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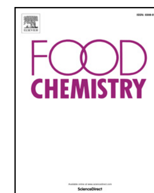

A shotgun method for high throughput screening microcystins in *Margarya melanioides* on a triple quadrupole tandem mass spectrometry

Shiyan Li[a,b,1], Yiwei Cui[a,1], Yang Wang[b], Zhiyuan Dai[a], Qing Shen[a,*]

[a] *The Joint Key Laboratory of Aquatic Products Processing of Zhejiang Province, Institute of Seafood, Zhejiang Gongshang University, Hangzhou 310012, China*
[b] *Aquatic Products Quality Inspection Center of Zhejiang Province, Hangzhou 310012, China*

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ABSTRACT

A selective and sensitive procedure for determining seven hazardous microcystins in *Margarya melanioides*, using tandem mass spectrometry, is presented. The product ion m/z 135.2 was selected as representative for precursor ion scanning and used to increase overall sensitivity of this shotgun method. The method was validated, and recoveries ranged from 69.3 to 87.9%, with the relative standard deviation less than 10%. Limits of detection and quantitation were less than 3.1 and 9.3 ng·mL^{-1}, respectively. A total of 60 samples were tested using this method. 38 samples were found to be contaminated with microcystins, among which there are 31 samples whose estimated daily intake (EDI) was 0–7.8 times higher than the tolerable daily intake (TDI). This method would be useful for carrying out direct, ultra-rapid quantification of microcystins in foods, and is of significant interest for environmental and food safety applications.

1. Introduction

Microcystins are potent hepatotoxins produced by a number of planktonic cyanobacteria genera, such as Anabaena, Anabaenopsis, Nostoc, Planktothrix and Microcystis (van Apeldoorn, van Egmond, Speijers, & Bakker, 2007). The usual structure of microcystins is cyclo (-d-Ala–l–X–d-erythro-methylAsp–l-Z-Adda–d-Glu-N-methyldehydro-Ala) where Adda stands for the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid. More than 80 structural analogues have been identified, varying in degrees of methylation, hydroxylation, epimerization, peptide sequence and toxicity (Amado, & Monserrat, 2010). Exposure to microcystins represents a health risk to aquatic organisms, wild life, domestic animals, and humans upon drinking contaminated water or ingesting these algae (Malbrouck, & Kestemont, 2006). Microcystins toxicity is based on their potent inhibition of protein phosphatases 1 and 2A. Microcystins can act as tumor promoters and have been shown to induce oxidative DNA damage in the human hematoma cell line HepG2 (Zegura, Zajc, Lah, & Filipic, 2008). The World Health Organization has published a provisional guideline value for microcystin-LR in 1 µg·L^{-1} of drinking water. It was also reported that the tolerable daily intake (TDI) value of 0.04 µg·kg^{-1} body weight per day, which was the estimated daily intake of microcystins for an adult weighing 60 kg ingesting 300 g of edible organs of aquatic animals (Chorus & Bartram, 1999).

There has been much research carried out in the development of analytical methods for microcystins in foods, such as bioassays based on cultured bacterial cells, enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis, liquid chromatography with UV or mass spectrometric (MS) detection (Devlin et al., 2013; Li et al., 2014; Mountfort, Holland, & Sprosen, 2005; Onyewuenyi, & Hawkins, 1996; Parker, Stutts, & DeGrasse, 2015; Roy-Lachapelle, Fayad, Sinotte, Deblois, & Sauvé, 2014). Lawton et al. developed a microtox bioluminescence assay for preliminary screening of cyanobacterial blooms and microcystin-sourced toxicity (Lawton, Campbell, & Codd, 1990). Liu, Xing, Yan, Kuang, and Xu (2014) used an ELISA and immunochromatographic strip for highly sensitive detection of microcystin-LR with a cut-off value of 1 µg·L^{-1}. Tong et al. (2010) analyzed trace levels of microcystin isomers in crude algae samples using a rapid and effective method based on capillary zone electrophoresis (CZE) coupled with electrospray ionization mass spectrometry (ESI-MS). LC–MS, which involves separation on a C18 sorbent column followed by ESI and detection using MS, has increased in popularity because it provides specificity and good sensitivity. Mekebri, Blondina, and Crane (2009) developed and validated a LC-ESI–MS/MS method to identify and quantify trace levels of cyanotoxins or microcystins in water, bivalves and fish tissue, which enabled quantification of six microcystins (microcystin-LA, LF, LR, LW, RR and YR) in a single chromatographic run. However, these methods are based on targeted analysis and cannot

