutyric acid dihydrochloride (DAB ≥ 95 %) and N-β-aminoethylglycine (AEG ≥ 98 %) were supplied by Sigma Aldrich (Darmstadt, Germany).

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 μg·mL^{-1} MC-LR in methanol, 25 μg·mL^{-1} MC-RR in 50 % aqueous MeOH, 50 μg·mL^{-1} NOD in 50 % aqueous MeOH, 25 μg·mL^{-1} CYN in MeOH, 1000 μg·mL^{-1} ANA in water. Stock solutions of 1000 μg·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 μg·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), isopropanol (IPA) and methanol (MeOH) were purchased from VWR (Radnor, PA, USA). Ethanol (EtOH), ammonia solution ($NH_3{\cdot}H_2O$) (30 % assay) and ammonium acetate ($NH_4CH_3CO_2$) was purchased from Merck (Darmstadt, Germany). Formic acid (FA) and acetic acid (AA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) was obtained from PanReac-Química (Madrid, Spain). Ultra-pure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the study.

Oasis MCX cartridges (150 mg, 6 mL) from Waters (Milford, MA, USA) and Strata-X cartridges (200 mg, 6 mL) supplied by Phenomenex (Torrance, CA, USA) were used for cyanotoxin extraction from samples. SPE tube adapters from Supelco Inc. (Bellefonte, PA, USA) were used. CLARIFY polytetrafluoroethylene (PTFE) hydrophilic filters (0.2 μm × 13 mm) were employed to filter the extracts.

2.2. Instrumentation

Experiments were performed with an Agilent 7100 CE system coupled to a triple quadrupole 6495C mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization source operating in positive ionization mode (ESI+). Sheath liquid was supplied with a 1260 Infinity II Iso Pump. MS data were collected and processed by MassHunter software (version 10.0).

Separations were carried out in bare fused-silica capillaries (90 cm of total length, 50 μm I.D., 375 μm O.D.) from Polymicro Technologies (Phoenix, AZ, USA).

A pH-meter (Crison model pH 2000, Barcelona, Spain), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), an analytical balance with 0.0001 g precision (Sartorius; Goettingen, Germany), a multi-tube vortexer (model BV1010 from Benchmark Scientific; Sayreville, USA), a

Universal 320R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH; Bielefeld, Germany), a LaboGene Scanvac CoolSafe 4 L freeze dryer (American Laboratory Trading, USA), a Visiprep solid-phase extraction unit from Supelco (Bellefonte, PA, USA) and a polytron (Kinematia, AG, Luzern, Switzerland) were also employed.

2.3. Sample preparation

2.3.1. Pretreatment of water samples

Water samples collected in June 2022 and November 2022 from different freshwater swamps named El Portillo (Castril), Canales (Güéjar Sierra), Bermejales (Arenas del Rey), Cubillas (Albolote) and Quéntar (Quéntar), located in Granada (Andalucía, Spain) were considered in this study. Samples were collected in amber glass bottles. After sampling procedure, the pH and the conductivity were measured. Conductivity ranged between 136.6 and 555.0 $\mu S \cdot cm^{-1}$ and pH from 8.3 to 8.6. The content of sulphates, chlorides, nitrates, fluorides, and phosphates was determined by ion chromatography (Table SD1). Water samples were filtered through a 0.22 μm cellulose acetate membrane filter to remove suspended particles and they were kept at 4 °C until analysis.

Aliquots of 25 mL of freshwater samples, previously acidified to pH 3 with HCl 37 %, were placed in volumetric flasks and spiked at the desired analyte concentration levels for optimization studies.

2.3.2. Pretreatment of spinach samples

Fresh spinach leaves were bought from a local supermarket (Granada, Spain) and kept at 4 °C until lyophilisation. First, we checked whether lyophilisation could affect analytes content. A portion of 4 g of chopped fresh spinach leaves were spiked at 0.675 $\mu g \cdot Kg^{-1}$ for ANA-a, BMAA and MC-RR; 0.9 $\mu g \cdot Kg^{-1}$ for AEG, DAB and MC-LR; and 3.825 $\mu g \cdot Kg^{-1}$ for CYN and NOD. It was lyophilized and compared to 4 g of chopped fresh spinach sample spiked after the lyophilisation process at the same concentration levels. In order to verify that average areas results were homogeneous, a *t*-test with $n = 9$ was applied resulting a t-value (AEG: 0.83, DAB: 2.03, BMAA: 0.51, ANA-a: 1.33, MC-RR: 1.42, NOD: 0.68, MC-LR: 0.94, CYN: 0.43) lower than t tabulate (2.12, α=0.5) for all analytes; concluding that lyophilisation does not affect analyte content in the sample.

Approximately 300 g of chopped fresh spinach leaves were placed in a freeze dryer for 5 h. After lyophilisation, they were milled to powder and kept conveniently isolated in a dry place.

Solid-liquid extraction (SLE) was used to extract cyanotoxins from spinach samples. Aliquots of 50.0 mg of lyophilized spinach were weighed in 15 mL plastic tubes and spiked at the desired analyte concentration level. After fortification, samples were shaken in a vortex mixer (1 min) and left in the dark for at least 2 h to guarantee contact of the analytes with the matrix. Then, 6 mL of 80 % aqueous MeOH were added to the centrifuge tube containing the lyophilized spinach. It was homogenized by polytron for 30 s at 1000 rpm, sonicated for 10 min, and vortexed in the orbital shaker for 10 min at 2500 rpm. The mixture was centrifuged at 4500 rpm for 10 min, the supernatant was collected, acidified to pH 3 with 5 μL HCl 37 % and diluted up to 16 mL with ultrapure water.

