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Quantitative Analysis of Cyanobacterial Toxins by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

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Microcystins (MCs) are a growing problem in drinking water supplies worldwide. Common analytical techniques used to determine MC concentrations have several shortcomings, including extensive sample handling and lengthy analysis times. A simple, rapid method for quantitation of MCs by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is presented. Four potential internal standards were tested, including an ^{15}N-labeled MC. For MC-LR in mixed standard solutions, a linear range of 0.11−5.0 μM (R^2 = 0.98) was achieved, with a method detection limit (MDL) of 0.015 μM. Matrix effects due to extracted cell components decreased the MC-LR linear range slightly to 0.19−5.0 μM (R^2 = 0.99), with MDL = 0.058 μM. Extensive analysis of possible internal standards indicates that nodularin was preferred over [^{15}N]$_{10}$-microcystin-YR or angiotensin I. The ionization efficiency and analyte−analyte suppression for four MCs of varying polarity are presented; the three polar congeners exhibited good ionization efficiency and acceptable levels of analyte−analyte suppression. These results indicate that MALDI-TOF MS represents a viable alternative for the quantitative measurement of MCs in field samples.

Cyanobacterial blooms are an escalating problem in waters around the world. These organisms often produce toxins that exhibit acute and chronic effects on humans and wildlife through damage to the nervous system and liver.[1] The most common cyanotoxins in freshwater systems are the hepatotoxic microcystins (MCs) produced by several cyanobacterial genera. MCs are cyclic peptides containing seven amino acids, including five that are nonproteinogenic (Figure 1a); at least 90 congeners have been identified to date.[2] Amino acid variations are known at every position, with substitutions at positions 2 and 4 giving rise to the naming system. The congener with leucine (L) at position 2 and arginine (R) at position 4 is known as microcystin-LR (MC-LR). Congeners vary from nontoxic to highly toxic and exhibit molecular weights ranging from 900 to 1120 Da.[3,4]

Analysis of water samples for these toxins is a key component in the public health management of harmful algal blooms. Available analytical techniques for detection and quantitation of MCs include high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assays (ELISA), and protein phosphatase inhibition assays (PPIA). The results must be interpreted with caution, however, since these techniques have limitations. Quantitation via UV detection is inherently problematic due to the differing absorption coefficients of MC congeners (Blom et al.[5]) and the fact that very few standards are available. HPLC, LC−MS, and ELISA provide limited information on potential toxicity of the sample since congeners often generate responses not correlated with their mouse bioassay toxicity.[6−9] Additionally, when HPLC, ELISA, and PPIA have been used to analyze identical samples, the results sometimes show poor agreement between the methods, particularly when congeners other than MC-LR are present.[10−12] A further difficulty affecting all three methods is the need for extensive sample handling and lengthy analysis times. Development of alternative analytical methods could improve our prediction and response to harmful algal blooms.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has the potential to overcome many of these disadvantages. It is an extremely rapid, high-resolution, sensitive technique that requires little sample handling, is tolerant of contaminants, has low sample consumption, and allows identification of toxin congeners. Most compounds form singly charged molecules, offering simpler spectra than electrospray ionization mass spectrometry (ESI-MS) in many cases. Modern software allows high-throughput, multiplexed analyses. These advantages have led researchers to utilize MALDI-MS in the detection of

