Analysis of multi-class cyanotoxins in fish tissue. Application to fish from Greek lakes

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Abstract

The analysis of cyanotoxins in aquatic organisms, particularly in fish tissue, has lately received increasing interest, due to public health and environmental concerns. This study presents novel, efficient and sensitive analytical methods for the simultaneous determination of multi-class cyanotoxins i.e Cylindrospermopsin (CYN), Anatoxin-a (ANA-a) and 12 Microcystins (MCs), in freshwater fish. Prior to LC-MS/MS analysis, several combinations of extraction solvents at different pH were tested, for the efficient extraction of selected cyanotoxins from fish tissue (muscle and liver). Various treatment techniques were employed in order to release the fraction of MCs that is conjugated to proteins. Protein precipitation and hexane washing of lipids were also tested along with different SPE materials, in order to further eliminate matrix interferences. Evaluation of LC-DAD and LC-MS/MS chromatograms, using identical chromatographic conditions, revealed the co-elution of several matrix components with the targeted compounds, which induced increased matrix suppression, decreased sensitivity and overall efficiency of the method. Optimized pretreatment procedures are proposed, presenting drastic elimination of matrix effects and optimum extraction and recovery for each cyanotoxin group. The developed analytical methods were validated and subsequently used for the evaluation of the diversity, abundance and accumulation of cyanotoxins in fish samples from various Greek lakes.

Keywords: cyanotoxins, LC-MS/MS, fish tissues, matrix effect

Acknowledgments: The authors would like to sincerely acknowledge COST Action ES1105 "CYANOCOST".

1. Introduction

Cyanotoxins comprise a large variety of compounds with various physicochemical properties and different modes of toxicity. Microcystins (MCs) are hepatotoxic cyclic peptides (Merel *et al*., 2013), Cylindrospermopsin (CYN), is an alkaloid cyanotoxin with cytotoxic, dermatotoxic, hepatotoxic and possibly carcinogenic potency (De La Cruz *et al*., 2013), while the alkaloid Anatoxin-a (ANA), also known as "Very Fast Death Factor", is a bicyclic amine with acute neurotoxicity (Osswald *et al*., 2007).

Exposure of fish to cyanotoxins can occur through contact of their gill epithelium with water or oral consumption of cyanobacteria and can affect their growth, development, histology and reproduction (Deng *et al*., 2010; Guzmán-Guillén *et al*., 2015a; Malbrouck and Kestemont, 2006; Osswald *et al*., 2013; Rymuszka and Sierosławska, 2011; Sieroslawska and Rymuszka, 2015). Cyanotoxins may also be distributed and accumulated in various fish organs (Chen *et al*., 2006; Romo *et al*., 2012). Recent studies have shown that consumption of aquatic organisms which have accumulated cyanotoxins may pose a potential risk to human health ((Hardy *et al*., 2015).

A number of investigations have been carried out in the past, related to the analysis of MCs, CYN and ANA in fish, using various pretreatment methods and analytical techniques using LC-MS/MS (Bogialli *et al*., 2006; Cadel-Six *et al*., 2014; Guzmán-Guillén *et al*., 2017; Guzmán-Guillén *et al*., 2015b; Karlsson *et al*., 2005; Neffling *et al*., 2010). Nevertheless, the accurate and reliable determination of cyanotoxins in trace amounts at such complicated matrices poses several challenges, such as the efficient extraction and recovery of the targeted compounds, the evaluation of the matrix effect in each type of sample (liver, muscle or fish brain), the possible conjugation of certain types of cyanotoxins with the matrix compounds and the validation of the proposed methods. Finally, MCs, CYN and ANA are chemical compounds with physic-chemically diverse structures and properties, therefore the development of complementary treatment methods and the subsequent simultaneous determination of multiclass toxins, is of great importance.

The scope of this study was (a) to develop novel and sensitive analytical methods for the determination of multi-class cyanotoxins i.e CYN, ANA-a and 12 MCs, in freshwater fish tissues (liver and muscle), (b) to evaluate the matrix (fish liver and muscle) effect on cyanotoxin analysis, as well as certain pretreatment techniques for its elimination, and (c) to investigate the presence of cyanotoxins in a selection of fish originating from various Greek lakes.