2.4. SPE procedure

A tandem dual-cartridge solid phase extraction (SPE) for multiclass cyanotoxin analysis by HILIC-MS/MS, previously developed in our lab [30] was applied with some modifications. Two cartridges, a Strata-X and an Oasis® MCX connected in series, enables the simultaneous extraction and preconcentration of cyanotoxins with very different physicochemical properties. The cartridges were conditioned and activated separately with 3 mL MeOH followed by 3 mL deionized water at pH 3. After that, the cartridges were connected: the Strata-X cartridge, capable of retaining low polar and moderately polar compounds (MCs, NOD, CYN and ANA-a) was configured first in the line of sample flow. The MCX cartridge that can retain 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 was placed below. 25.0 mL of samples were loaded at a flow rate of 1 $mL \cdot min^{-1}$. Afterwards the cartridges were washed with 2 mL of 30 % MeOH in water. Then, dried under vacuum for 1 min. Before the elution step, the order of the cartridges was reversed, with MCX at the top and Strata-X at the bottom. Elution of the analytes was carried out using 5 mL of 10 % $NH_3 \cdot H_2O$ in MeOH. The eluate was evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was re-dissolved with 250 μL of 50 % MeCN 5 mM ammonium acetate 7 mM acetic acid. The final extract was filtered, transferred to a glass insert and analysed by the proposed CE-MS/MS method.

2.5. CE separation

New capillaries were conditioned with methanol for 10 min, ultrapure water for 15 min, 0.1 M NaOH for 20 min, 0.1 M HCl for 20 min and BGE for 1 h at 1 bar pressure and 20 °C [37]. At the beginning of each day, the capillary was flushed with BGE for 30 min at 1 bar and 20 °C. To obtain an adequate repeatability between runs, capillary was rinsed with BGE for 5 min at 1 bar and 20 °C at the beginning of each run. At the end of each working day, capillary was cleaned with ultrapure water for 5 min, followed by air for 10 min at 1 bar and 20 °C to dry it.

CE separation was carried out in bare fused-silica capillaries (90 cm total length) at 20 °C. BGE consisted of 2 M FA and 20 % MeCN. A constant voltage of 30 kV (normal polarity) was applied. Samples were hydrodynamically injected for 40 s at 50 mbar (34 nL of sample volume).

Preconcentration strategies were employed to improve method sensitivity and efficiency. Injection solvent consisted of 50 % MeCN, 5 mM ammonium acetate and 7 mM AA, resulting in an apparent pH of 4.5. On the other hand, a plug of 1.2 M FA for 10 s at 50 mbar as acidic barrage was added after sample injection. After that, BGE was injected for 10 s at 50 mbar to improve injection precision and avoid current disruptions.

2.6. MS/MS parameters

Sheath-liquid consisting of a mixture of 50:49.95:0.05 (v/v/v) MeOH:H_2O:FA was provided at a flow rate of 15 $\mu L \cdot min^{-1}$, with a 1:100 splitter. The mass spectrometer was operated in positive ionization mode (ESI+) under multiple reaction monitoring (MRM) conditions. 2000 V were applied in both capillary and nozzle. Other electrospray parameters at optimum conditions were: nebulizer pressure 10 psi; dry gas flow rate 11 $L \cdot min^{-1}$ and dry gas temperature 150 °C; sheath gas flow 5 $L \cdot min^{-1}$ and sheath gas temperature 195 °C. MS/MS experiments were performed by fragmentation of the molecular ions $[M+H]^+$ or $[M+2H]^{2+}$, which were selected as the precursor ions in all cases. MRM scan with two-time segments was employed: from 0.1 to 11.0 min transitions corresponding to BMAA, AEG, DAB and ANA-a were monitored; from 11 min to the end MC-RR, NOD, MC-LR and CYN transitions were monitored. Collision energies (V) were set between 10 and 80 depending on the analyte, and product ions were analysed in the range of 43.9–336.0 *m/z*. Optimized MS/MS transitions parameters are summarized in Table SD2.

3. Results and discussion

3.1. Optimization of electrophoretic conditions

The cyanotoxins involved in this work have different physicochemical properties including size, polarity, and pKa (Table SD3). MC-RR, MC-LR and NOD are large and hydrophobic cyclic peptides, ANA-a and CYN are relatively polar alkaloids, and BMAA and its two

structural isomers, DAB and AEG, are small polar and water-soluble amino acids. Several separation challenges were found in this study. On the one hand, the isomers have the same molecular mass, and they present the same molecular ions and MS/MS transitions, which means that they cannot be differentiated by MS. We needed to ensure a different migration time to quantify each of them, ensuring a resolution between peaks equal to or greater than 1.5. On the other hand, MCs and NOD are large molecules with a high molecular mass and *m/z*. Moreover, CYN has a sulphonic group that is always ionised in solution, having a pKa of −1.5 (Table SD3). At acidic pH it will be a neutral zwitterionic molecule.