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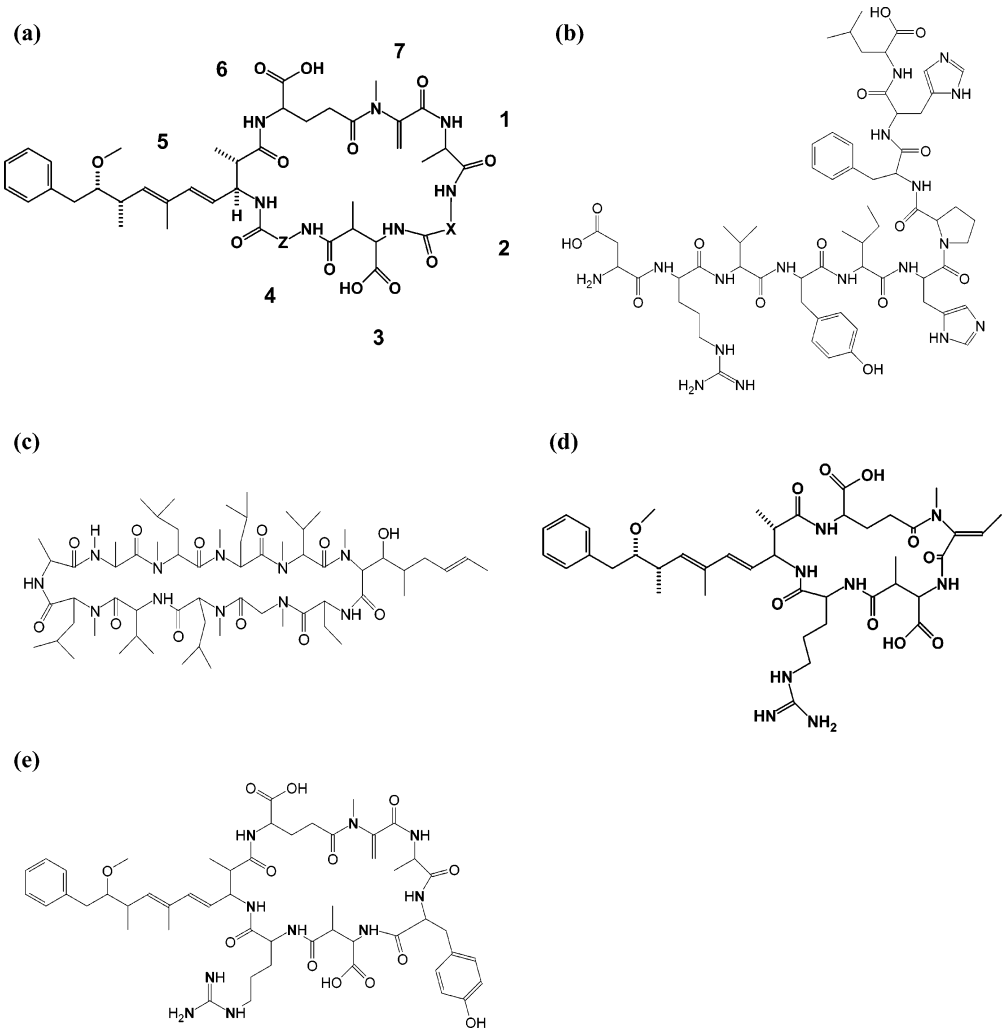

Figure 1. (a) Generic structure for MC congeners is *cyclo*-(D-alanine[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-glutamate[6]-Mdha[7]) in which X and Z are variable L-amino acids, D-MeAsp[3] is D-*erythro*-β-methylaspartic acid, Adda[5] is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha[7] is *N*-methyldehydroalanine; (b) angiotensin I; (c) cyclosporin A; (d) nodularin; (e) ^{15}N-MC-YR.

MCs.[11,13−15] In each case, however, MALDI-MS was utilized only for qualitative detection and identification of toxin congeners; PPIA, ELISA, and/or HPLC were used when quantitation was desired. To our knowledge, quantitation of cyanobacterial toxins using MALDI-MS has not been reported.

Unfortunately, MALDI-MS quantitation has been problematic since its early days. Unreliable spot-to-spot and shot-to-shot repeatability is widely accepted,[16−18] leading most researchers to utilize an internal standard (IS) for quantitation. IS compounds must be carefully selected to closely match the solubility, partition coefficient/crystallization process, and desorption/ionization behavior of the target analyte(s).[19,20] A stable isotope-labeled IS has often been recommended as the ideal choice.[21−23]

The limited focus of previous MALDI research on qualitative identification of MCs without quantitation has resulted in very little information on MC ionization behavior during MALDI analysis. Variations in both ionization efficiency (IE) and susceptibility to analyte−analyte suppression are likely due to the structural differences between congeners. Ionization efficiency is influenced to a significant degree by the ionization pathway preference (protonation vs cationization). However, it can also be affected by analyte−analyte or matrix−analyte suppression.[24] Matrix-mediated proton-transfer reactions in solution and in the gas phase may enhance the signal of analytes with higher proton affinities at the expense of those with lower affinities (analyte suppression) and may also provide a pathway for the cationization reactions

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typically seen in MALDI analysis. Analyte−analyte suppression can occur in two broad categories: (1) both analyte A and analyte B are likely to experience protonation as the dominant pathway, and (2) A is likely to be protonated, whereas B is more likely to form a sodium or potassium adduct.

Since sodium affinities are generally lower than proton affinities, a mixture of basic and nonbasic peptides should exhibit dominant suppression of the nonbasic peptides. The actual proton and sodium affinities for specific analytes and matrixes as well as the solution pH will affect the ionization pathways,[25] providing a possible explanation for the lack of a single, universal set of "best" MALDI sample preparation conditions.

The goal of this study was development of a simple, rapid method for analysis of cyanobacterial toxins using readily available equipment—including a low-end linear mass spectrometer, standard sample plates, and matrix compounds and additives that are easily obtained and prepared—in order to maximize applicability of the method in other laboratory settings. Limited sample handling, including minimal sample cleanup prior to analysis, was also targeted. This paper presents such a method for the MALDI-TOF mass spectrometric analysis of cyanobacterial toxins.

