JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Analysis of Trace Microcystins in Vegetables Using Solid-Phase Extraction Followed by High Performance Liquid Chromatography Triple-Quadrupole Mass Spectrometry

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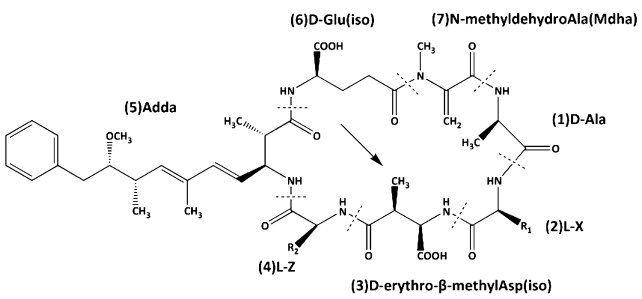
Supporting Information

ABSTRACT: A selective and sensitive method for the simultaneous detection of three common and hazardous microcystins (microcystins-LR, -RR, and -YR) in various vegetables was established using solid-phase extraction followed by high performance liquid chromatography coupled with mass spectrometry. The methanol−water proportion ratio of the extraction solvent and its acidity, as well as the efficiencies of solid-phase extraction, were evaluated to optimize a pretreatment procedure for extracting the microcystins from 10 vegetable matrices. The limits of detection and quantitation were below 7.5 μg/kg (dw) and 25 μg/kg (dw), respectively, in different vegetable matrices. The recoveries of the microcystins in the 10 vegetable matrices ranged from 61.3 to 117.3%, with RSDs of 0.2−18.3%. The established method was used to analyze 28 field vegetable samples collected from the sides of Lake Dianchi, and microcystin-RR was found in almost all samples at concentrations of 36.4−2352.2 μg/kg (dw).

KEYWORDS: *microcystins, vegetable, HPLC-MS/MS, simultaneous analysis, matrix effect*

INTRODUCTION

Microcystins (MCs) are biotoxins generated by cyanobacteria in eutrophic fresh water bodies. Microcystins have the common cyclic peptides 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 that give the name to the molecule (Figure 1); for example, LR, RR, and YR represent -Leu- and -Arg-, two -Arg-, and -Tyr- and -Arg-, respectively. In the molecular structure, Adda, D-MeAsp, and Mdha, respectively, represent (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid, 3-methylaspartic acid, and *N*-methyldehydroalanine.[1,2] More than 80 isoforms of microcystins have been identified. Microcystins are hepatotoxins and tumor promoters due to their strong potent inhibition of protein phosphatases and the effect on cell signaling pathways.



Name	R_1 (X)	R_2 (Z)
MC-LR	Leucine(L)	Arginine(R)
MC-RR	Arginine(R)	Arginine(R)
MC-YR	Tyrosine(Y)	Arginine(R)

Figure 1. General structure of microcystins.

Exposure to hepatotoxin-contaminated water or food can lead to liver failure in animals and fish, as well as to human primary liver cancer.[3−6] The high tumor incidence in some regions of China, such as Haimen City in Zhejiang Province and Qidong City in Jiangsu Province, may be related to long-term exposure of humans to trace microcystins through aquatic product food.[7]

In recent years, with the rapid economic development and the increase in water consumption, water eutrophication occurred frequently in China. Cyanobacterial blooms have become more serious, and almost all major freshwater resources, involved with rivers, lakes, drinking water reservoirs, ponds, and water ditches, are affected.[8−12] Microcystins were detected ubiquitously in surface water bodies at relatively high concentrations, from several μg/L to dozens of μg/L, compared with a water standard limit of 1.0 μg/L in China. Among more than 80 variants of MCs, MC-LR, one of the most lethal toxins,[13] as well as MC-RR and MC-YR are the three most common and dangerous forms found in cyanobacterial blooms in freshwaters.

Received: July 11, 2014
Revised: November 13, 2014
Accepted: November 13, 2014
Published: November 13, 2014

dx.doi.org/10.1021/jf5033075 | *J. Agric. Food Chem.* 2014, 62, 11831−11839

MCs in aquatic environments may be transferred to farmland through agricultural irrigation using toxin-contaminated water and fertilization using harvested toxic cyanobacterial paste.[14] In order to reduce the water contamination, thousands of tons of cyanobacterial blooms have been mechanically salvaged from the eutrophicated water in the past decade in China. Some cyanobacterial blooms from this collection were discharged as organic fertilizers directly onto the farmlands near the waters. Following transfer of the cyanobacterial blooms and the toxin-contaminated water to farmlands, a considerable quantity of toxins is released to farmlands. High levels of MCs, ranging from 2.1 to 6.6 ng/g and 1.4 to 21.3 ng/g, were detected in the cropland soil near Taihu Lake and Dianchi Lake in China, respectively.[15,16]

Previous studies have shown that the morphological characteristics and the productivity of crop plants were affected when exposed to microcystins.[17,18] Furthermore, the exposure of edible crops to microcystins would cause human health risks, as terrestrial crops can take up and accumulate these compounds in the roots, stems, and leaves.[19−21] MCs accumulation in crops (such as vegetables) irrigated with toxin-contaminated water or fertilized with toxic cyanobacterial blooms could introduce MCs to the human food chain and consequently pose a hazard health risk to humans. Recent studies have reported that the consumption of vegetables (for example, salad lettuce) is another important exposure route of MCs to humans, in addition to aquatic products and drinking water exposure.[20,22,23]