First, the pH of the BGE was studied between 1 and 12. Better separation and peak shapes were achieved with an acidic BGE (pH 1.5 – 3.0). FA and AA were tested at concentrations ranging between 0.1–2.0 M achieving in all cases higher current intensities (50–11 μA for FA and 10–3 μA for AA), lower pH (1.58–2.8 for FA and 2.11–2.86 for AA) and better resolution between DAB and AEG when FA was used, according to previously published data [40]. Increasing the FA concentration also improved isomer separation and the analysis time was barely affected. Organic modifiers (MeCN, EtOH, IPA, MeOH) were tested in a range of 10 %−50 % and we observed that adding 20 % MeCN the resolution of DAB increases without extending the analysis time. EtOH, IPA and MeOH also showed a good resolution of the isomers, but they were discarded because the analysis time was at least 6.5 min longer. Final conditions were 2 M FA containing 20 % MeCN as a compromise between resolution, analysis time and electric current.

Regarding cassette temperature, 30, 25, 20 and 15 °C were tested. Temperatures of 15 and 20 °C showed an increase in DAB resolution, but 15 °C led to longer analysis time. An optimum temperature of 20 °C was selected considering analysis time and isomer resolution. The interaction between capillary length and separation voltage was also studied starting from 70 cm and 20 kV. By increasing the capillary length up to 90 cm, an acceptable resolution between isomers was achieved. In fact, with this length, it was possible to increase the separation voltage up to 30 kV to reduce the analysis time, and the resolution and electric current were still satisfactory (Fig. 1).

Under optimum conditions, baseline resolution of 8 cyanotoxins was achieved in 21 min, with a separation current of 28 μA.

3.2. Optimization of detection parameters

Sheath liquid composition is a key aspect in CE-MS/MS hyphenation, as it must be carefully selected to achieve a stable electrospray and good sensitivity [43]. MeOH, EtOH, IPA and MeCN were evaluated as organic solvents, and AA and FA as acids for sheath liquid composition. MeOH and FA showed the best results in terms of S/N, particularly for those compounds less prone to ESI ionization such as CYN. Regarding acid concentration, slight differences were found between 0 % and 0.05 % FA, most likely due to our already very acidic BGE, which makes higher concentrations pointless. MeOH concentration was tested in a range from 20 to 80 %, and finally 0.05 % FA in 50 % MeOH was selected as it showed the optimal S/N of CYN. Dry gas temperature was evaluated from 100 to 300 °C and was kept at 150 °C following the S/N criteria. Sheath liquid flow rate plays an important role to ensure electrospray stability and, therefore, it influences repeatability. Values from 2 to 25 $\mu L \cdot min^{-1}$ were studied. A flow rate below 5 $\mu L \cdot min^{-1}$ led to an unstable electrospray, and values higher than 15 $\mu L \cdot min^{-1}$ affected NOD repeatability. Moreover, increasing sheath liquid flow and sheath gas temperature values implied a higher sensitivity but produced a loss of resolution between DAB and AEG; so we increased their values to the point where we could keep their resolution above 1.5. Finally, 195 °C was selected for sheath gas temperature and 15 $\mu L.min^{-1}$ for sheath liquid flow rate. The nebulizer pressure was studied between 8 and 14 psi. Above 10 psi, the spray stability decreased, inducing poor repeatability in the migration times and loss of resolution. Best compromise between repeatability, resolution, and S/N ratio was obtained when a nebulizer pressure of 10 psi was applied. Regarding the dry gas flow, 11 $L.min^{-1}$ was selected because higher values caused current disruptions and lower values were not allowed in our instrument.

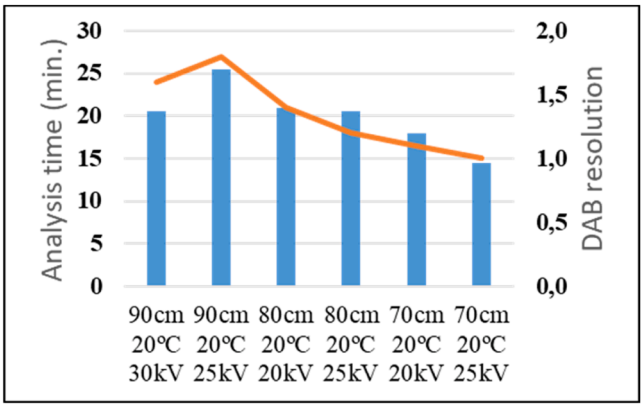

Fig. 1. Optimization of electrophoretic separation. Blue bars correspond to analysis time (min) and orange line to DAB resolution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MS/MS transitions were also studied. First, the SCAN mode was used to select the precursor ion. It was observed that the protonated molecules $[M+H]^+$ were the most abundant for all analytes, with the exception of MCs, which mainly formed diprotonated ions $[M + 2H]^{2+}$ (Table SD2). Then, the main fragment ions were investigated and two identification ions and one quantification ion were selected for each analyte to ensure a reliable determination. Optimum collision energy was studied to obtain the highest signal in each case. Finally, dwell time for each transition was also optimized varying from 40 to 150 ms, depending on the analyte, to guarantee a minimum data acquisition of 10 points per peak. MS/MS detected ions of the studied analytes are found in Table SD2.

For the three isomers only one identification ion is shown in Table SD2. The ion 101.9 *m/z* was included as identification ion initially for the three isomers, as commonly described in bibliography [42]. Nevertheless, problems regarding analyte signals were found with sample impurities when this ion was included in the MRM method. A similar situation was found for ANA-a first identification ion *m/z* 149.1 in spinach analysis, showing a large interference after the analyte signal. In this product ion search, different and unique identification ions were achieved for DAB and BMAA, allowing us to have another crucial point to differentiate the isomers aside from their migration time. However, we still have fragments in common for the three isomers in the proposed method (Fig. 2).

