DEVELOPMENT AND VALIDATION OF AN SPE-LC-MS/MS METHOD FOR THE SIMULTANEOUS DETERMINATION OF MULTICLASS CYANOTOXINS IN WATER

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ABSTRACT

Cyanobacteria are present in surface waters and under favorable environmental conditions can form extensive algal blooms and release hazardous toxic compounds (cyanotoxins), posing a significant risk to aquatic habitats and human health. Cyanotoxins comprise a large group of organic compounds, with a variety of physicochemical properties, chemical structures (alkaloids, cyclic peptides, amino acids, lipopolysaccharides etc) and toxic activity (hepatotoxic, neurotoxic, cytotoxic, dermatotoxic). In this study, we present the development and optimization of a fast and sensitive analytical method for the preconcentration and simultaneous determination of multi-class cyanotoxins in water.

The targeted compounds were: Cylindrospermopsin, Anatoxin-a, Nodularin and 12 Microcystins ([D-Asp3]MC-RR, MC-RR, MC-YR, MC-HtyR, [D-Asp3]MC-LR, MC-LR, MC-HilR, MC-WR, MC-LA, MC-LY, MC-LW and MC-LF). For the chromatographic separation of the compounds a reversed phase column was used (Atlantis T3, 100x2.1 mm, 3μm, Waters). A gradient elution program was used with mobile phase acetonitrile (A) 5-100% and (B) water, both containing 0.5% formic acid, at a flow rate of 0.2 $mL.min^{-1}$, resulting in analysis time of 43 min. Ionization was positive ESI in multiple SRM mode. Solid Phase Extraction included two tandem SPE cartridges, Oasis HLB (200mg, 6cc, Waters) and porous graphitic carbon (Hyper PGC, 200mg, Thermo) for efficient extraction of the target toxins from water. SPE factors studied were the proportion of methanol – dichloromethane, the presence of formic acid in the elution solvent and the volume of elution solvent.

Based on the obtained results, the use of mixed SPE sorbents enhances the overall efficiency of the method for most of the compounds. Mean recoveries ranged 71.4% to 94.5% except for Anatoxin-a, MC-WR, MC-LW and MC-LF which were slightly reduced. Repeatability and reproducibility of the method was satisfying.

Keywords: Cyanotoxins, SPE, LC-MS/MS, Microcystins, Cylindrospermopsin, Anatoxin, Method optimization, Method validation

1. Introduction

Cyanobacteria can form under favorable environmental conditions extensive algal blooms and they can release hazardous toxic compounds, which comprise a large group of compounds, with a variety of structures (alkaloids, cyclic peptides, amino acids, lipopolysaccharides etc) and toxic activity (hepatotoxic, neurotoxic, cytotoxic, dermatotoxic) (De La Cruz *et al*., 2013; Kaushik and Balasubramanian, 2013). As a result the simultaneous chromatographic separation and determination of these compounds in environmental matrices, pose significant challenges, due to the diversity of their properties and structures. The scope of this study was the development and optimisation of a fast, robust and sensitive multi-toxin analytical method for the preconcentration and unambiguous determination of various cyanotoxins in water (Kaloudis *et al*., 2013; Triantis *et al*., 2010).

2. Materials and methods

Compounds included Cylindrospermopsin, Anatoxin-a, Nodularin and 12 Microcystins ([D-Asp3]MC-RR, MC-RR, MC-YR, MC-HtyR, [D-Asp3]MC-LR, MC-LR, MC-HilR, MC-WR, MC-LA,

MC-LY, MC-LW and MC-LF). A reversed phase chromatographic column (Atlantis T3, 100x2.1 mm, 3μm, Waters) coupled with a triple quadrupole mass spectrometer (LC-MS/MS) was used for their chromatographic separation, employing a gradient elution program with acetonitrile (A) 5-100% and (B) water, (0.5% formic acid), at a flow rate of 0.2 mL.min^{-1}. Positive ESI ionization in multiple SRM mode was used, resulting in analysis time of 43 min. ESI operating conditions were optimized for the precursor and product ions of all compounds, while deuterated phenylalanine PHE-d5, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2,3,5-Trimethylphenyl methyl carbamate (2,3,5-TMC) and Leucine Enkephaline (LE) were examined for their application as surrogate and internal standards.

Solid Phase Extraction assembly included two tandem SPE cartridges, Oasis HLB (200mg, 6cc, Waters) and porous graphitic carbon (Hyper PGC, 200mg, Thermo) for the successful extraction of the toxins from water. Examined factors included the proportion of methanol – dichloromethane, the presentence of formic acid in the elution solvent and the volume of the elution solvent. In all experiments, 400ml of sample were used, with 1% MeOH, adjusted at pH 11. Elution did not include washing steps. Sample reconstitution was realised using 400μl of H_2O:MeOH (95:5).