EXPERIMENTAL SECTION

Materials. Microcystin-LR, microcystin-LF, nodularin, and cyclosporin A were obtained from Alexis (San Diego, CA); microcystin-YR and microcystin-RR were from CalBioChem/EMD Biosciences (La Jolla, CA); ethanol was from Pharmco (Brookfield, CT); HPLC-grade methanol and acetonitrile were from Mallinckrodt Baker (Phillipsburg, NJ); α-cyano-4-hydroxycinnamic acid, trifluoroacetic acid, ^{15}N-sodium nitrate (98+ atom %), zinc sulfate heptahydrate, and human angiotensin I were from Sigma-Aldrich (St. Louis, MO). All materials were used as received. The stable isotope-labeled $[^{15}N]_{10}$-microcystin-YR was prepared in-house as described below. Deionized (DI) water (>18 MΩ·cm resistivity) was used for all solutions.

Preparation of the Stable Isotope Internal Standard. Stable isotope-labeled MCs were prepared in vivo using batch culture of *Microcystis aeruginosa* UTEX LB2664 grown in BG-11 medium modified to replace all nitrogen-containing components except sodium nitrate, for which the ^{15}N-labeled equivalent was used.[26] Briefly, 10 mL of BG-11 stock salt solution and 0.4 g ^{15}N-$NaNO_3$ were added to 990 mL of DI water; the pH was adjusted to 7.1 with 1 M HCl. The resulting media was autoclaved in a 2.8 L Fernbach flask and inoculated with 100 mL of healthy stationary phase culture. Cultures were incubated at room temperature in natural light and were swirled once daily to keep cells from settling. Cells were collected, lyophilized, and extracted via probe sonication using 75% MeOH; extracts were analyzed using HPLC with ESI-MS and photodiode array (PDA) detection (239 nm) to identify MCs.

$[^{15}N]_{10}$-Microcystin-YR (^{15}N-MC-YR), the most abundant congener in the extracts, was selected for further purification via solid-phase extraction (Waters Oasis HLB/500 mg/6 mL). Preparative HPLC-UV was conducted using a Phenomenex Bondclone 10 C18 column (300 mm × 7.8 mm i.d.), mobile phase (A) 0.1% TFA/H_2O, (B) 0.1% TFA/ACN, gradient 71% B to 74% B over 22.5 min, flow rate of 2.4 mL per min. Analytical HPLC was performed using an Ace 5 C18 column as previously described.[27] No other MC congeners were detected when this purified IS was analyzed using MALDI-MS and HPLC-PDA.

Sample Preparation. Several sample preparation techniques were evaluated for their precision and sensitivity for the analysis of MCs by MALDI-TOF MS (data not shown). Best results were obtained when 10.0 μL sample aliquots were combined with 1.0 μL of an internal standard/$ZnSO_4 \cdot 7H_2O$ (IS/Zn) mixture and 10.0 μL of matrix, bath sonicated (50 W, 3 min), spotted on the MALDI sample plate, and allowed to crystallize at ambient conditions.

Two different matrix preparations were tested. The normal matrix was α-cyano-4-hydroxycinnamic acid (CHCA), 15 mg/mL in 60% EtOH/36% ACN/4% DI water, providing average matrix/analyte molar ratios of 2000. Samples were also tested in a different matrix ($CHCA_{cell}$) to assess the impact of cyanobacterial cell components on the analysis of field samples. A healthy batch culture of nontoxic *M. aeruginosa* UTEX LB 2386 in BG-11 medium was diluted 1:100 with tap water to mimic field conditions and filtered in 50 mL aliquots onto 25 mm, 1.2 μm nylon filters. The filters were frozen for at least 24 h, then combined with 1 mL of CHCA matrix to lyse the cells.[28,29] After removal of the filter and centrifugation, the resulting supernatant (CHCA matrix solution + cell components, $CHCA_{cell}$) was used as an alternate MALDI matrix. The high organic content of the matrix solvent in both cases provided rapid (<2 min) drying time and improved crystal homogeneity.

Samples were prepared in 50% MeOH/0.1% TFA. Unless specified otherwise, standard solutions were prepared at concentrations of 0, 0.1, 0.3, 0.5, 1.0, 1.8, 2.5, 3.0, 4.0, and 5.0 μM for *each* MC (i.e., $[MC]_{total}$ from 0 to 20 μM when four MC congeners were present). This range extended from the general limit of detection to a level sufficient to bracket typical field samples. The IS/Zn mixture contained 50 μM IS/50 mM Zn^{2+} when used in CHCA matrix and 100 μM IS/200 mM Zn^{2+} when used in the $CHCA_{cell}$ matrix. Zinc ions were used to simplify the adduct pattern by eliminating sodium and potassium adducts as described elsewhere;[30] a more concentrated IS/Zn solution was required to achieve the same effects when additional cell components from $CHCA_{cell}$ were present.