3.3. Optimization of the on-line preconcentration strategy

On-line sample preconcentration, in which analytes are focused into a narrow zone after sample injection, is an effective approach for increasing method sensitivity. Among these methodologies, pH-junction focusing is one of the simplest alternatives in which a difference in the pH between the BGE and the injection solvent induces a change in the charge of the analytes, creating an analyte concentrated zone where they get stacked [35]. Considering the analyte pKas (Table SD3) and the apparent pH of the BGE (1.7), more basic solutions were tested as sample solvent: 5 mM ammonium acetate + 50 mM AA (pH 3.5), 5 mM ammonium acetate + 7 mM AA (pH 4.5), and 5 mM ammonium acetate (pH 5.5). Solutions with higher pH involved a complete loss of resolution between DAB and AEG. Enrichment factors were similar for the three sample solvents tested (from 1.0 for CYN to 1.37 for BMAA), but at pH 4.5 a higher theoretical plate count was obtained. Field amplified sample stacking (FASS) was also tested. FASS is based on the different

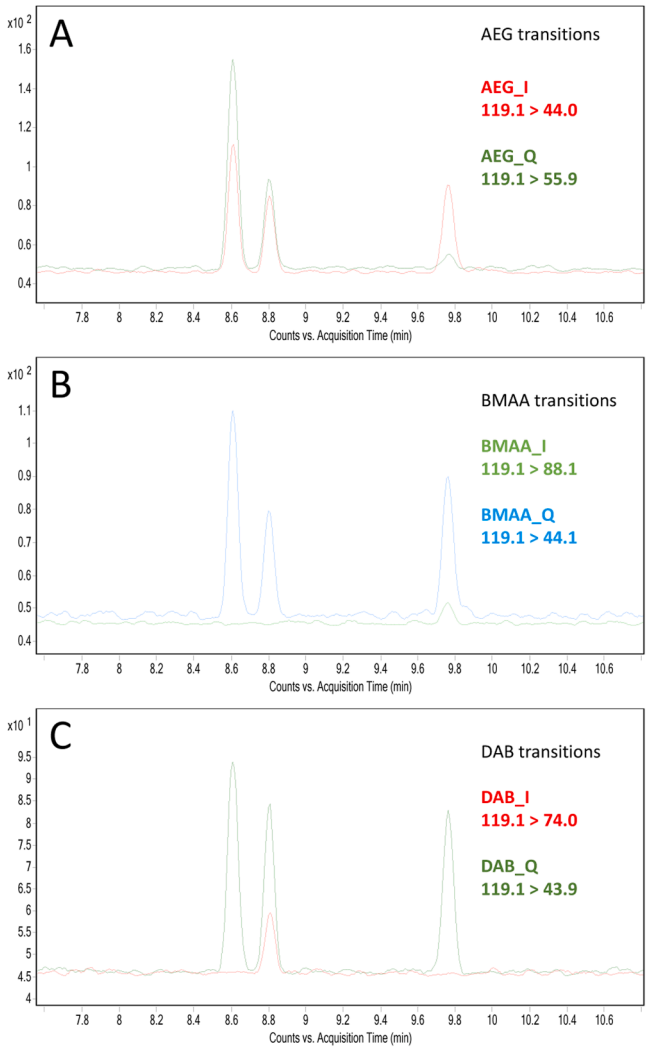

Fig. 2. Extracted ion electropherograms of the three isomers at 4 μg·L^{-1}. Identification and quantification transitions of AEG (A), BMAA (B) and DAB (C).

conductivity of the BGE and the injection solvent which leads to different electrophoretic mobilities. Water, 50 % MeCN, 50 % MeOH, 50 % EtOH and 50 % IPA were tested. Enrichment factors were slightly higher for MeCN (from 1.0 CYN to 1.6 ANA). Then, three different percentages (25, 50, 70%) were tested and a higher enrichment factor was obtained for 50 % MeCN. Better peak shape for CYN, the last migrating compound, was obtained when an acidic barrage was added after the injection of the sample. This step has an effect similar to pushing 'lagging' and weak ions to the detector [36]. Finally, 1.2 M FA in ultrapure water without any organic modifier was selected as acidic barrage as showing a significant push on the last analyte to reach the detector, which does not improve increasing FA concentration.

Final stacking conditions include an injection solvent at pH 4.5 consisting of 50 % MeCN, 5 mM ammonium acetate and 7 mM acetic acid, and an acidic barrage after sample injection of 1.2 M FA. With this stacking strategy, we could raise the injection time from 20 s to 40 s at 50 mbar keeping the isomer resolution and obtaining an enrichment factor of about 4 times (Fig. 3).

3.4. Characterization of the method in reservoir water samples

The tandem-SPE followed by CE-MS/MS methodology developed was applied to waters from five different reservoirs located at the province of Granada (Spain): El Portillo, Bermejales, Canales, Quéntar and Cubillas. Conductivity, pH and content of anions in water samples are summarized in Table SD1.

3.4.1. Calibration curves and analytical performance characteristics of the method

The composition and amount of matrix in a real sample, together with the properties of the analytes studied can impact the quantification greatly. Mild or no matrix effects simplify the analysis enormously if external calibration obtained with standard solution can be used.