The increasing awareness of the human health hazard and occurrence of cyanobacterial toxins in plants highlights the need for highly sensitive and selective analytical methods to examine MCs in plants. The methods commonly used to examine MCs can be categorized as those used for screening and quantitation. The former consisted of enzyme-linked immunosorbent assays (ELISA) and protein phosphatase inhibition assays (PPIA), which detect MCs in samples with high sensitivity. However, they cannot discriminate among MC analogues. The latter methods utilized high performance liquid chromatography (HPLC) combined with ultraviolet (UV) or mass spectrometric detection. Compared to ELISA and PPIA, the recently developed reverse-phase HPLC method can separate toxins and identify each microcystin variant. HPLC with a UV detector has been used for the identification and quantitation of microcystins through the characteristic UV spectra absorption of conjugated Adda diene, trypotophan, or tyrosine at 238, 223, or 232 nm, respectively.[24] However, identifying the target MCs based on chromatographic retention time alone is prone to false positives due to the complicated sample matrices and the similar UV spectra among MCs, which can also interfere with quantitation. Furthermore, the sensitivity of HPLC-DAD is not sufficient to detect trace amounts of MCs in plants.

HPLC coupled with mass spectrometry is an effective and powerful technology to detect the trace organic toxins in complex matrices, as it can provide the necessary sensitivity and selectivity. The feasibility of HPLC−MS/MS used to identify and quantitate trace amounts of microcystins in water and aquatic products, such as fish, shrimps, clams, and crabs, has been described in previous reports.[25−28] However, to our knowledge, simultaneous assays of trace amounts of microcystin variants in vegetable matrices using HPLC−MS/MS have not been reported. In previous studies, the detection of MCs in vegetables was performed using ELISA or HPLC-UV methods, which analyzed total MCs and did not distinguish among MC variants.[17,18]

This study was performed to establish a reliable, sensitive, and convenient method for the determination of MC variants in vegetables. The microcystins selected in our study were the toxins detected most frequently in previous environmental analyses, which included MC-LR, MC-RR, and MC-YR.

Overall, a sensitive and selective method was established for the simultaneous analysis of microcystin variants in various vegetable matrices using solid-phase extraction and HPLC-MS/MS in positive-ionization mode. This method comprised sample treatment procedures and methodologies, was validated with accuracy, precision, and matrix effect, and was applied to assay the MCs in real vegetables with acceptable results.

MATERIALS AND METHODS

Reagents. The microcystin standards were bought from Taiwan Algal Science (Taoyuan, Taiwan) and Enzo Biochem (NewYork, NY), respectively, with purities >95%. Single and mixture microcystin standard stock solutions were prepared in methanol and stored in a refrigerator. Matrix-matched standard solutions were generated by adding serially diluted standard mixture to the microcystin-free (blank) vegetables (lettuce, water spinach, cabbage, choi sum, carrot, turnip, potato, pumpkin, cucumber, and eggplant). The microcystin-free vegetables detected without the target micocystins were purchased from an organic vegetable production base, which was irrigated with clean water and fertilized without the paste of cyanobacterial bloom. All the standard solutions were stored in a refrigerator and used within 3 months.

HPLC-grade acetonitrile and methanol were purchased from Fisher Co. (Fair Lawn, NJ). The solid-phase extraction (SPE) system with 24-port vacuum manifolds was bought from Sigma-Aldrich (St. Louis, MO). The Sep-pak C_{18} cartridges and Oasis HLB were obtained from Waters Corporation (Milford, MA). The cartridge was pretreated with 3 mL of methanol and 6 mL of ultrapure water sequentially before sample extraction. Trifluoroacetic acid (TFA) (≥99%) was purchased from Aladdin Reagent Company (Shanghai, China). All other reagents were analytical grade. Ultrapure water used throughout the entire analytical process was obtained from a Unique-R20 system (Research Scientific Instruments Corporation, Xiamen, China).

Instrument and Apparatus. The Applied Biosystems API 4000Q-Trap triple-quadrupole mass spectrometer (Applied Biosystems, FosterCity, Canada) equipped with an electrospray ionization (ESI) source was used in this study. The liquid chromatography system was an Agilent 1100 system equipped with a G1379A degasser, G1311A binary pump, G1313A autosampler and G1316A column oven (Agilent Technologies, Palo Alto, CA). HPLC separation was performed using a 150 mm × 2.1 mm i.d., 5 μm, Eclipse Plus C_{18} column (Agilent Technologies, PaloAlto, CA).

Recommended Procedure. The tested vegetable samples (lettuce, water spinach, cabbage, choi sum, carrot, turnip, potato, pumpkin, cucumber and eggplant), with moisture content of 80.9−95.4%, were collected and immediately transported to the laboratory under the cool conditions, then the fresh vegetables were lyophilized and ground to powder (0.45 mm). Two g (accurate to 0.01 g) of the lyophilized powder of vegetable sample was extracted using 10 mL of acidified methanol aqueous solution (methanol/water/TFA, 80/19.9/0.1 or 80/19/1, v/v/v) by 20 min of vortexing