MALDI Mass Spectrometry. Analyses were conducted using a Voyager-DE linear MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a 337 nm pulsed nitrogen laser. The high-voltage power supply was allowed to warm up for at least 30 min prior to collection of data. A 100-well, stainless steel MALDI target was polished with "pol" metal polish (Electron Microscopy Sciences, Hatfield, PA) prior to use, providing a somewhat hydrophobic surface coating to aid in beading of samples during crystallization. The sample plate was optimized to correct the plate-to-detector flight distance for each sample position on the plate, providing more accurate mass determination.

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Spectra were obtained in positive ion linear mode with accelerating voltage 20 kV, grid voltage 19 kV, guide wire 10 V, delayed extraction time 225 ns, low-mass gate setting m/z 400, and digitizer vertical scale set to 1000 mV. Operating pressure in the source was maintained near 4×10^{-7} torr. External mass calibration using nodularin (NDN, m/z 825.44) and angiotensin I (AngI, m/z 1296.48) bracketed the MC molecular weight range without causing analyte suppression. The number and position of laser firings was optimized using the strategy of Bucknall and Fung.[22] Each sample was spotted as seven replicate 2.0 μL aliquots on the prepared target and allowed to air-dry at ambient conditions. The laser fired 150 shots per sample spot accumulated from five firing positions; each data point is reported as the arithmetic mean of the replicate spots (1050 total laser shots per sample).

Statistical Treatments. The method detection limit (MDL) was determined by analyzing seven replicates of the blank (x_0) and a sample near the estimated detection limit (x_s, 0.1 μM). The calculation was adapted from Taylor and Cihon[31] using the following equation:

$$\mathrm{MDL} = (3.14s - b)/m \quad (1)$$

where s is the standard deviation of the x_s replicates, b and m are the intercept and slope of a regression in the region from x_0 to x_s, and 3.14 is the one-tailed t-value at the 99% confidence level. The limit of quantitation (LOQ) was similarly calculated using a multiplier of 10 to ensure that measurements are well removed from the random error of a blank result. Precision was calculated as the coefficient of variation (CV) expressed as a percentage:

$$\mathrm{CV} = (s/\bar{X}) \times 100 \quad (2)$$

where s is the standard deviation of a set of replicates and $\bar{X}$ is the mean.

All calibration curves were fitted to a linear regression using the method of least-squares. The resulting lines were evaluated using joint confidence ellipses for slope and intercept (JCE) as described by Mandel and Linnig.[32] Briefly, the ellipses were defined using equations of the form

$$N(x - b')^2 + 2S(x - b')(y - m') + Q(y - m')^2 = 2Fs^2 \quad (3)$$

where N is the number of pairs of x, y experimental values used to generate the regression, b' and m' are estimates of the intercept and slope, respectively, for the regression, $S = \Sigma x$, $Q = \Sigma x^2$, F is the critical value with $(2, N - 1)$ degrees of freedom at the 0.05 level, and s is the standard error of estimate. The equations were graphed using Graphmatica (kSoft Inc., Walnut Grove, CA); relative areas of the resulting oblique ellipses (e.g., Figure 2) were calculated using Microsoft Excel. These ellipses represent the 95% confidence region for the slope/intercept pair; therefore, a smaller elliptical area indicates a regression with less uncertainty.

Quantitation throughout was based on the $[M + H]^+$ peak only, without inclusion of isotope peaks or adduct peaks. This approach is supported by data presented elsewhere.[30] Mathematical outliers were rejected based on Dixon's "Q" test at the 95% confidence level as modified by Rorabacher.[33]

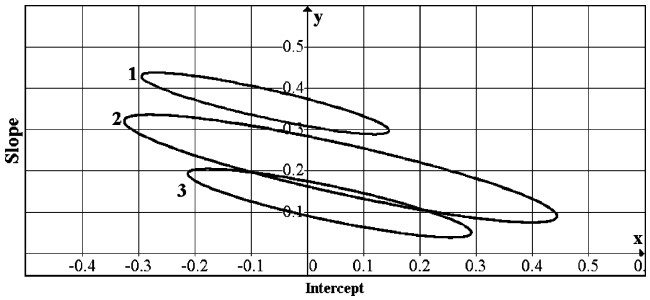

Figure 2. The 95% joint confidence ellipses for MC-LR calibration curves in $CHCA_{cell}$ using (1) nodularin, (2) angiotensin I, and (3) ^{15}N-MC-YR. Each ellipse is centered on the estimated slope/intercept pair for the regression.