To test whether the standard calibration curves can be used to quantify these cyanotoxins in reservoir water samples, two calibration curves were established: a matrix-matched calibration curve obtained by spiking tandem-SPE extracts of water samples from El Portillo, as the representative matrix and an external calibration with standard solutions.

The external calibration curves were established for six different concentration levels corresponding to 1.5, 3.0, 6.0, 9.0, 12.0 and 15.0 μg·L^{-1} for AEG, DAB, BMAA, ANA-a MC-RR and MC-LR; and 15.0, 30.0, 60.0, 90.0, 120.0 and 150.0 μg·L^{-1} for NOD and CYN for standard solution prepared in methanol:ultrapure water (1:1, v/v). The concentration ranges tested for matrix-matched calibration in reservoir water are listed in Table 1, considering the pre-concentration carried out with the SPE cartridges (from 25.0 mL to 250 μL). Three samples per each concentration level were prepared and injected in triplicate ($n = 9$). The peak area as signal response versus analyte concentrations was

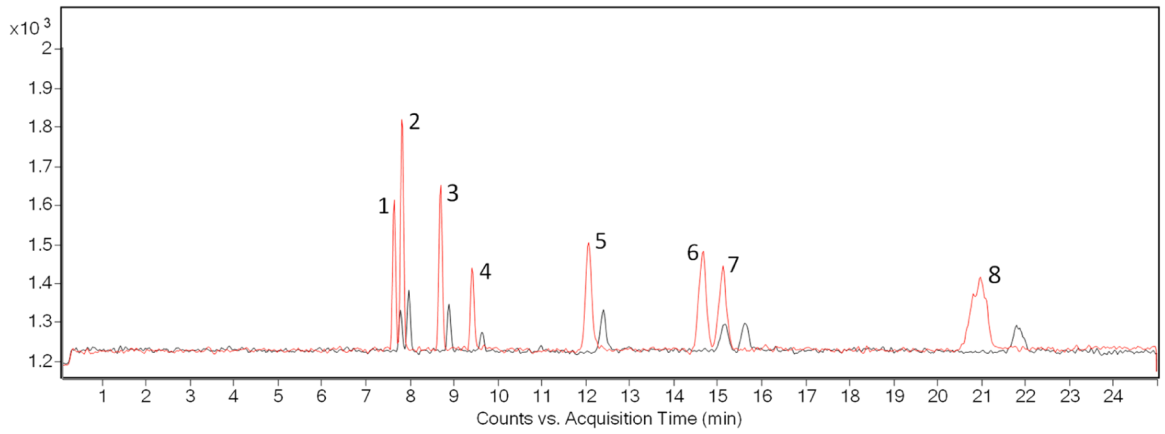

Fig. 3. Total ion electropherogram 1: AEG; 4 μg·L^{-1}. 2: DAB; 4 μg·L^{-1}. 3: BMAA; 4 μg·L^{-1}. 4: ANA-a; 3 μg·L^{-1}. 5: MC-RR; 5 μg·L^{-1}. 6: NOD; 70 μg·L^{-1}. 7: MC-LR; 5 μg·L^{-1}. 8: CYN; 70 μg·L^{-1}. Black line: without on-line preconcentration. Red line: after applying the combination of the on-line preconcentration strategies; signal increases up to about 4 times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Performance characteristics for the proposed tandem-SPE-CZE-MS/MS method in reservoir water.

Analyte	Linear range ($\mu g \cdot L^{-1}$)	LOQ ($\mu g \cdot L^{-1}$)	LOD ($\mu g \cdot L^{-1}$)	R^2
AEG	0.020–0.150	0.020	0.006	0.992
DAB	0.023 −0.150	0.023	0.007	0.993
BMAA	0.022 −0.150	0.022	0.007	0.992
ANA-a	0.016 −0.150	0.016	0.005	0.994
MC-RR	0.034 −0.150	0.034	0.010	0.994
NOD	0.270 −2.500	0.270	0.081	0.998
MC-LR	0.048 −0.150	0.048	0.015	0.991
CYN	0.340 −2.500	0.340	0.102	0.993

monitored. A blank sample was also analysed, and no interfering signals were detected at the retention time of the analytes (Fig. 4). Calibration parameters for matrix-matched calibration are shown in Table 1.

In all cases, acceptable linearity was achieved, with coefficient of determination (R^2) values ranging from 0.991 to 0.998. Statistical parameters were calculated by least-square regression. LODs and LOQs were calculated as the lowest concentration which produces a signal three or ten times above noise signal, respectively. The LOD obtained for all analytes is at least one order of magnitude lower than the value of 1 $\mu g \cdot L^{-1}$ recommended as a safe limit by the World Health Organization (WHO) [44]. These results are the lowest reported to date for cyanotoxin analysis by CE-MS/MS [37,38,39,40].

A t-Student test was used to compare the slopes of the external calibration and the matrix-matched calibration curves. In all cases, the calculated t-value was below the theoretical t-value for a confidence level of 95 %. Therefore, no significant differences were observed for the slopes. This means that the proposed method does not show matrix effects for the reservoir water tested.

3.4.2. Matrix effect

To confirm the absence or presence of a matrix effect a study was carried out in the different reservoirs sampled. Matrix effect was calculated as the relationship between the signal of a blank sample spiked after the extraction procedure and the signal of a standard solution (Table 2) with the same concentration of analytes. No significant matrix effect was found for water samples from El Portillo, Canales and Quéntar. However, the samples from Bermejales and Cubillas showed a signal suppression for BMAA at high concentrations, probably due to their higher conductivity values (367 and 555 $\mu S \cdot cm^{-1}$, respectively) related to their higher amount of ions.