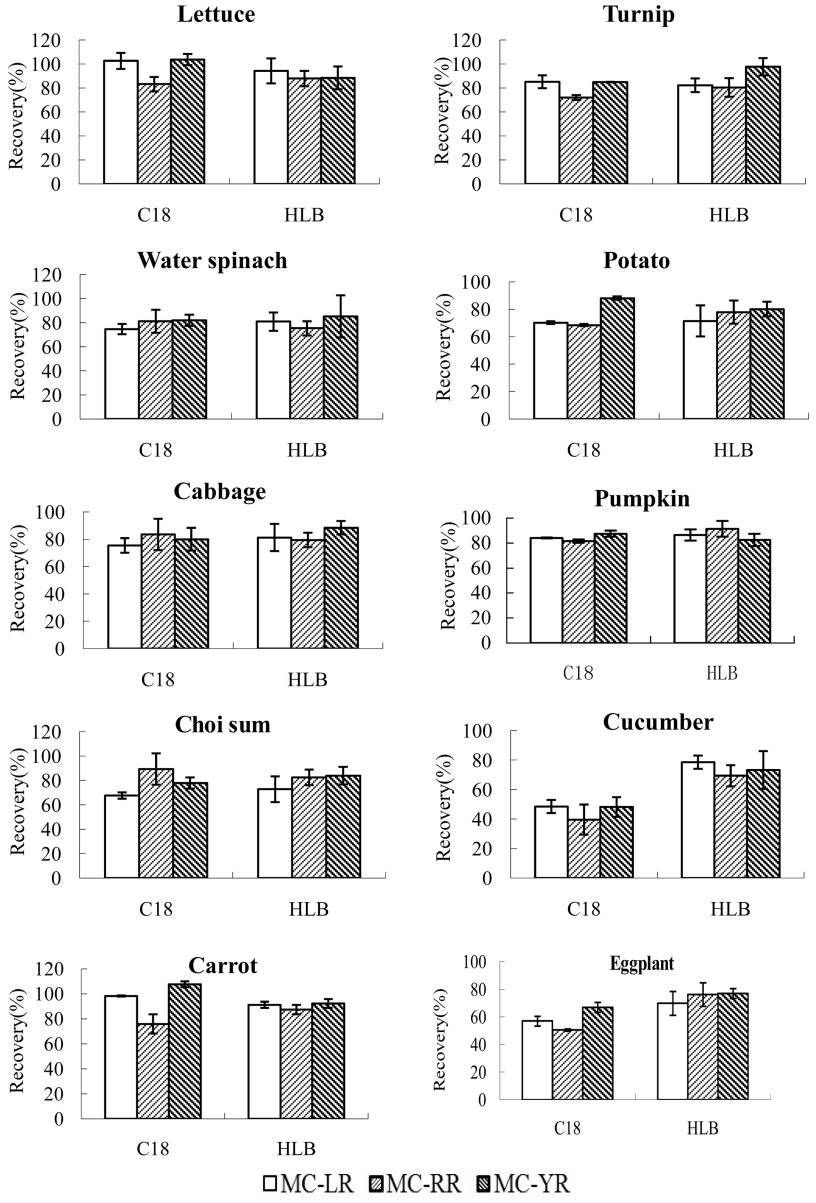


Figure 2. Effect of sorbents (C_{18} and HLB) on the recovery of microcystins in different vegetables (n = 5).

extraction and 10 min of ultrasonic extraction, followed by centrifugation at 10000 rpm and 10 °C for 10 min. The supernatant was collected and the vegetable residue was extracted again with 10 mL of acidified aqueous methanol. The supernatants were combined and concentrated to ~2 mL using a rotary evaporator below 40 °C under reduced pressure. The obtained extract was introduced into the C_{18} (HLB cartridge was used for cucumber and eggplant sample) cartridge (500 mg/6 mL) for solid-phase extraction cleanup. The effluent was collected and reintroduced into a C_{18} (or HLB) cartridge for re-extraction. Then the cartridge was rinsed with 5 mL of ultrapure water and vacuum-dried. It was then eluted with 5 mL of acidified aqueous methanol (methanol/water/TFA, 80/19.9/0.1, v/v/v) and the resulting eluate was filtered through a 0.22 μm filter and concentrated to 1 mL using a nitrogen stream for LC-MS/MS detection. Quantitation was performed using matrix-matched standard calibrations.

Microcystin-free vegetables were used as blanks for validation experiments and matrix-matched standard calibrations. Fortification of the vegetable samples for recovery studies was carried out by delivering 0.4 mL of mixed microcystin standard solutions at 0.1, 1, and 5 μg/mL in methanol to 2 g of lyophilized vegetable samples to obtain 20, 200, and 1000 μg/kg dw fortification levels (roughly equivalent to 1, 10, and 50 μg/kg fresh weight fortification levels, respectively). The fortified samples were left for 12 h at room temperature prior to the sample extraction procedure.

Chromatographic and Mass Spectrometric Conditions. *HPLC Operating Conditions.* The mobile phase consisted of acetonitrile (solvent A) and water containing 0.2% (v/v) formic acid (solvent B), the flow rate was 0.3 mL/min. The optimal gradient program started with 20% acetonitrile at the time of injection and increased linearly to 80% acetonitrile over 2 min, which was kept for 4.5 min, and then brought back to 20% acetonitrile over 0.1 min and held for 9.4 min until the next injection. The column oven temperature was maintained at 35 °C. The sample injection volume was 5 μL. All the three microcystins were eluted within 6.0 min.

dx.doi.org/10.1021/jf5033075 | J. Agric. Food Chem. 2014, 62, 11831−11839

MS Operating Conditions. The ESI source in positive multiple-reaction-monitoring (MRM) mode was used for the identification and quantitation of the three microcystin variants. The monitoring parameters were optimized as follows: ion source spray voltage (IS), 5500 V; atomization temperature (TEM), 450 °C; atomization gas pressure (GAS1), 55 psi (nitrogen); heated auxiliary gas (GAS2), 50 psi (nitrogen); air curtain gas pressure (CUR), 25 psi (nitrogen); collision flow ratio (CAD), high.

