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Ultra-fast analysis of anatoxin-A using laser diode thermal desorption-atmospheric pressure chemical ionization-tandem mass spectrometry: Validation and resolution from phenylalanine

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ABSTRACT

A novel approach for the analysis of the cyanobacterial toxin, anatoxin-a (ANA-a), in an environmentally relevant matrix, using laser diode thermal desorption-atmospheric pressure chemical ionization-tandem mass spectrometry (LDTD-APCI-MS/MS) is presented. The ultra-fast analysis time (15 s/sample) provided by the LDTD-APCI interface is strengthened by its ability to remove interference from phenylalanine (PHE), an isobaric interference in ANA-a analysis by MS/MS. Thus the LDTD-APCI interface avoids the time consuming steps of derivatization, chromatographic separation or solid-phase extraction prior to analysis. Method development and instrumental parameter optimizations were focused toward signal enhancement of ANA-a, and signal removal of a PHE interference as high as 500 μg/L. External calibration in a complex matrix gave detection and quantification limit values of 1 and 3 μg/L respectively, as well as good linearity ($R^2 > 0.999$) over nearly two orders of magnitude. Internal calibration with clomiphene (CLO) is possible and method performance was similar to that obtained by external calibration. This work demonstrated the utility of the LDTD-APCI source for ultra-fast detection and quantification of ANA-a in environmental aqueous matrices, and confirmed its ability to suppress the interference of PHE without sample preparation or chromatographic separation.

1. Introduction

Anatoxin-a (ANA-a) is a neurotoxic alkaloid compound produced by many freshwater cyanobacteria genera, namely *Anabaena*, *Aphanizomenon*, *Cylindrospermum*, *Microcystis*, *Oscillatoria* and *Planktothrix* among others (Osswald et al., 2007). This neurotoxin was the first cyanotoxin structurally characterized and was found to be a low molecular weight (M W = 165.24 g/mol) bi-cyclic secondary amino molecule, which is also named more systematically: 2-acetyl-9-azabicyclo (4,2,1) non-2-ene (Fig. 1) (Devlin et al., 1977). It is readily soluble in natural waters by protonation of the amino function (pK_a 9.4), generating the cationic species (Wonnacott and Gallagher, 2006). ANA-a is stable in acidic media (pH $\leq$ 3), but degrades rather rapidly in alkaline conditions and is light sensitive (Stevens and Krieger, 1991).

This potent toxin mimics the effect of the neurotransmitter acetylcholine by competition for binding to acetylcholine-receptors. ANA-a has a greater affinity for nicotinic acetylcholine-receptors than acetylcholine, and is not hydrolyzed by acetylcholinesterase, resulting in overstimulation of respiratory muscles and suffocation of

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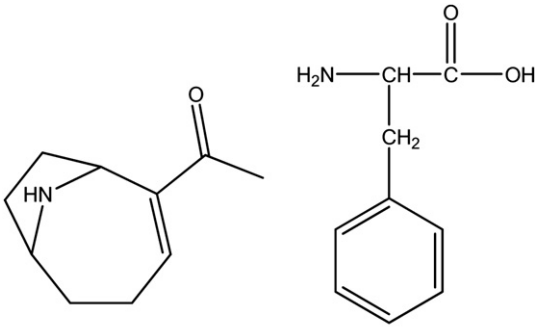

Fig. 1. ANA-a and PHE structures and corresponding molecular weights (M.W.).

exposed individuals (Swanson et al., 1986). An LD_{50} value of 200–250 μg/kg was determined by intraperitoneal injection on mice and as of now, suggested guideline values addressing tolerable ANA-a concentration in drinking and recreational water range from 1 to 12 μg/L (Codd et al., 2005; Fawell et al., 1999; Svrcek and Smith, 2004).