RSD(%) results for each water reservoir were as follows: 1.8 to 9.7 % (L1) and 1.6 to 7.1 % (L2) for El Portillo; 3.6 to 11.5 % (L1) and 2.7 to 7.3 % (L2) for Canales; 1.8 to 9.4 % (L1) and 2.8 to 7.6 % (L2) for Quéntar; 3.6 to 8.5 % (L1) and 3.0 to 7.3 % (L2) for Bermejales; and 3.6 to 11.5 % (L1) and 2.7 to 7.3 % (L2) for Cubillas.

3.4.3. Recovery and precision assays

Reservoir waters were divided into two groups depending on matrix effect for BMAA. The first group consisted of samples from El Portillo, Canales and Quéntar with no significant matrix effect; Cubillas and Bermejales samples, with signal suppression for high BMAA concentration, were in the second group. Recovery experiments were carried out for El Portillo, representing the first one and Bermejales for the second group, spiked at the 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 analysed by CE-MS/MS in triplicate ($n = 9$). In all cases a blank sample was analysed to check the absence of matrix compounds co-migrating with cyanotoxin peaks. Recoveries (%) were estimated as the ratio 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 from 75.9 to 99.4 % for the first group. For the second group, values ranged from 53.5 to 105.0 % (Table SD4). The lower recoveries were obtained for DAB (58.17–53.54 %). Loss of this analyte when using Oasis MCX cartridges SPE protocol has already been described in bibliography for natural water samples [45]. This fact can be related to the presence of cations in the matrix competing for active ion-exchange sites. Previously, lower recoveries (70.6–94.7 % for non-significant ME samples; and 16.2–101.0 % for Cubillas and Bermejales) were shown for these analytes analysing the same reservoir water matrices by HILIC-MS/MS where no washing step is added in the tandem SPE extraction protocol [30].

The precision of the method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability was assessed over three samples for each concentration level and analysed in triplicate ($n = 9$) in the same day under the same conditions. Intermediate precision was evaluated with a similar procedure, with five samples analysed in triplicate for five consecutive days ($n = 15$). The results, expressed as RSD of the peak areas (Table SD4), show satisfactory precision with values lower than 9.8 for intra-day and lower than 13.7 for inter-day precision, expressed as RSD (%). These values are similar to those obtained previously by HILIC-LC-MS/MS for the same water samples [30].

3.5. Characterization of the method in spinach samples

The optimized SLE-SPE-CZE-MS/MS method was evaluated in terms of linearity, limits of detection, limits of quantification, extraction recovery, matrix effect, and precision in spinach samples.

When we tried to work with fresh spinach, we found it difficult to

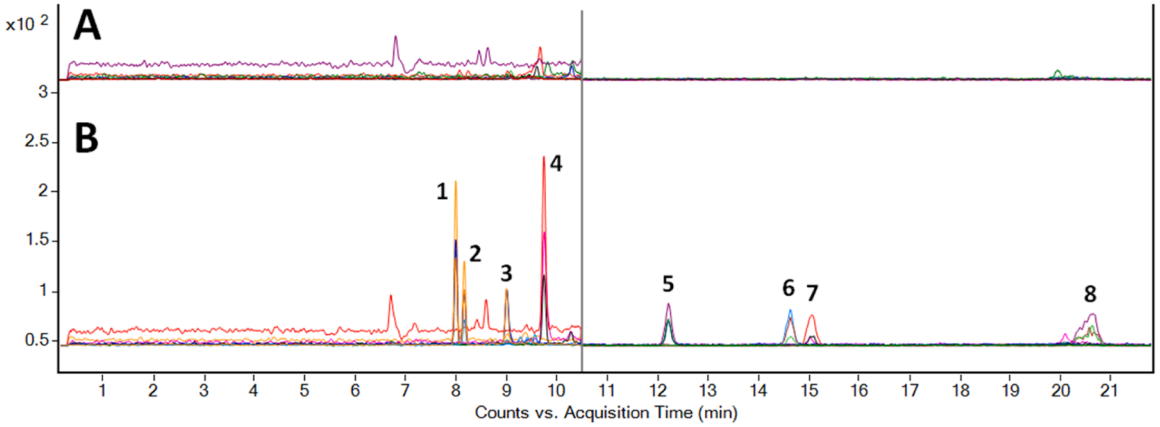

Fig. 4. Extracted ion electropherograms of a blank of reservoir water sample (A) and a reservoir water sample spiked at: 1: AEG; 0.04 $\mu g \cdot L^{-1}$. 2: DAB; 0.04 $\mu g \cdot L^{-1}$. 3: BMAA; 0.04 $\mu g \cdot L^{-1}$. 4: ANA-a; 0.03 $\mu g \cdot L^{-1}$. 5: MC-RR; 0.05 $\mu g \cdot L^{-1}$. 6: NOD; 0.70 $\mu g \cdot L^{-1}$. 7: MC-LR; 0.05 $\mu g \cdot L^{-1}$. 8: CYN; 0.70 $\mu g \cdot L^{-1}$. (B).

Table 2
Matrix effect in the determination of cyanotoxins by tandem-SPE-CZE-MS/MS in reservoir water samples.