Table 1. Comparison of Calibration Curve Parameters Using Two Laser Power Settings and Using Peak Area vs Peak Height[a]

	MDL (μM)[b]	range (μM)[c]	R^2	regression eq	av CV (%)[d]	relative JCE area[e]
Laser Power (Arbitrary Units)						
2400	0.312	0.99−5.0	0.98	$y = 0.340x + 0.005$	35.8	0.0207
2600	0.075	0.24−5.0	0.99	$y = 0.293x - 0.013$	11.9	0.0018
Relative Response						
peak area	0.091	0.29−5.0	0.99	$y = 0.376x - 0.015$	14.8	0.0029
peak height	0.075	0.24−5.0	0.99	$y = 0.293x - 0.013$	11.9	0.0018

[a] All MCs mixed in $CHCA_{cell}$ matrix; nodularin used as internal standard. Results shown are for MC-LR; other MCs, IS choices, and CHCA matrix showed similar trends. [b] Method detection limit. [c] Quantitative range beginning with the limit of quantitation (LOQ). [d] Coefficients of variation are averages of the CVs for the data points making up each regression curve. [e] Relative area of the joint confidence ellipse for slope and intercept.

RESULTS AND DISCUSSION

MALDI Conditions. Laser intensity is a crucial parameter to optimize during MALDI method development. Laser intensities near the threshold are often utilized to avoid fragmentation and detector saturation that can result in loss of signal intensity.[28] However, improvements in the quality of MALDI spectra are possible when operating substantially above the laser threshold.[34] In this study, the threshold laser intensity was determined to be near 2400 (arbitrary units; available range of 0−4600) for MC standards at the low end of the desired concentration range. Increasing the laser intensity to 2600 resulted in an approximately 10-fold increase in signal strength. Calibration curves taken at these two settings were compared to determine the optimal laser intensity for this method (Table 1). All relevant parameters were improved with the 2600 setting, which was selected for the remainder of the study.

Relative Response. A significant concern in MALDI-MS quantitation is the poor shot-to-shot reproducibility of analyte

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Table 2. Precision Obtained for MC-LR Using Peak Height and Peak Height Ratio

[MC-LR] (μM)	MC-LR PH CV (%)[a]	MC-LR PH/NDN PH CV (%)[b]
0.1	21.2	16.5
0.3	24.0	19.7
0.5	30.3	17.6
1.0	12.4	13.2
1.8	22.2	10.6
2.5	20.3	8.0
3.0	25.3	10.6
4.0	14.7	7.6
5.0	19.4	3.7
av	21.1	11.9

[a] Coefficients of variation (CV) for the MC-LR peak height. [b] CVs of the MC-LR/NDN peak height ratio.

signal intensities. Relative responses (analyte/IS) are often used to overcome this difficulty. Table 2 shows results from a MC-LR/NDN mixture; on average, the relative response CVs were 43.6% lower than those for MC-LR peak height alone. Results for other MC congeners and ISs showed the same trends, indicating a significant improvement in precision when using relative response.

Nelson and McLean[35] report that normalized signal intensity (peak height ratio) is more suitable than use of peak area ratio for MALDI quantitation since the integration range and baseline choice used to generate peak areas can be variable. This is particularly true when isotope peaks or adducts cannot be fully resolved, as when operating in the linear (nonreflectron) mode. Peak heights and peak areas for mixed MC solutions were recorded and used to generate two separate calibration curves (Table 1). The R^2 values were very similar, whereas the MDL and linear range were better when using peak heights. The area slope was 28% higher than the height slope; this increase in sensitivity might be expected to improve the analysis. However, the average CV of the data points was 24% higher; this inferior precision was reflected in the area of the joint confidence ellipse (JCE), which is 61% larger for area than for height. These factors combine to indicate that peak heights are the best choice for quantitation of MCs. Relative responses (MC peak height/IS peak height, or "PHR") were thus used throughout the study.

Ionization Behavior of MCs. Three polar MC standards (MC-LR, MC-RR, MC-YR) and one nonpolar MC (MC-LF) were tested alone and in combination to assess their ionization behavior during MALDI analysis. The three polar MCs produced strong $[M + H]^+$ signals as expected from protonation of the basic arginine side chains.[36] Nonpolar MC-LF generated weak signals for either $[MCLF + Na]^+$ (m/z 1008.5) or $[MCLF + H]^+$ (m/z 986.5); the presence of $[MCLF + H]^+$, unusual for nonpolar peptides, was probably due to the use of Zn^{2+} to suppress adduct formation.[30] It has been reported[37,38] that MC-RR and its desmethyl variants preferentially form $[M + 2H]^{2+}$ during ESI-MS; the doubly charged peak was not observed during this study.