* Corresponding author at: Institute of Seafood, Zhejiang Gongshang University, No.149, Jiaogong Road, Hangzhou, China.
E-mail addresses: sq@zjgsu.edu.cn, leonqshen@163.com (Q. Shen).
[1] Contributed equally to this work.

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quantify isomers in the absence of reference substances. Therefore, a non-targeted and high-throughput method for screening of microcystins is needed urgently in order to evaluate the safety of a given foods and beverages.

Shotgun methods are a popular technique for rapid screening of non-targeted compounds in a variety of samples. They are commonly used in genetics, proteomics, and lipidomics. For example, shotgun cloning is typically used for full genome sequencing. Similarly, bottom-up shotgun proteomics techniques for identifying proteins in complex mixtures have been explored. Wolters, Washburn, and Yates (2001) described an automated method for shotgun proteomics, multidimensional protein identification technology (MudPIT), which can separate thousands of peptides, improving the analysis of proteomes by identifying proteins of all functional and physical classes. Shen et al. (2012) developed an efficient shotgun lipidomics strategy for rapid phospholipid profiling of viscera from three fish species, and some specific phospholipids can be used as potential markers for fish species differentiation. Jin, Li, Feng, et al. (2017) and Jin, Li, Guo, et al. (2017) used shotgun lipidomics to analyze phospholipids from *Hypophthalmichthys nobilis* on the basis of zwitterionic hydrophilic interaction liquid chromatography solid-phase extraction.

The rapid development of mass spectrometry methods makes analysis of non-targeted analogs possible, especially compounds with the same or similar moieties. Because microcystins have the same parent molecule, Adda, it is theoretically possible to establish a shotgun method for high-throughput screening of microcystins. In this study, a sensitive and selective shotgun method was established for ultra-rapid screening of microcystins using precursor-ion scanning (PreIS) in positive-ion mode. The method was validated for sensitivity, precision, recovery, and matrix effects, and applied for the detection of microcystins in food samples with good results.

2. Materials and methods

2.1. Chemicals and reagents.

Microcystin-RR, YR, LR, LA, LF, LY, and LW (purity > 95.0%) were purchased from Alexis (Lausen, Switzerland), and the chemical structures are shown in Fig. S1. Standard working solutions (concentrations ranging from 0.1 to 200 ng·mL^{-1}) were prepared freshly by diluting stock solutions with LC mobile phase. Chromatographic grade acetonitrile (ACN), methanol (MeOH), and formic acid were obtained from Merck, Co. Inc. (Darmstadt, Germany). High purity water with a resistivity of 18.2 MΩ·cm was obtained using a Milli-Q system (Millipore, Bedford, USA).

2.2. Sample preparation.

A total of 60 batches of sample (*M. melanioides*) were caught in Jinghang Grand Canal, Hangzhou, at different collecting sites. They were authenticated by Dr. Haixing Wang (Zhejiang Province Key Lab of Anesthesiology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University). All the samples were handled carefully to reduce stress and humanely killed using liquid nitrogen freezing. The tissue (100 g) was collected and homogenized using an electric blender (company name, city, country). 2 g of the homogenate was put in an oven and heated to 80 °C for 5 min. Then, the homogenate underwent ultra-sound assisted extraction in triplicate with 10 mL aqueous methanol (methanol/water/TFA, 80/19.9/0.1, v/v/v) for 15 min using a Model VCX-130 ultrasonic probe (20 kHz, 130 W, 50% amplitude power) (Sonics & Materials Inc., Newton, USA). After extraction, the mixture was centrifuged at 9000 r/min for 10 min to separate liquid phase, while the solid residue was re-extracted for another two times. The liquid extracts were combined and condensed to 1 mL by nitrogen flow for further analysis.