Techniques for analyzing ANA-a have been reviewed and range from biological methods to chromatographic methods, both in gas and liquid phase, using electron capture, UV, fluorescence or mass spectrometry (MS) for analyte detection (Osswald et al., 2007). Bioassays using different test organisms are no longer in use for the analysis of ANA-a since they lack specificity, require a large amount of standard material to be effective and are prone to ethical criticisms. The chromatographic methods coupled with spectroscopic detection are not sensitive enough, and necessitate derivatization to improve their detection limits. The coupling with MS allows for better sensitivity and specificity, however the essential amino acid phenylalanine (PHE) (Fig. 1) is an isobaric interference in MS detection of ANA-a, using single or triple quadrupole(s) instruments (Gugger et al., 2005). Multiple options have been described to remove this interference (Furey et al., 2005): (i) derivatization of ANA-a and PHE with 4-fluoro-7-nitro-2,1,3-benzoxadiazole to alter significantly the compounds' chromatographic behavior, (ii) selective methylation of PHE with diazomethane prior to LC-MS analysis (iii) use of other MS techniques such as hybrid quadrupole time-of-flight (QqTOF) or quadrupole ion-trap (QIT) providing respectively higher mass resolution or intrinsic mass fragmentation pathway. Otherwise, sufficient chromatographic resolution of PHE from ANA-a, without derivatization, was achieved with high performance LC (Bogialli et al., 2006) and ultra-performance LC (Oehrle et al., 2010) with retention times around 8 and 3 min respectively. Despite these performances achieved by chromatography, costly solvent and columns are necessary and the analysis time is counted in minutes.

An MS interface called LDTD (Laser Diode Thermal Desorption), which is combined with atmospheric pressure chemical ionization (APCI), has shown great potential to reduce analysis time to seconds by removing the chromatographic step and introducing charged analytes directly into the mass spectrometer (Fayad et al., 2010; Segura et al., 2010; Wu et al., 2007) The details and of this method and the fundamental principles of LDTD-APCI are described in detail through a specific optimization of instrumental parameters for the analysis of feminizing hormones (Fayad et al., 2010). Herein we report the first application of the LDTD-APCI source, coupled to MS/MS, for the detection and quantification of cyanotoxin ANA-a in a complex matrix, eliminating the PHE interference without any laborious and time-consuming sample preparation. Optimization of instrumental parameters for simultaneous detection of ANA-a and removal of PHE interference, are demonstrated. Method validation, including evaluation of detection and quantification limits, linear dynamic range, accuracy and precision are reported. We also explore the suitability of clomiphene (CLO) as an internal standard (IS) for internal calibration. This new method generates high-throughput results (roughly an order of magnitude faster than LC alternative) and could be adapted and applied for environmental and food safety screening purposes.

2. Materials and methods

2.1. Chemical and reagents

ANA-a/fumarate salt was obtained from Tocris Bioscience (Ellisville, MO, USA). L-PHE, 99% and CLO/citrate salt were obtained from Sigma–Aldrich Chemical Co. (Oakville, ON, Canada). Sodium hydroxide (certified A.C.S.) and glacial acetic acid (HPLC) were purchased from Fisher Scientific (Whitby, ON, Canada). Compressed air (Ultra Zero Certified grade; $\leq$ 2 ppm water), used as carrier gas, was obtained from MEGS Specialty Gases, Inc. (St-Laurent, QC, Canada). Methanol (MeOH) of HPLC grade was acquired from J.T. Baker (Phillipsburg, NJ, USA).

2.1.1. Stock solution and bloom matrix preparation

One mg ANA-a/fumarate salt was dissolved in 10 mL deionized/distilled water (dd-H_2O), containing 0.05 M glacial acetic acid, (pH $\sim$ 3), and was stored at -20 °C. Earlier studies showed that ANA-a is stable for 3–4 months under these conditions (Araoz et al., 2005; Ghassempour et al., 2005). All working solutions and dilutions of ANA-a were prepared daily with the final pH adjusted to 11.5 with NaOH (5 mM). Diluted solutions of ANA-a were prepared in dd-H_2O and MeOH (50:50, v/v). The artificial bloom matrix was made by mixing, in equal proportion, a pure culture media (ASM-1) (Gorham et al., 1964), containing a non-toxic strain of *Microcystis aeruginosa*, and river water. Before mixing, freeze-thawing of the culture media was repeated three times to lyophilize cells (Baker et al., 2001), followed by filtration using 0.45 μm nitrocellulose membranes (Millipore, Billerica, MA, USA). Working solutions of ANA-a in bloom matrix were also prepared daily (pH 11.5) with the filtrate. Diluted solutions of ANA-a were prepared in bloom matrix and MeOH (50:50, v/v). For internal calibration, CLO in bloom matrix and MeOH (50:50, v/v), was added to solutions at 10 μg/L.

2.2. Analysis with LDTD-APCI-MS/MS

Compound desorption and ionization were performed with the T-960 LDTD-APCI ionization interface model,