Data Analysis. Microcystin concentrations from LC-MS/MS analysis were determined using Analyst software(version 1.4, Applied Biosystems). The data were exported to Microsoft Excel 2003 (Microsoft Co., Redmond, WA, USA) to calculate the average and to perform statistical (ANOVA) analysis.

RESULTS AND DISCUSSION

Optimization of Sample Pretreatment. Ten vegetables—lettuce, water spinach, cabbage, choi sum, carrot, turnip, potato, pumpkin, cucumber, and eggplant—were selected for the study to represent tropical and subtropical vegetables commonly consumed in China. Ten vegetables were chosen to examine the matrix effects of each vegetable on the determination of MCs in vegetables because the matrix components in the samples can affect the analyte signals. It is believed that the same species of vegetables should have similar matrix effects. Thus, lettuce, water spinach, cabbage, and choi sum could represent green leafy vegetables with high content of chlorophyll.[29] Carrot, turnip, and potato were the representatives of root vegetables, while pumpkin, cucumber, and eggplant represent fruit vegetables.

Microcystin variants in vegetable samples were usually present at trace or even ultratrace amounts, and the vegetable matrix effect may interfere with the HPLC separation and reduce the sensitivity of the mass spectrometry detector; therefore, analyte enrichment and sample cleanup were required prior to HPLC-MS/MS analysis. During the sample treatment process, lyophilization was used to dehydrate the vegetables and concentrate microcystins, with less loss of analyte under vacuum and cool conditions. Furthermore, the volumes of reagents used for sample extraction decreased by 80% compared to the fresh vegetable samples, which could also reduce the time required for the extract to pass through the cartridge.

Previous studies suggested that an acidified aqueous methanol solvent was suitable for the extraction of both hydrophilic and hydrophobic microcystins.[30] Therefore, the extraction efficiencies of solvents at several methanol−water ratios (60%, 80%, and 100% methanol) and acidities (1%, 0.1%, and 0.01% TFA, v/v) were evaluated.

Extraction with 80% methanol provided recoveries higher than 67.6% in all vegetable samples for the three target MCs, while extraction with 60% and 100% methanol yielded low recoveries of 42.6% and 45.3% for MC-LR, 48.1% and 48.8% for MC-RR, and 44.4% and 45.5% for MC-YR in the vegetable samples, respectively. In most of the selected vegetable matrices, the highest extraction efficiencies for the three microcystins were obtained by addition of 0.1% TFA (v/v) in aqueous methanol (19.9/80, v/v) solution, while the highest extraction efficiencies of MC-LR in potato and choi sum, of MC-RR in cucumber and pumpkin, and of MC-YR in water spinach and cabbage were obtained by addition of 1% TFA (v/v) in aqueous methanol.

In the sample cleanup and preconcentration step, two commonly used sorbents, Oasis Sep-Pak C_{18} and HLB cartridges, which could simultaneously retain nonpolar to moderately polar toxins from complicated matrices,[31] were tested in various vegetable matrices to optimize extraction and concentration efficiency. As shown in Figure 2, both recovery and RSD were satisfied when Sep-Pak C_{18} or Oasis HLB was used in lettuce, water spinach, cabbage, choi sum, carrot, turnip, potato, and pumpkin samples. However, the C_{18} cartridge was selected as a sorbent for the above-mentioned eight species of vegetable because it was relatively cheap compared with the HLB cartridge. Nevertheless, microcystin recoveries were very poor (less than 60%) when the C_{18} cartridge was used for cucumber and eggplant samples, suggesting that interfering impurities in the matrices were not completely eliminated. Otherwise, when HLB was used in these matrices, both recoveries (>70%) and RSD were acceptable in terms of removing pigments from cucumber and eggplant (Figure 2). Therefore, C_{18} (500 mg/6 mL) was used to clean up lettuce, water spinach, cabbage, choi sum, carrot, turnip, potato, and pumpkin samples, while HLB (500 mg/6 mL) was chosen to purify cucumber and eggplant in the study.

In the elution step, the retained microcystins should be completely eluted using as little volume of eluent as possible. Microcystins are relatively polar molecules with hydrophobic regions, such as the Adda residue or hydrophobic substituents of amino acids. Some investigations found that the efficiency of eluting solvents depended on various factors, such as the water content of the sample and eluent polarity.[32,33] Thus, various aqueous/methanol ratios from 10% to 100% methanol were tested for elution of the adsorbed microcystins from the cartridges. When the solutions (5 mL) contained less than 50% methanol, they could not remove the target microcystins. The pure methanol resulted in more efficient elution of MC-LR and MC-YR, but the yield of MC-RR, a strong polar, was not sufficient. However, the desired elution yields of the three microcystin variants were obtained when using 80% methanol. Therefore, 5 mL of 80% methanol aqueous solution was optimal to elute the tested microcystin variants from the cartridges.