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Table 3. Comparison of Linear Regression Data for Microcystins Individually and in Mixed Solutions Using Matrix with and without Cell Components[a]

	MDL (μM)[b]	range (μM)[c]	regression eq	R^2	IE[d]
Individual MC Standards (0−5.0 μM)					
in CHCA					
MC-LR	0.081	0.26−5.0	$y = 0.155x - 0.020$	0.98	1.00
MC-RR	0.030	0.10−5.0	$y = 0.186x + 0.050$	0.99	1.41
MC-YR	0.066	0.21−5.0	$y = 0.572x - 0.100$	0.98	2.27
MC-LF	3.0	nq[e]	nq[e]	nq[e]	<1[f]
in CHCA$_{cell}$[g]					
MC-LR	0.14	0.60−5.0	$y = 0.0443x + 0.003$	0.95	1.00
MC-RR	0.12	0.37−5.0	$y = 0.110x - 0.001$	0.98	1.73
MC-YR	0.13	0.41−5.0	$y = 0.221x - 0.098$	0.95	3.35
MC-LF	nd[h]	nd[h]	nd[h]	nd[h]	nd[h]
Mixed MC Standards (0−5.0 μM Each, MC_{total} = 0−15.0 μM)					
in CHCA					
MC-LR	0.015	0.11−5.0	$y = 0.149x + 0.001$	0.98	1.00
MC-RR	0.048	0.10−5.0	$y = 0.192x + 0.010$	0.99	1.23
MC-YR	0.058	0.19−5.0	$y = 0.216x + 0.030$	0.97	1.38
in CHCA$_{cell}$[g]					
MC-LR	0.058	0.19−5.0	$y = 0.106x + 0.001$	0.99	1.00
MC-RR	0.071	0.23−5.0	$y = 0.140x + 0.001$	0.99	1.35
MC-YR	0.023	0.19−5.0	$y = 0.161x + 0.009$	0.99	1.51

[a] Nodularin used as internal standard. [b] Method detection limit. [c] Quantitative range beginning at the limit of quantitation (LOQ). [d] Normalized ionization efficiency compared to MC-LR under each set of conditions. [e] MC-LF was not quantifiable within the tested range. [f] MC-LF was not detected within the tested range. [g] CHCA matrix containing extracted cyanobacterial cell components. [h] Not quantifiable, but very low based on MDL.

The three polar congeners generated good linear regressions when tested in CHCA matrix and $CHCA_{cell}$ (Table 3). MC-LF was not quantifiable in standard mixtures and is therefore unlikely to be quantifiable in field samples; this should be expected with other nonpolar MCs as well. Since the vast majority of the field samples tested worldwide[4] exhibit predominantly polar MCs, this is not a significant problem with this method. However, it should be kept in mind when comparing MALDI-MS results with those obtained using other techniques.

Ionization efficiency, or the ease with which molecules are ionized during MALDI analysis, can be compared for various analytes to gain insights into a method. The slope of the calibration curve can be used to approximate the IE across the linear range. By definition, the PHR is used to calculate this slope whenever an IS is used, thus linking the slope to ionization of the IS as well as the analyte. To avoid this complication, Table 3 reports the normalized IE using the slopes for peak height (rather than PHR) versus concentration under various conditions. The order of IE was MC-LF < MC-LR < MC-RR < MC-YR under all conditions. The very low IE of MC-LF is related to the lack of available protonation sites as discussed above, and the lower IE of MC-LR compared to MC-RR and MC-YR is in line with the presence of a nonpolar amino acid (leucine) at position 2 (Figure 1).

Suppression of analyte ionization under various conditions is reported in Table 4, again using slopes for peak height versus concentration. In normal CHCA matrix, analyte−analyte suppression is evident when mixed standards are compared to individual standards; the response curve slopes for mixed standards are only 46% to 76% of those for individual standards; the order of susceptibility to suppression by other MCs was MC-LR <

Table 4. Suppression Coefficients for Polar Microcystins upon Addition of Other MCs or Cell Components[a]

	MC-LR	MC-RR	MC-YR
	Mixed/Individual[b]		
in CHCA	0.76	0.66	0.46
in $CHCA_{cell}$	2.67	2.08	1.20
	$CHCA_{cell}$/CHCA[c]		
when alone	0.24	0.30	0.36
in mixed MCs	0.86	0.95	0.94

[a] Peak height (not PHR) vs concentration regressions were used. [b] Calculated by dividing the slope for each MC in mixed standards by the slope of each individual MC standard curve. [c] Calculated by dividing the appropriate regression slope obtained in CHCA matrix by that obtained in $CHCA_{cell}$ matrix.