Analyte	Matrix effect (%)									
	El Portillo		Canales		Quéntar		Bermejales		Cubillas	
	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2
AEG	−3.5	−1.0	−1.8	4.5	−3.4	2.7	−2.7	−0.7	−3.9	−0.4
DAB	0.4	1.5	−0.6	2.4	1.7	0.6	−0.8	−1.4	−2.3	−0.5
BMAA	−3.8	−2.3	−3.1	−0.8	−4.1	−0.6	−1.8	−27.9	−4.7	−17.5
ANA-a	−1.0	−0.3	5.3	−1.5	7.5	−2.8	1.5	3.0	0.6	1.0
MC-RR	0.8	2.8	4.8	2.2	1.8	2.2	0.0	−1.0	3.6	2.8
NOD	2.1	−1.6	4.0	−1.6	−0.4	−0.6	−0.5	−0.4	3.0	1.4
MC-LR	0.6	−0.9	8.2	2.3	6.8	−0.1	1.0	2.8	2.3	−1.1
CYN	0.1	−0.8	5.0	2.2	1.0	2.7	2.4	4.7	6.9	0.3

L1: 0.05 $\mu g \cdot L^{-1}$ for AEG, DAB, BMAA, ANA-a, MC-RR and MC-LR; 0.4 $\mu g \cdot L^{-1}$ for NOD and CYN.
L2: 0.12 $\mu g \cdot L^{-1}$ for AEG, DAB, BMAA, ANA-a, MC-RR and MC-LR; 2.0 $\mu g \cdot L^{-1}$ for NOD and CYN,.

mill and homogenize the sample, so we worked with lyophilized material.

3.5.1. Calibration curves and analytical performance characteristics

Procedural calibration was performed at concentration levels ranging from 0.60 to 2.40 $\mu g \cdot kg^{-1}$ for AEG, DAB and MC-LR; 0.45 to 1.80 $\mu g \cdot kg^{-1}$ for ANA-a, MC-RR and BMAA; and 1.28 to 8.00 $\mu g \cdot kg^{-1}$ for NOD and CYN. Procedural calibration involves the analysis of samples spiked before the sample treatment. This calibration, which compensates not only the matrix effect, but also the sample treatment losses, ensure the reliability of the quantification. Three samples were spiked at each concentration level, treated according to the SLE and SPE sample treatment, and analysed in triplicate by the proposed CZE-MS/MS method. Peak area was selected as the analytical response and considered as a function of the analyte concentration on the sample. LODs and LOQs were calculated as specified in previous sections. As shown in Table 3, the values for the coefficient of determination (R^2) were higher than 0.990, except for AEG. LODs and LOQs values obtained for MC-LR, MC-RR and CYN are similar to those obtained previously for lettuce [46]; but significantly better than those reported for MC-RR and MC-LR in spinach [47]. Fig. 5 shows an extracted ion electroferogram of a spinach sample blank (A) and a spiked spinach sample (B).

3.5.2. Matrix effect and recovery assays

The matrix effect in spinach samples was calculated for the eight cyanotoxins as previously described for water samples. The most pronounced ME is observed in DAB followed by AEG and CYN, with signal enhancement for DAB, and signal suppression for AEG and CYN. For the other cyanotoxins the value found was $\leq$ 10 % (Table SD5). Compared with previous studies, our results were similar or even slightly better for CYN, MC-LR and MC-RR [46,47].

Recoveries for the eight cyanotoxins were also calculated to evaluate the efficiency of the sample treatment (SLE-SPE) by comparing peak areas of blank samples spiked before and after the sample treatment (Table SD5). Quantitative extractions at both concentration levels were achieved for BMAA, NOD and MC-LR. AEG and CYN showed the lowest recovery values (from 65.5 % to 72.6 %). DAB, ANA-a and MC-RR showed recovery values higher than 81.0%.

The results obtained evidence the need to use a procedural calibration for the quantitative analysis of cyanotoxins in spinach samples.

Satisfactory precision was also obtained, with RSD% from 1.1 to 11.9, despite the complexity of the sample. Our results were better than previously reported for MC-RR, MC-LR and CYN in vegetable samples [46], and similar to those reported for several microcystins in spinach samples [47].

Table 3
Procedural calibration curve for the determination of cyanotoxins by SLE-SPE-CZE-MS/MS in spinach matrix.

Analyte	Linear range ($\mu g \cdot Kg^{-1}$)*	LOQ ($\mu g \cdot Kg^{-1}$)*	LOD ($\mu g \cdot Kg^{-1}$)*	R^2
AEG	0.14–2.40	0.14	0.04	0.988
DAB	0.15–2.40	0.15	0.04	0.992
BMAA	0.18–1.80	0.18	0.05	0.990
ANA-a	0.10–1.80	0.10	0.03	0.997
MC-RR	0.13–1.80	0.13	0.04	0.993
NOD	0.78–8.00	0.78	0.23	0.991
MC-LR	0.15–2.40	0.15	0.05	0.992
CYN	0.65–8.00	0.65	0.19	0.991

* Fresh weight (f.w.).

4. Conclusions

For the first time, a method based on the use of CE-MS/MS for the simultaneous detection and quantification of eight cyanotoxins belonging to three different classes: cyclic peptides (MC-LR, MC-RR, NOD), alkaloids (CYN, ANA-a) and non-protein amino acids (AEG, DAB, BMAA) has been developed. Considering that the preconcentration strategies affect each analyte differently, a combination of pH-junction and FASS has been used. In addition, an acidic barrier was introduced after sample injection to improve the peak shape of CYN, which is last migrating compound. The optimized method shows acceptable linearity, sensitivity, and precision for all analytes.

