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Analysis of trace microcystins in vegetables using matrix solid-phase dispersion followed by high performance liquid chromatography triple-quadrupole mass spectrometry detection

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ABSTRACT

A selective and sensitive method for the simultaneous determination of five common microcystins (MC-LR, -RR, -YR, -LW, -LF) in various vegetables was established using Matrix Solid-Phase Dispersion (MSPD) followed by high performance liquid chromatography-mass spectrometry detection. To optimize the pretreatment procedure for extracting the microcystins from three vegetable matrices (tomato, cucumber and spinacia, which represented different colors), Box-Behnken design was employed to optimize the main factors affecting the extraction efficiency, including sample/dispersant mass ratio, as well as the proportion mixture dispersant and the volumes of solvent. Based on the optimum conditions, the intra-day and inter-day variability for each microcystins in all vegetable samples were less than 8.6%, with the recoveries ranging from 71.9% to 96.5%. The limits of detection and quantitation of the method were 13.0 μg/kg (dw) and 43.0 μg/kg (dw), respectively. The established method was successfully applied to the analysis of MCs in vegetable samples which were collected by Lake Taihu. The procedure promising to be relative small sample size and short pretreatment time for the assay that can be used for monitoring MCs concentrations in vegetables.

1. Introduction

Lake Taihu is the third largest freshwater lake in China. With the rapid economic development over the past 20 years, Lake Taihu has been subjected to serious eutrophication, which has caused frequent outbreaks of cyanobacteria blooms every year. Cyanotoxins, produced by cyanobacteria, have become increasingly perceived as a global water-quality issue. Due to their increasing severity, cyanobacterial blooms have become a major problem in freshwater lake ecosystems all over the world [1]. Among cyanotoxins, microcystins (MCs) are the most widespread and concerned category, which have costed most study efforts [2]. In the molecular structure of MCs, more than 90 isoforms have been isolated and identified to date, among these, MC-LR is the most toxic and common studied variant [3,4]. MCs are stable cyclic heptapeptides with the structure of cyclo (-D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha), in which X and Z are variable L-amino acids to the molecule [5]. Multiple investigations have confirmed that MCs are primarily hepatotoxins that can cause apoptosis, necrosis, hemorrhage, and also inflammation of hepatocellular carcinoma which mainly based on the inhibition of protein phosphatases in aquatic animals and higher plants and the effects on cell signaling pathways [6–8]. Microcystins can accumulate in the environment because of their stability (with half-lives ranging from days to weeks) [9–11]. Water supply reservoirs used for irrigation are sometimes contaminated with toxic cyanobacteria and may contain high concentrations of MCs [12–15]. Recent studies have shown that MCs can be retained and accumulated in terrestrial plant tissues when irrigated with contaminated water [16–20]. In response to the threat of this exposure, the World Health Organization recommends a human Tolerable Daily Intake (TDI) limit of 0.04 μg MC-LR kg^{-1} body mass d^{-1} [21–25].

In the analysis of MCs, the key procedures are the extraction, cleanup, and preconcentration of the samples. Various sample preparation techniques have been employed for the extraction of MCs, such as liquid–liquid extraction (LLE) and solid phase extraction (SPE). A review of the literature reveals only solid–liquid extraction (SLE) combined with SPE clean-up for determination of MCs residues in vegetables [26–30]. C_{18} cartridges and Oasis HLB cartridges were the commonly used cartridges for the extraction of MCs. Although these methods can obtain satisfactory results, the SPE procedure requires multistep, time-consuming operations and the commercial SPE cartridges are expensive. The matrix solid-phase dispersion (MSPD) technique is a well-known preparation method that comprises

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sample homogenization, cellular disruption, fractionation and purification in a single process. Additionally, it has been pointed out that MSPD requires only a small sample size as well as a small amount of solvent [31–34].

In this study, an effective and efficient strategy for developing an extraction methodology is using experimental design methods. Design of Experiments (DOE) is one of the numerous applications for an assessment and improvement of method robustness, the effect of each factor is measured simultaneously in combination with other factors via a multivariate method. It evaluates the small changes influence in parameter values around a specified set of conditions. Therefore, all interactions among several factors should be taken into account and the reported factor effects should be an average value for the whole domain, representing the experimental design space more completely [35]. Several statistical DOE methods, such as Box-Behnken design (BBD), Fractional Factorial design (FFD), Central Composite design (CCD) and Plackett-Burman design (PBD) were applied for chromatographic method development and robustness testing. BBD can be used to perform the optimization for the separation step of chromatographic methods in pharmaceutical analysis. In a recent study the application of BBD in analytical chemistry is still rare [36]. FFD can be applied to optimize the important factors in sample preparation procedure for fast screening, it is very efficient for reducing the number of experimental analyses and particularly favorable when there exist limitations that can be performed high cost or small amount of samples [37–39]. CCD delivers high quality predictions in studying linear, quadratic and interaction effect factors which influence a system, while interactions are unobserved in the normal orthogonal design and single factor tests. This design can still be estimated to performs well in case of loss one or two observations, and parameters of the assumed model without much loss of efficiency [40]. The PBD was preliminary applied for the detection of significant factors on method efficiency, as interaction effects are assumed to be negligible and only main effects are estimated. It was proved to be an efficient tool with good precision for robustness study selecting the critical factors with a small number of experimental analyses for analytical method validation [41].