3. Results and discussion

Based on the obtained results, chromatographic separation of selected cyanotoxins was efficient with high resolution values (Figure 1) in overall 43 min.

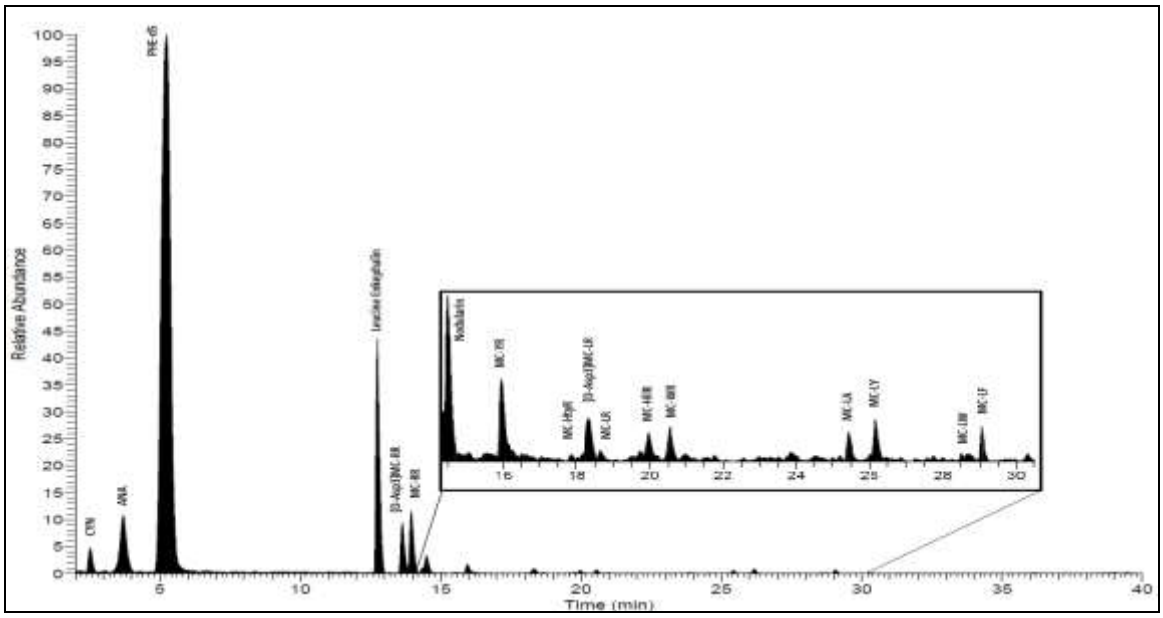

Figure 1: TIC of LC-MS/MS MRMs chromatogram of 12 MCs, NOD, CYN, ANA, Phe-d5 and LE (IS) at 100 μgL^{-1}

A separate assessment for the efficiency of different cartridges was carried out. Results indicated that the group of compounds containing Microcystins, Nodularin and Anatoxin-a are mainly retained by Oasis HLB cartridges while Cylindrospermopsin is better retained by PGC cartridges. Based on the obtained results, the use of a dual SPE assembly, enhances the overall efficiency of the method for the majority of the compounds, despite the variations in their physicochemical properties.

After careful consideration, optimized initial sample pH was 11, in order to maximize the retention of Anatoxin-a. For the elution of the compounds from the mixed SPE assembly different elution solvents and their mixtures were assessed (0-40% DCM in MeOH in different volumes of 5, 8 and 10mL). Optimum overall elution was achieved using 10mL elution mixture containing 40% DCM in MeOH.

An overall evaluation of the most effective reconstitution solvent was realized, taking into account the effect of the presence of methanol in the overall chromatographic separation of the compounds. Methanol contents higher than 8% in the sample solvent negatively affects the chromatographic separation, resulting in partial chromatographic elution of Cylindrospermopsin and Anatoxin-a with the dead volume. Moreover, additional tests indicate that the presence of methanol in the reconstitution solvent positively affects the overall recoveries of Microcystins. Taking these results into account, the reconstitution solvent of the final extract was established as 95%Water – 5% Methanol. Finally the sonication time of the reconstituted solution was assessed and was optimized at 5min.

Mean recoveries ranged 71.4% to 94.5% except for Anatoxin-a, MC-WR, MC-LW and MC-LF which were slightly reduced. $\%RSD_R$ ranged 5.8 to 24.9%, with the exception of MC-WR, MC-LY, MC-LW and MC-LF which tended to be less reproducible. LOD values ranged from 1ng/L to 5 ng/L.

Validation of the method included repeatability and reproducibility studies for two consecutive days at the level of 100ng.L^{-1}.

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