2. Materials and Methods

2.1. Standards and reagents

Standards of MC variants [D-Asp3]MC-LR, [D-Asp3]MC-RR, MC-WR, MC-HtyR, MC-HilR,MC-LY, MC-LW and MC-LF were supplied by ENZO Life Science (Lausen, Switzerland). MC-RR, MC-LR, MC-YR and MC-LA standards were supplied by Sigma-Aldrich (Steinheim, Germany). CYN was purchased from Abraxis (Warminster, USA) and ANA-fumarate from TOCRIS Bioscience (Bristol, UK). All substances had purity >95%. Methanol (MeOH) of HPLC grade (99.99%) and hexane of analytical grade were obtained from Fischer Scientific (Leics, UK), acetonitrile (ACN) of gradient grade for HPLC (≥99.9%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). High purity water (18.2 $M\Omega.cm^{-1}$) was produced on-site using a TEMAK ® SA ultra-pure system. Sodium hydroxide (NaOH) 2 M used for sample pH adjustment, was prepared from NaOH pellets (purity 98%) purchased from Sigma-Aldrich (Steinheim, Germany). Acetic acid and Formic acid (FA) (>98%) were purchased by Riedel-de Haën (Seelze, Germany).

2.2. Fish sampling and preparation

For the development of the analytical methods, aqua cultured rainbow trout (*Oncorhynchus mykiss*) was purchased from the Greek market. The optimised methods were applied on fish samples, ie roach (*Rutilus rutilus),* white roach *(Alburnoides prespensis),* carp *(Cyprinus carpio)* and catfish *(silurus glanis)* were collected from Lakes Kastoria (9/2016 and 9/2016) and lake Karla (4/2015) during the presence of cyanobacterial bloom.

The fish were filleted, using a sterile knife, separating the gills, heart, liver and muscle. Part of the tissues was preserved at 4^0C for further pretreatment and analysis, while another part of them was lyophilized prior to pretreatment and analysis.

2.3. Sample extraction and treatment

For the optimization of a pretreatment method developed for cyanotoxins' determination, pre-weighed amounts of fish tissue (0.5g lyophilized or 2g untreated) were initially spiked with the appropriate amount of a mixture of cyanotoxins (including CYN, ANA, MC-LR and MC-RR) and they were subsequently extracted using several combinations of extraction solvents (MeOH, ACN, butanol, water) at different pH (addition of Formic acid). Extraction was facilitated by incubation, ultrasonication and homogenation steps, in order to obtain the maximum amount of cyanotoxins from fish tissue. The efficiency of each treatment procedure was evaluated based on the calculated recoveries of the spiked samples. Additionally, the matrix effect during each extraction procedure was calculated based on the recovery of cyanotoxins from an extracted blank sample (rainbow trout), which was spiked with an appropriate mixture of cyanotoxins.

During method development, several extraction parameters were evaluated in order to optimize the simultaneous extraction of target toxins: (a) sonication techniques, (b) extraction efficiency of various organic solvents, (c) composition of extraction solvent, (d) volume of extraction solvent, (e) mass of analyzed sample.

For the elimination of matrix components several additional techniques were employed. Protein precipitation at low temperature and in the presence of inorganic salts, was carried out, in order to separate the rich protein fraction of the extract, especially in the cases of the more aqueous extracts. Extra steps were also added for lipid separation using hexane washing. Finally, centrifugation and various filters (Nylon 0.45μm from Whatman, PVDF 0.45μm from Whatman, PTFE 0.45μm from Valuprep and Fiber glass 0.47μm from Millipore filters) were also tested, for the maximum separation of suspended solids and possible colloid formation. The effect of matrix components after the application of different extraction and pretreatment techniques, was evaluated by comparing LC-DAD and LC-MS/MS chromatograms under identical chromatographic conditions.

Several SPE materials (HLB, C18, graphitized carbon) were employed after the extraction procedures, in order to isolate the target compounds, further eliminate matrix content and pre-concentrate the toxins.

Finally two extraction/treatment procedures were developed, offering maximum recoveries of the selected toxins and minimum matrix effect.