Table 5. Comparison of Calibration Curve Parameters for MC-LR in a Solution of Mixed MCs Using Three Internal Standard Choices[a]

	MDL (μM)[b]	range (μM)[c]	regression eq	R^2	relative JCE area[d]
		In CHCA			
AngI	0.16	0.51−5.0	$y = 3.36x - 0.49$	0.97	2.6010
NDN	0.057	0.18−5.0	$y = 1.13x - 0.09$	0.99	0.0938
15N-MC-YR	0.070	0.22−5.0	$y = 2.80x - 0.12$	0.96	2.7740
		In $CHCA_{cell}$			
AngI	0.20	0.64−5.0	$y = 0.195x + 0.098$	0.88	0.0745
NDN	0.13	0.41−5.0	$y = 0.340x + 0.005$	0.98	0.0244
15N-MC-YR	0.21	0.67−5.0	$y = 0.119x + 0.050$	0.89	0.0336

[a] Using mixed MC standards (only the data for MC-LR is shown). [b] Method detection limit. [c] Quantitative range beginning at the limit of quantitation (LOQ). [d] Relative areas of the joint confidence ellipses for slope and intercept.

MC-RR < MC-YR. Interestingly, the results are reversed in $CHCA_{cell}$ matrix, with the mixed standards exhibiting steeper slopes (increased peak heights) compared to the individual standards. The reasons for this effect are presently unknown, although buffering effects provided by the cell components may be a possible explanation.

Rogatsky and Stein[39] contend that assessment of matrix effects (the effects of substances present in naturally occurring samples, including other cell components) is critical to method development in mass spectrometric methods due to possible suppression of analyte ionization by matrix substances. The effect of added cell components was tested by analyzing identical standard solutions in both CHCA and $CHCA_{cell}$ matrixes. These results, shown in Table 4, reveal the suppressive effects of cell components on the MC response. The use of $CHCA_{cell}$ decreased the slopes of the individual regressions to only 24% (MC-LR) to 36% (MC-YR) of those obtained using CHCA. The MDL and LOQ values also increased (Table 3), shortening the linear range. This suppression was also evident in mixed standards to a lesser degree.

The factors leading to suppression by cell components were not probed in detail but could include alteration of the sample pH, addition of other biological peptides to the sample, or the presence of excess cellular salts. Since analytes are reported to retain their solution charge state during crystallization,[40] alteration of the sample pH could exert significant effects on the ionization of analytes and even alter the sample crystallization process. Nontargeted peptides added to the sample will decrease the matrix/analyte ratio and may out-compete the target analytes for protons, either of which can result in suppression of analyte ionization. High concentrations of ionic salts are known to suppress analyte signals[28] by interfering with formation of the matrix crystal lattice. Regardless of the reasons, an awareness of the suppressive effects of cyanobacterial cell extracts on MCs is essential for accurate quantitation of field samples.

Internal Standard Selection. The selection of an IS was a critical part of the method development process. Four compounds (Figure 1b−e) were selected for testing; they exhibited a range of potential ionization behaviors, cost, and convenience while providing molecular weights within 200 Da of the MC mass range. AngI (m/z 1296.7) and cyclosporin A (CsA, m/z 1203.8) are commercially available and relatively inexpensive; NDN (m/z 825.4), a brackish-water cyanobacterial toxin not present in the freshwater samples tested in our lab, is commercially available but costly; ^{15}N-MC-YR (m/z 1056.5) is not commercially available. Due to the frequent literature preference for stable isotope-labeled ISs as previously discussed, the lengthy in-house production of ^{15}N-MC-YR was considered a worthwhile investment of time.

Each potential IS (5.0 μM) was tested in CHCA and $CHCA_{cell}$ to provide an indication of ionization behavior. The three arginine-containing peptides exhibited significantly stronger IE than the nonpolar CsA (20-fold for AngI and NDN, 8-fold for ^{15}N-MC-YR). This was particularly evident in $CHCA_{cell}$ matrix where CsA was not detectable; the likely cause is suppression by basic peptides in the cell extract, analogous to the results for MC-LF. Since CsA did not give a quantifiable response when mixed with extracted cell components, it would not be of practical use with field samples and was dropped from the remainder of the study.