Liquid Chromatography. The reverse-phase HPLC system with a C_{18} stationary phase and an acetonitrile in the water mobile phase is a typical method of separating microcystin variants.[34] Two Agilent Eclipse Plus C_{18} columns, both 150 mm × 2.1 mm i.d., but with different particle sizes of 5 and 3.5 μm were compared for separation of the target microcystins. A better reproducibility and sensitivity of the three target microcystins was observed when separated with the 5 μm particle size column compared to the 3.5 μm particle size column. Thus, the Agilent Eclipse Plus C_{18} column with 5 μm particle size was selected.

The mobile phase, which consists of acetonitrile gradients in the presence of organic acid, such as formic acid, is capable of separating microcystin variants with greater chromatographic efficiency than the neutral mobile phase because the acid could improve the protonation of carboxylic acid groups in the toxins and decrease the interactions between basic groups of the microcystin molecule and the silanol groups coated on the particle surface in the column.[35] The effects of the formic acid addition ratio in the mobile phase on the MCs defect sensitivity were evaluated. It can be found that a mobile phase containing 0.2% formic acid provided the highest sensitivity. Under this

condition, the liquid chromatography elution order was MC-RR, MC-YR, and MC-LR.

Mass Spectrometry. Individual standard solutions of each microcystin were infused into the ESI source to create an ion transition under the positive-ionization mode. In the full-scan mass spectrum, the protonated molecules ($[M + H]^+$) of m/z 996.1 and 1046 were produced by MC-LR and MC-YR, respectively, and the double-charged protonated molecular ion $[M + 2H]^{2+}$ of m/z 520 was produced by MC-RR, which contains two basic Arg residues in the molecule. The protonated molecule was selected as a precursor ion and was subjected to fragmentation. The fragment ions of m/z 135 and 213 were the major product ions for the three target microcystins and used as the quantitative and qualitative ions, respectively. The product ion mass spectra of MC-LR, MC-RR, and MC-YR are shown n Figure 3. The fragment ion of m/z 135 was identified as the $[\text{phenyl-CH}_2\text{–CH(OCH}_3)]^+$ ion formed by cleavage of the Adda residue between C-8 and C-9, and m/z 213 was $[\text{Glu-Mdha+H}]^+$ formed by the cleavage of the α-linked glutamic acid (D-Glu) and *N*-methyldehydroalanine (Mdha) residue.[36] Two major ion pairs for each analyte and the optimal MS/MS parameters for each ion pair are described in Table 1.

Method Validation. *Matrix Effects.* The matrix effect (ME), which impacts the quantitation greatly, depends on various factors, including the instrument, the type and amount of matrix, the treatment procedure, and the properties of the analyte.[37,38] The matrix effect in vegetable matrices was studied by comparing the slopes of the calibration curves of standards in methanol and sample matrices. The following expression was used to quantitate signal suppression or enhancement for each microcystin:

$$ME = 100 \times \left(\frac{slope_m}{slope_s} - 1 \right)\%$$

The slope_m and slope_s represent the slopes of the matrix-matched and solvent-only (matrix-free) calibration curves, respectively. The matrix effect was considered mild when it ranged from −20% to −10% or from +10% to +20%, medium when it ranged from −50% to −20% or from +20% to +50%, and strong when it was below −50% or above +50%. If it ranged from −10% to +10%, the effect could be ignored. These results reveal the suppressive effects of vegetable components on the tested microcystins in the range of −63% to −2%. The matrix effects of three tested microcystins in lettuce (−13 to −5%) and carrot (−10 to −2%) could almost be ignored, but those in water spinach, choi sum, turnip, cucumber, and eggplant matrices showed medium suppression (−50 to −20%). A strong signal-suppressive effect was detected in potato and pumpkin for MC-RR and in cabbage and pumpkin for MC-YR. Generally, matrix effects originated from insufficient elimination of sample endogenous materials such as pigments and fatty acids. The coexistence of the impurities would affect the ionization efficiency and weaken the response of the analytes. Therefore, in order to get more accurate results, the matrix-matched calibration curves should be used for quantitation due to the existence of matrix effects.

Linearity and Limit of Quantitation (LOQ). The linearity and LOQ were obtained using the peak areas of the product ions acquired from the MS/MS mode. The matrix-matched calibration curves were constructed using least-squares linear regression analysis of the matrices (lettuce, water spinach, cabbage, choi sum, carrot, turnip, potato, pumpkin, cucumber, and eggplant) spiked with MC-LR, MC-RR, and MC-YR at 5–2500 ng/mL. The correlation coefficients (R^2) were higher than 0.9876 in all cases, and the limit of detection (LOD) was below 7.5 μg/kg, while the LOQ was 1.6–10.0 μg/kg (dw), 6.3–25.0 μg/kg (dw), and 5.6–25.0 μg/kg (dw) for MC-LR, MC-RR, and MC-YR in different vegetable matrices, respectively. Regarding specificity, no responses were detected in the