In the case of MCs extraction was carried out with a solvent mixture MeOH:H_2O (90:10 v/v), in the presence of 0.1% FA, in a microwave water bath at 30^0C for 30min. Centrifugation at 14000g was employed and subsequent liquid/liquid extraction of the extract with hexane, removed the lipids present in the sample. The procedure was followed by SPE pretreatment with C18 SPE cartridges and elution with a mixture of MeOH:H_2O (80:20 v/v). The solutions were filtered (PVDF filters 0.45μm from Whatman were selected), dried under nitrogen and reconstituted in a predefined amount of MeOH:H_2O. Tests were performed in duplicate, for each tested value of parameters.

In the case of CYN and ANA, the common extraction/treatment procedure that was developed included extraction with a solvent mixture of ACN:H_2O (70:30, v/v), in the presence of 0.5% acetic acid in a microwave water bath (same as MCs). Centrifugation at 14000g was employed and subsequent liquid/liquid extraction of the supernatant with hexane, was also carried out. The procedure was followed by SPE pretreatment with a sequential double Oasis HLB and Graphitized Carbon cartridge assembly, based on the method proposed by Zervou et. al. (Zervou *et al*., 2017).

For both methods reconstituted solutions were transferred to an autosampler glass vial and analyzed by LC-ESI-MS/MS and LC-DAD.

2.4. LC-DAD and LC-MS/MS analysis

CYN, ANA-a, and 12 MCs ([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) were chromatographically separated with a reverse phase

column Atlantis T3 (2.1 x 100mm, 3μm, Waters) with a gradient elution program of ACN – water (Zervou *et al*., 2017).

LC–MS/MS was carried out on a Finnigan TSQ Quantum Discovery Max triple-stage quadrupole mass spectrometer (Thermo, USA), equipped with electrospray ionization (ESI) source. Separation of target analytes was achieved with a Finnigan Surveyor LC system, equipped with a Finnigan Surveyor AS autosampler (Thermo, USA).

Determination of cyanotoxins in LC-MS/MS was achieved by monitoring three different precursor to fragment ion transitions, in positive ESI mode using Multiple Reaction Monitoring (MRM). Identification of the target toxins was achieved by: (a) t_R of compounds, (b) three characteristic precursor/product ion transitions and (c) two calculated ratios of precursor to product ion transitions. The developed method achieves 5.5 identification points (IPs), fulfilling the confirmation criteria according to the European legislation concerning the performance of analytical methods (Commission, 2002).

LC-DAD analysis was carried out on a Waters Alliance chromatographic system (e2695 separation module) coupled with 2998 PDA Detector. The same chromatographic conditions and column were used as in the case of LC-MS/MS. The matrix components were observed under various detector wavelengths and chromatograms obtained from standard and spiked samples were overlayed, in order to evaluate the elution times and profile of the matrix in each extraction step.

3. Results and Discussion

This study presents novel, efficient and sensitive analytical methods for the simultaneous determination of multi-class toxins in lake water fish, using a combination of extraction and clean-up steps, prior to LC-ESI-MS/MS.

CYN and ANA-a presented lower retention times during chromatographic separation, due to their decreased hydrophobicity. Evaluation of LC-DAD and LC-MS/MS chromatograms revealed that these compounds co-eluted with several matrix components, which induced increased matrix suppression, decreased sensitivity and affected method trueness (significantly lower % recoveries). The calculated matrix suppression observed for CYN and ANA using only extraction without any additional treatment steps reached 85% and 40%, respectively. MCs were chromatographically eluted far later and the matrix suppression reached a maximum of 21% for MC-LR.

An optimized pretreatment procedure was proposed, presenting the maximum elimination of matrix effect on CYN and ANA-a. The extracts were analyzed using the multi-class chromatographic method proposed. The overall optimized analytical method presented recoveries exceeding 65% and 70% for CYN and ANA respectively. For MC-LR the recovery was higher than 80%.

Finally, several fish samples, originating from various lakes of Greece, were analyzed with the developed method. The study is in progress. The diversity, abundance and accumulation of toxins in fish muscle and liver will be evaluated and an overall assessment of the risks associated to human consumption will be attempted.

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