**Simultaneous determination of BMAA, 2,4-DAB, Saxitoxin and neoSaxitoxin in water, using SPE HILIC-LC-MS/MS.**

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Cyanobacteria are common aquatic microorganisms, able to form extentive blooms under favorable conditions. They can release hazardous toxic compounds (cyanotoxins), with a variety of chemical structures and modes of toxic activity (dermatotoxins, hepatotoxins, neurotoxins etc), which can significantly affect aquatic habitats and human health. Cyanotoxins, such as BMAA (b-N-methylamino-L-alanine) and its structural isomers, eg 2,4-DAB (2,4-diaminobutyric acid), with increased polarity and lower molecular weight as well as STX (Saxitoxin) and neoSTX (neoSaxitoxin), with hydrophilic properties are of particular interest, since they present challenges in their isolation, chromatographic separation and identification. BMAA, is a low molecular weight neurotoxin, which has been associated to progressive neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Alzheimer’s disease and Parkinson’s disease. BMAA’s structural isomer 2,4-DAB is frequently misidentified as BMAA, presenting false positive results. STX and neoSTX are potent neurotoxins produced by various cyanobacteria. This study presents the development and optimization of a sensitive analytical method for the preconcentration and simultaneous determination of BMAA, DAB, STX and neoSTX in water.

Separation of the compounds was achieved using a SeQuant ZIC-HILIC (2.1x150mm, 3.5μm, 100Å) chromatographic column. A gradient elution program was used with mobile phases ACN and water with ammonium acetate buffer, at a flow rate of 0.2 mL.min-1. Retention times were: BMAA tR = 8.3min, DAB, tR=9.3min, neoSTX tR = 23.6min και STX tR = 24.8min. Positive ESI in multiple SRM mode was employed. Identification of BMAA was based on the transitions of precursor ion *m/z* 119 > 76, 88, 102, while for 2,4 DAB it was based on *m/z* 119> m/z 56, 74 and 102. STX identification relied on the transitions or precursor *m/z* 300 to the product ions *m/z* 110, 179 and 204 while for neoSTX, identification was based on the fragmentation *m/z* 316 > 164, 220 and 298. Internal standards d3-BMAA and d3-DAB were used for analytical quality assurance and determination of recovery losses. For the optimization of SPE, various experimental parameters were studied, including initial sample pH (2-7), SPE cartridge material (cationic, mixed mode, C18), elution solvents and their mixtures as well as reconstitution solvent and its acidic content.

Results showed that for optimum recovery of all compounds the following method was selected: water sample pH was adjusted to 3. Sample was consequently divided in two parts, followed by two parallel individual SPE methods. For BMAA and 2,4-DAB determination, cationic Oasis MCX (Cation Exchange) 60mg/3cc (Waters) SPE cartridges were employed, using MeOH with 5% NH4OH as elution solvent. For STX and neoSTX, C18 (500mg – 6 ml) (Waters) SPE cartridges were used, followed by extraction using a mixture of methanol/water (with formic acid). Eluates were combined and dried under nitrogen flow at 400C. Recoveries at two different concentration levels (20 and 100 ng/L) for BMAA and 2,4-DAB, were 60-65% and 51-58%, respectively. STX and neo-STX presented recoveries above 50% for all concentration levels. Repeatability for intra-day samples ranged 10-23% for all compounds. Linearity for BMAA and 2,4-DAB compounds extended 2-100μg/L, while for STX and neoSTX it ranged 2-200μg/L. LODs of the method were in the range of 1.5-2.5ng/L. Overall, the developed method achieved sensitive and accurate determination of the targeted cyanotoxins, providing adequate separation between BMAA and its main structural isomer 2,4-DAB, as well as closely eluting STX and neoSTX in one run.

**Keywords**: BMAA, DAB, Saxitoxin, neoSaxitoxin, SPE, HILIC-LC-MS/MS, cyanotoxins

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