2.3. Solid phase extraction

The crude extract was cleaned-up using a solid-phase extraction HLB cartridge (500 mg/6 mL). The cartridge was rinsed with 5 mL of ultrapure water and vacuum-dried before the samples were eluted with 5 mL of acidified aqueous methanol (methanol/water/TFA, 80/19.9/0.1, v/v/v). The resulting eluate was passed through a 0.22 µm filter (company name, city, country) and concentrated to 1 mL under nitrogen prior to microcystins detection.

2.4. HPLC conditions

Result from the shotgun method were compared with those from the recommended HPLC-MS/MS method, which was performed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA). Microcystins were separated on a XSelect HSS T3 column (2.1 mm × 150 mm, 3.5 µm I.D., Waters, USA). The mobile phase consisted of water (A) and acetonitrile (B) (both containing 0.1% formic acid (v/v)). The flow rate was 0.3 mL·min^{-1}. The injection volume was 5 µL. After each run, the column was equilibrated for 5 min to the initial conditions. The temperature of column and sample were kept at 30 and 40 °C, respectively. A linear gradient elution program was applied as follows: initial, 25% B; 1.0 min, 25% B; 5 min, 95% B; 9 min 95% B; 10 min, 25% B; 16 min, 25% B. The run time was 16 min.

2.5. MS/MS parameters

The 4000 QTrap system was operated using an electrospray ionization (ESI) interface in positive mode as follows: voltage of the turbo ion spray source was 4500 V and the temperature was kept at 550 °C. The nitrogen gas pressures for GS1 and GS2 source gases, curtain gas, and collision gas were 3.45×10^5 Pa, 2.76×10^5 Pa, 2.42×10^5 Pa, and "medium", respectively. For each microcystin, the declustering potential (DP) and collision energy (CE) were optimized separately using a manual tuning procedure. The time window for each ion transition was 30 ms, and the total cycle time for all ion transitions was 1 s. Instrument control and data acquisition were accomplished using Analyst software version 1.5.1 (ABSciex, Foster City, CA).

2.6. Ethics statement

The study has been approved by the Ethical Committee of the Zhejiang Gongshang University. All the samples (*M. melanioides*) were handled carefully to reduce stress and humanely killed as soon as possible after capture.

3. Results and discussion

3.1. Ionization state and effect of acid

The microcystins standard working mixture was injected into the ESI source using a syringe pump at a flow rate of 10 µL·min^{-1} for Q1 scanning, product-ion scanning, and PreIS. Full scans of microcystins, under the positive ESI mode, were acquired for the selection of precursor ions, according to the relative intensities of multiple charged ions. As shown in Fig. 1, microcystins can be ionized in both singly and doubly charged forms. Fig. 1A shows the doubly charged microcystins in the *m/z* range 450–600. The peak *m/z* (520.3) was dominant, followed by peaks at *m/z* 531.3, *m/z* 509.8, and *m/z* 534.8, which were identified as $[RR + 2H]^{2+}$, $[RR + H + Na]^{2+}$, $[LR + H + Na]^{2+}$, and $[YR + H + Na]^{2+}$, respectively. Because of the basic guanidine group on the side chain, Arg-containing microcystins (microcystin-RR, YR, and LR), especially microcystin-RR, which contains two Arg residues, were easily ionized to protonated ions, sodium, and/or potassium adducts, and dominated the mass spectrum. The other four microcystins -LF, LA, LY, and LW were also doubled charged with comparatively low

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