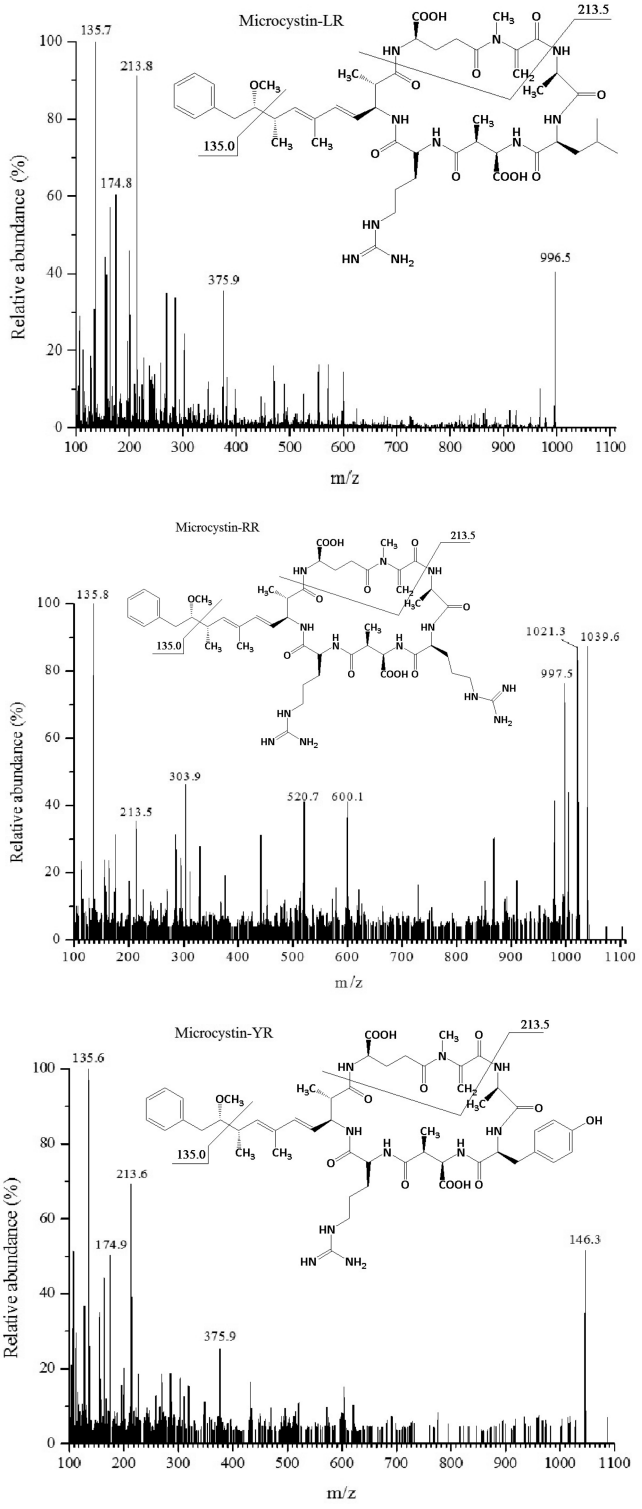


Figure 3. MS/MS spectra of the three target microcystins.

dx.doi.org/10.1021/jf5033075 | J. Agric. Food Chem. 2014, 62, 11831–11839

Table 1. Mass Spectrometry Characteristics of the Target Microcystins as Determined Using LC-MS/MS

Compound	Mol wt	Retention time (min)	Precursor ion	Product ion	DP[a]	CE[b]
Microcystin-LR	995.2	5.5	996.1 $[M + 1]^+$	135[c]/ 213[d]	96/91	100/85
Microcystin-RR	1038.2	5.2	520 $[M + 2]^{2+}$	135/213	90/90	56/56
Microcystin-YR	1045.2	5.4	1046 $[M + 1]^+$	135/213	150/150	100/90

[a]DP, decluster potential. [b]CE, collision energy voltages. [c]Quantitative ion. [d]Qualitative ion.

Table 2. Recoveries and RSD for the Target Microcystins from Different Matrices at Three Fortified Levels

		MC-LR			MC-RR			MC-YR		
Matrix	Fortification level (μg/kg dw)	Recovery (%)	RSD[a] (%)	RSD[b] (%)	Recovery (%)	RSD[a] (%)	RSD[b] (%)	Recovery (%)	RSD[a] (%)	RSD[b] (%)
Lettuce	20	61.3	9.1	9.8	83.9	11.5	12.1	116.1	6.4	6.9
	200	102.6	6.6	8.7	83.2	6.1	8.2	103.8	4.7	5.3
	1000	95.2	4.7	7.3	92.5	4.8	5.9	93.3	5.0	5.8
Water spinach	20	85.5	5.8	4.2	62.1	16.4	13.8	91.2	0.8	4.9
	200	74.7	4.3	6.9	81.2	9.6	9.7	82.0	4.7	8.5
	1000	77.6	7.2	6.8	65.6	3.5	10.1	81.8	8.8	8.5
Cabbage	20	78.4	3.4	5.5	117.3	4.3	6.7	97.0	16.7	12.3
	200	65.4	5.3	9.4	83.5	11.5	9.9	79.9	8.3	13.5
	1000	82.2	6.8	10.2	70.4	6.9	7.9	82.5	7.8	9.1
Choi sum	20	102.5	0.6	6.7	79.4	7.9	8.5	84.0	17.9	11.6
	200	67.6	2.6	3.8	89.3	12.8	10.5	67.8	4.6	18.3
	1000	68.3	2.1	11.2	70.0	9.1	11.4	68.0	2.9	10.9
Carrot	20	107.9	8.5	4.4	85.9	11.2	10.9	100.3	7.4	11.3
	200	98.3	0.5	3.9	76.0	7.7	9.8	107.6	2.3	5.6
	1000	89.3	4.0	5.0	92.7	11.4	8.9	111.4	4.0	6.3
Turnip	20	81.7	4.7	5.1	72.2	6.8	9.9	86.7	5.4	9.2
	200	85.2	5.4	3.8	72.1	2.0	6.9	84.9	0.2	2.1
	1000	72.9	2.6	8.1	71.5	3.5	7.2	81.5	0.7	1.6
Potato	20	70.2	5.3	2.4	68.9	4.5	6.4	95.3	7.3	5.6
	200	70.2	11.0	9.5	68.4	0.8	2.1	88.1	1.2	4.1
	1000	74.1	4.3	5.8	79.6	2.9	2.9	78.2	1.6	7.5
Pumpkin	20	93.7	9.8	9.2	77.8	9.3	10.4	92.9	4.9	6.5
	200	84.0	0.4	4.3	81.5	1.4	3.6	87.5	2.4	3.2
	1000	91.7	3.1	11.6	87.3	5.4	6.9	90.7	2.9	4.6
Cucumber	20	74.1	4.3	8.9	79.6	2.9	5.4	78.2	1.6	2.4
	200	78.5	4.5	9.4	69.4	1.2	7.1	73.2	12.8	14.3
	1000	76.1	4.4	6.6	79.3	4.0	4.2	78.2	6.6	5.9
Eggplant	20	75.0	9.4	8.4	81.9	8.7	8.8	83.9	7.3	9.4
	200	69.9	8.6	8.2	76.3	8.5	9.4	77.0	3.6	5.4
	1000	68.1	0.7	6.7	87.5	7.0	9.1	70.6	0.8	6.9