However, to the best of our knowledge, few studies have reported the enrichment of MCs in vegetables by using MSPD. Therefore, the purpose of this study was to develop a sensitive, accurate, and facilitated method for the determination of five MCs (included MC-LR, MC-RR, MC-YR, MC-LW, and MC-LF) in vegetables.

2. Experimental

2.1. Reagents and instruments

The microcystin standard (MC-RR, LR, YR, LW, LF, respectively, ≥ 95%) were bought from Enzo life sciences, Inc (New York, USA). Methanol (HPLC grade) was purchased from Merck KGaA (Germany). The solid-phase materials graphitized carbon black (GCB) and primary secondary amine (PSA) used for the matrix solid-phase extraction were obtained from Agela Technologies (Tianjin, China). Formic acid (> 99%) and Trifluoroacetic acid (TFA) (> 99%) were purchased from Aladdin Reagent Company (Shanghai, China). Deionized water was obtained from a Milli-Q waters system (Millipore, Bedford, USA). All samples were lyophilized by using vacuum-freezing drying equipment (ZL-12TD, ZOLLO, China) and weighed using precision balances with 0.1 mg accuracy electronic balance (Metler Toledo AL104, Shanghai, China). A MX-S basic vortex mixer (Sci Logex, USA) and a high-speed refrigerated centrifuge (CT18RT, TECHCOMP, China) as well as nitrogen evaporator (MODEL 5085, Organomation Associates Inc, USA) were used for the sample pretreatment. An Agilent 1260 HPLC system (Agilent Technologies, USA) coupled to an API4000 ESI-TQMS (AB SCIEX, USA) was used to detect MCs.

All the vegetable samples were collected by Lake Taihu.

2.2. Preparation of solutions

To prepare the individual stock solution of microcystins, 100 μg of each microcystins was dissolved in 100 mL of methanol and stored at −20 ℃. A mixed standard working solution of five MCs was prepared every day before the quality control assays by diluting each stock solution to a series of appropriate concentrations with methanol and ranging from 5.00 to 100.0 μg/L.

2.3. Sample preparation

The tested samples, including spinach, cucumber and tomato, were chosen to represent different color depths of vegetables, with moisture contents of 70.8–90.2%. They were collected and immediately transported to the laboratory under cool conditions, and then the fresh vegetables were cleaned, lyophilized, and ground to powder. 20.0–200.0 mg of powder was weighed (accurate to 0.001g) in a centrifugal tube which contained a certain amount of absorbent and MeOH, then vortex-mixed for 1 min (2000 rpm) followed by centrifugation at 10,000 rpm for 5 min. The supernatant was collected and then evaporated under dry nitrogen stream at room temperature till dryness. The residue was reconstituted in 1.00 mL MeOH and the solution was filtered through a 0.22-μm micropore membrane and then transferred into sampler vials and ready to be analyzed by HPLC-MS/MS detection.

Microcystin-free vegetables were used as blanks for validation experiments and matrix-matched standard calibrations for quantitation.

2.4. HPLC-MS/MS instrumentation and conditions

Qualitative and quantitative analyses of MCs were performed on an Agilent 1200 HPLC system coupled with a triple quadrupole mass spectrometer in positive ion mode of electrospray ionization. A C_{18} column (250 mm × 2.1 mm i.d., 5 μm, 100 Å Alltima) was operated at 30 °C to separate five MC compounds. The mobile phase solvents A and B were 0.1% formic acid in water and methanol, respectively, and the flow rate was 0.400 mL/min. The optimized gradient elution for solvent B was as follows: 0–1 min, 40%, 1–16 min, 80%, and followed by 4 min for equilibrium with 40% solvent B. The injection volume was 5.00 μL and all the five microcystins were well separated and eluted within 13.0 min.

Direct infusion MS/MS was used to obtain the mass transitions (see Table 1) for multiple reaction monitoring (MRM) of MCs based on their fragmentation patterns.

2.5. Optimization of the MSPD extraction

In this work, the proposed extraction method was based on the MSPD procedure. These target compounds were weakly bonded to the solid phase sorbent and extracted easily by using an elution solvent at room temperature. The sorbent and the elution solvent are the key factors for the extraction efficiency and the purity of the final extracts [42]. Compared with the commonly used adsorbents, two types of dispersants (PSA and GCB) were used to investigate the interference (such as pigment, steroids and fatty acids) in the vegetables which can influence the chromatographic behavior of the analytes. The ratio of PSA/GCB was a key parameter, and four different ratios (see Table 2) were investigated. A previous report has provided the evidence that the mass ratio of sample to dispersant was another parameter influencing the extraction efficiency of investigated compounds [43]. The mass ratio not only dominates the interface area between sample matrix and dispersant but also affects the elution process. Hence, the mass ratios of sample to dispersant (see Table 2) with the amount of samples varying from 0.02 to 0.20 g were studied in detail. For efficient extraction, the elution solvents must be capable of dissolving the target