The method was validated in reservoir water samples with better recoveries and smaller matrix effect than the multiclass method published previously in our group using HILIC-MS/MS. In fact, the matrix effect obtained was negligible for all of them, except for BMAA in Bermejales and Cubillas which have a higher ionic content. However, there are losses in the recovery of some analytes after tandem SPE. Consequently, we have proposed a method for the analysis of these cyanotoxins in water samples from five reservoirs with different ionic content, using the same calibration curve made with aqueous standards previously treated, in order to correct this loss. The limits of quantification achieved are below the levels recommended by the World Health Organization (WHO).

The study reports as well the only multiclass detection and quantification method of eight cyanotoxins in spinach samples, previously lyophilized. To date, only HPLC coupled to MS-MS has been used to analyse edible vegetables for cyanotoxins contamination. The results obtained for matrix effect and recovery assays show the need to use a procedural calibration for the quantitative analysis of cyanotoxins in spinach samples.

In summary, the methods described here, environmentally friendly and sustainable, are great candidates for their application in the monitoring of the presence of the target analytes in these matrices. Laboratories could benefit from the possibility of knowing the potential of an alternative and/or complementary technique such as CE coupled to mass spectrometry.

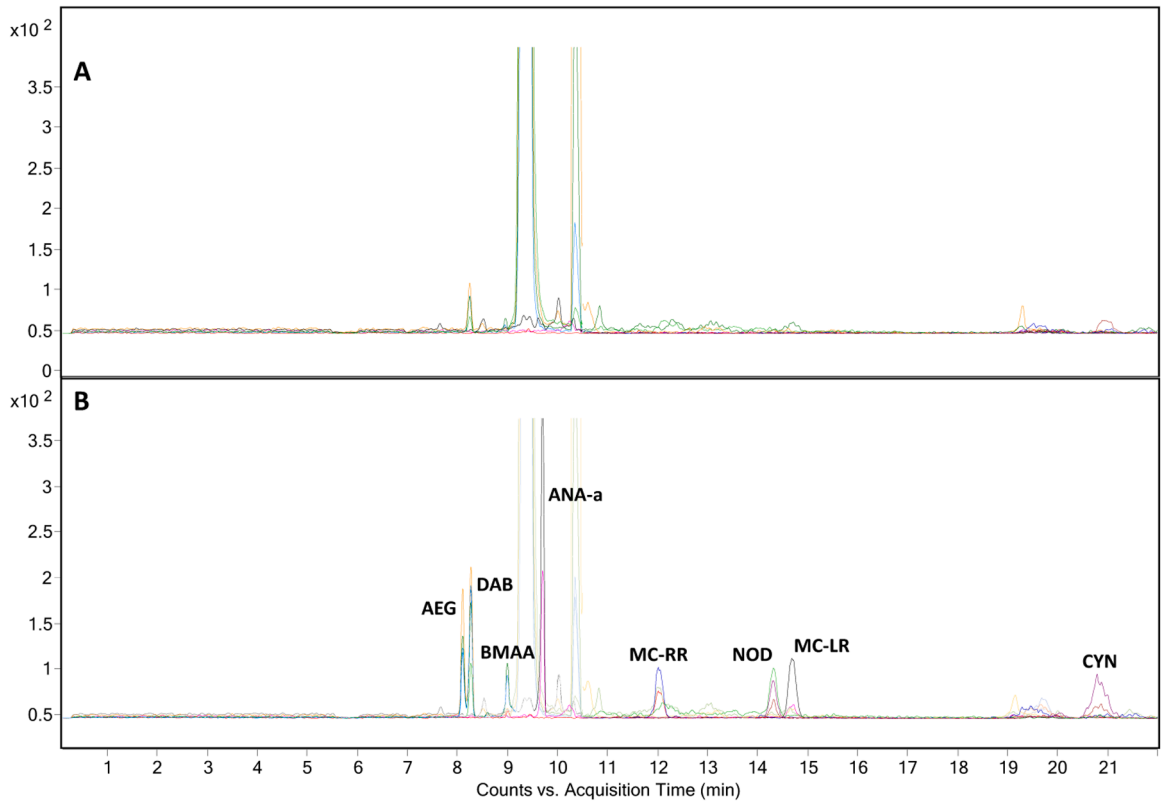

Fig. 5. Extracted ion electropherogram of a blank (A) and a spiked (B) spinach sample at: 1.8 μg·Kg^{-1} for BMAA, ANA-a, MC-RR; 2.4 μg·Kg^{-1} for AEG, DAB and MC-LR; and 4.8 μg·Kg^{-1} for NOD and CYN. Coloured lines corresponding to the analytes are emphasized in electropherogram (B).

CRediT authorship contribution statement

Rocío Carmona-Molero: Conceptualization, Investigation, Methodology, Writing – original draft. **M. Mar Aparicio-Muriana:** Formal analysis, Methodology, Visualization. **Francisco J. Lara:** Data curation, Supervision, Visualization, Writing – original draft. **Ana M. García-Campaña:** Project administration, Resources, Supervision. **Monsalud del Olmo-Iruela:** Investigation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data available in supplementary material.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.464666.

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