[a]Intraday precision (n = 5). [b]Interday precision (n = 15).

procedure blank or vegetable sample blank, suggesting the high specificity of MS-MS detection.

Accuracy and Precision. The recovery analysis was performed to evaluate the performance of the proposed method by analyzing 10 species of vegetable samples spiked with 20, 200, or 1000 μg/kg of MC-LR, MC-RR, and MC-YR. The precision of the method was expressed using the intraday and interday relative standard deviation (RSD). The intraday RSD was measured by comparing the standard deviation of the recovery rates of the spiked samples analyzed in the same day. The intraday RSD was determined by analyzing the spiked samples for three distinct days. Table 2 showed the detailed recovery and repeatability data for the microcystins in various vegetable matrices. The recoveries of the microcystins were in the range of 61.3−117.3% (n = 5), and the RSD of the intraday (n = 5) and interday (n = 15) ranged from 0.2−17.9% and 1.6−18.3%, respectively. The MRM chromatograms of the blank and the positive lettuce sample are shown in Figure 4, and this indicates that the proposed method can maintain blanks below a critical threshold.

Method Application. To demonstrate its functionality, the proposed method was applied to determine the field vegetable samples collected from the farmlands irrigated with Dianchi Lake water. Dianchi Lake is China's sixth largest fresh water lake, fed by several rivers and located at 1700 m above sea level in Yungui Plateau in southwest China. This lake is used to irrigate millions of hectares of grains and vegetables in a lush agricultural region in Yunnan Province, and it was formerly one of China's most important freshwater fisheries. With the rapid economic development over the past 30 years, the lake has been subjected to serious eutrophication, which has caused frequent outbreaks of cyanobacteria blooms during years.[39−41]

A total of 28 vegetable samples (8 lettuce samples, 3 water spinach samples, 12 cabbage samples and 5 choi sum samples) with moisture content ranging from 91.4% (water spinach) to 95.4% (lettuce) were collected in the winter of 2013 from

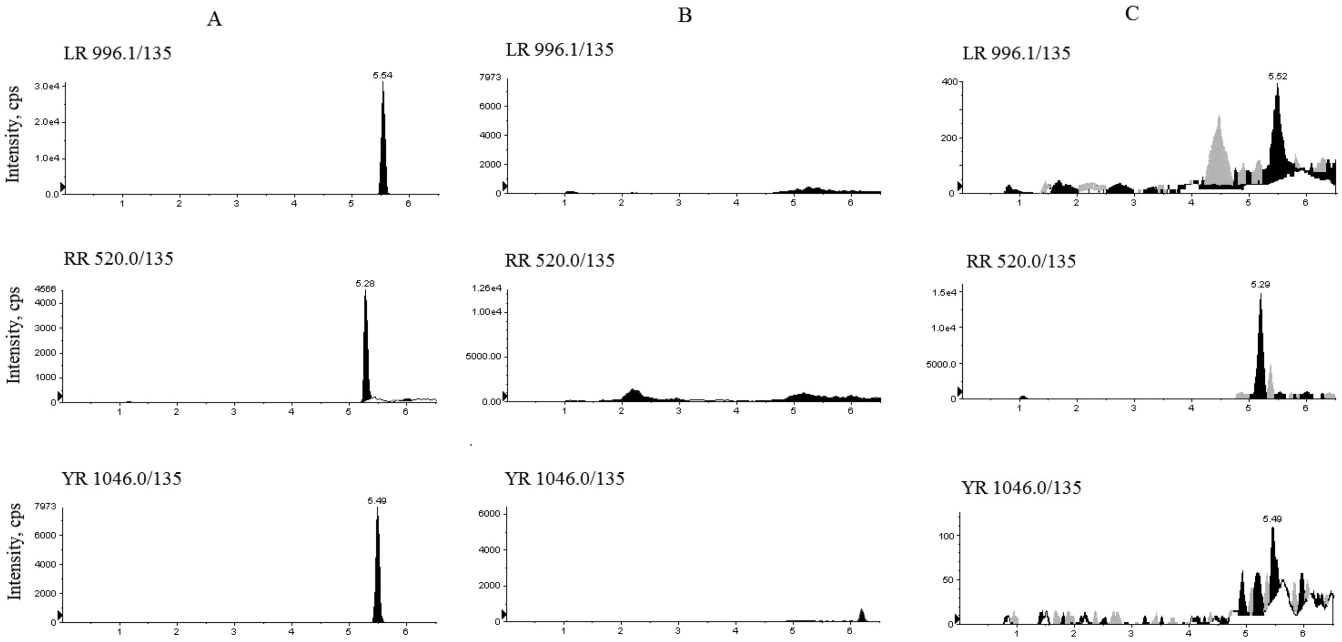


Figure 4. MRM chromatograms of (A) standard solution; (B) lettuce matrix; and (C) positive lettuce sample by HPLC-MS/MS.

Table 3. Concentrations of the Three Microcystin Variants in Vegetables

MC	Vegetable	Moisture content (%)	Sample no.	Positive sample no.	Range (dw[a]/fw[b]) (μg/kg)	Average (dw) ± SD (μg/kg)
MC-LR	Lettuce	95.4	8	0	ND[c]/ND	
	Water spinach	91.4	3	1	23.7/2.0	
	Cabbage	94.9	12	0	ND/ND	
	Choi sum	92.7	5	0	ND/ND	
MC-RR	Lettuce	95.4	8	8	46.1−2352.2/2.1−108.2	973.7 ± 849.4
	Water spinach	91.4	3	3	36.4−765.2/3.1−65.8	298.8 ± 330.6
	Cabbage	94.9	12	10	53.1−394.1/2.7−20.1	138.6 ± 54.4
	Choi sum	92.7	5	4	86.2−553.3/6.3−40.4	196.4 ± 189.0
MC-YR	Lettuce	95.4	8	1	22.8/1.0	
	Water spinach	91.4	3	0	ND/ND	
	Cabbage	94.9	12	0	ND/ND	
	Choi sum	92.7	5	0	ND/ND	
∑MCs	Lettuce	95.4	8	8	ND-2352.2/ND-108.2	
	Water spinach	91.4	3	3	ND-788.9/ND-67.8	
	Cabbage	94.9	12	11	ND-394.1/ND-20.1	
	Choi sum	92.7	5	4	ND-553.3/ND-40.4	

[a]dry weight. [b]fresh weight. [c]not detected.

farmlands along the lake shore, which were irrigated with the lake water. Three quality controls (QCs) at 20, 200, and 1000 μg/kg were included for each vegetable sample in the analysis sequence.

The concentrations of MCs in the analyzed samples are listed in Table 3. The detected frequencies of the target microcystins were 3.6%, 89.3%, and 7.1% for MC-LR, MC-RR, and MC-YR, respectively, in the Dianchi Lake vegetable samples. The most frequently detected microcystin in the vegetable samples was MC-RR, which was consistent with the results reported previously in Dianchi Lake water.[22] All the tested vegetable samples, with the exception of two cabbage samples and a choi sum sample, contained MC-RR at 36.4−2352.2 μg/kg (dw). The vegetable samples with high MC-RR concentrations, exceeding 1000 μg/kg, were cultivated in a greenhouse. One water spinach sample and one lettuce sample contained MC-LR and MC-YR with 23.7 and 22.8 μg/kg, respectively. Similar concentrations were found in various vegetables cultivated around Taihu Lake (the third largest freshwater lake in China) using an ELISA screening method (∑MCs concentration: 30−360 μg/kg).[14]

In summary, a sensitive, selective, and reliable method based on HPLC-MS/MS was developed through stepwise optimization for the individual analysis of three microcystins in common vegetables. This method requires less than 6 min to perform each sample analysis. Using a simple solid-phase extraction procedure consuming only 2 g of lyophilized vegetable samples for cleanup and analyte enrichment, the method can be used to simultaneously determine trace microcystin variants (MC-LR, MC-RR, and MC-YR) in various vegetables at μg/kg levels. The optimized method was applied to analysis of vegetables collected from farmlands irrigated with cyanobacterial bloom water. The results indicated that vegetables were contaminated with microcystins, of which MC-RR exhibited the highest concentration, 2352.2 μg/kg. More research is clearly needed to

evaluate the human health risk exposed to microcystins via edible terrestrial plants such as vegetables in the future.

■ ASSOCIATED CONTENT

Supporting Information

Table S1. Effects of formic acid on the microcystin detection sensitivity of MS. Table S2. Linear range, calibration curves, limits of detection (LOD), and limits of quantitation (LOQ) of microcystins in various vegetables (to be continued). Figure S1. Effect of water/methanol ratio on extraction efficiency of microcystins in different vegetables (n = 5). Figure S2. Effect of acidity on extraction efficiency of microcystins in different vegetables (n = 5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the National Natural Science Foundation of China (41301337, 41173101), Natural Science Foundation of Guangdong Province (2011020003196), the project of Science and Technology of Dongguan City (201210815000399), and the High-Level Talents Program of Guangdong Higher Education Institutions, granted to C.-H.M., Jinan University, and by a Scientific Research Innovation Project for Graduates without Requiring Entrance Examinations, granted to T.-F.S.

Notes

The authors declare no competing financial interest.

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