AngI, NDN, and ^{15}N-MC-YR were tested with mixed MC standards in both CHCA and $CHCA_{cell}$. PHR was plotted versus MC concentration and fitted to linear regressions. Table 5 shows the results for MC-LR; MC-RR and MC-YR exhibited similar trends. The joint confidence ellipses for MC-LR using $CHCA_{cell}$ and each of the IS choices are shown in Figure 2. In both matrixes, NDN had the lowest MDL and LOQ, highest R^2 value, and lowest JCE area. The use of NDN with field samples should thus allow detection of more congeners at lower concentrations than AngI or ^{15}N-MC-YR. The decline in the slope for $CHCA_{cell}$ compared to CHCA was less dramatic for NDN (70% decline) than for AngI (94% decline) or ^{15}N-MC-YR (96% decline).

To test the possibility that the decrease in slope observed for NDN (70%) was due to the increased NDN concentration used with $CHCA_{cell}$ (100 μM) versus CHCA (50 μM), identical standard curves were prepared in $CHCA_{cell}$ using 50, 100, and 200 μM NDN. The results (Table 6) indicate expected slope decreases of 58% to 60% due to increasing [NDN] from 50 to 100 μM. This slope decrease probably resulted mainly from increased NDN peak heights (thus mathematically reducing the PHR), with some analyte suppression by the additional NDN. Subtracting from the total decrease (70%) caused by $CHCA_{cell}$ for mixed standards yields

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Table 6. Effect of Nodularin Concentration on Microcystin Calibration Curves[a]

[NDN] (μM)	MC-LR regression eq	MC-LR R^2	MC-RR regression eq	MC-RR R^2	MC-YR regression eq	MC-YR R^2
50	$y = 0.264x + 0.014$	0.97	$y = 0.332x + 0.017$	0.99	$y = 0.403x + 0.073$	0.99
100	$y = 0.106x + 0.001$	0.99	$y = 0.140x + 0.001$	0.99	$y = 0.161x + 0.009$	0.99
200	$y = 0.044x + 0.004$	0.95	$y = 0.0531x + 0.0035$	0.96	$y = 0.0728x + 0.0032$	0.97

[a] Using mixed MC standards in $CHCA_{cell}$. Regressions are from plotting relative response (MC peak height/NDN peak height) vs MC concentration for each MC congener.

Table 7. Triplicate Calibration Curves Using Nodularin as Internal Standard[a]

	MDL (μM)[b]	range (μM)[c]	regression eq	R^2
		MC-LR		
run 1	0.10	0.33−5.0	$y = 0.160x - 0.007$	0.98
run 2	0.034	0.11−5.0	$y = 0.276x + 0.041$	0.98
run 3	0.096	0.31−5.0	$y = 0.312x - 0.051$	0.95
		MC-RR		
run 1	0.089	0.28−5.0	$y = 0.138x + 0.006$	0.96
run 2	0.092	0.29−5.0	$y = 0.214x + 0.082$	0.96
run 3	0.13	0.40−5.0	$y = 0.255x - 0.019$	0.97
		MC-YR		
run 1	0.10	0.32−5.0	$y = 0.112x + 0.002$	0.99
run 2	0.11	0.34−5.0	$y = 0.170x + 0.063$	0.97
run 3	0.092	0.29−5.0	$y = 0.181x + 0.011$	0.96

[a] Using mixed MC standards in $CHCA_{cell}$ matrix with NDN as the internal standard. [b] Method detection limit. [c] Quantitative range beginning at the limit of quantitation (LOQ).

a "net suppression" by the cell components. The net suppression of the MCs in $CHCA_{cell}$ was 10% (MC-LR, MC-YR) to 12% (MC-RR).

Surprisingly, the stable isotope-labeled ^{15}N-MC-YR was not the best IS for the analysis of MCs. It may be *too* similar to the MC standards in structure and polarity and thus more likely to participate in analyte−analyte suppression. Alternatively, the HPLC-purified ^{15}N-MC-YR may have contained impurities at levels too low to be detected using MALDI-MS or HPLC-UV but still capable of hindering quantitation.

Identical sets of mixed MC standards were prepared and tested on three consecutive days, using $CHCA_{cell}$ and NDN; results are shown in Table 7. The R^2 values ranged from 0.95 to 0.99; these were quite good considering the potential impact of cell components on quantitation. Since quantitation of field samples will occur by comparison of the peak height ratio MC/NDN for each MC peak observed, the variability of the slopes (by as much as a factor of 2) is a point of concern. There are several possible factors that may have contributed to these variations, including vacuum pressure within the ion source (affecting plume formation and charge-transfer interactions) and varying laboratory temperature, humidity, and ventilation conditions, which can dramatically alter the sample crystallization process. The possible impact of environmental conditions is supported by the fact that each MC showed the same trends (lowest slope on day 1, highest on day 3). This difficulty can be overcome by running a fresh calibration curve with each set of samples, a routine approach required with several other methods used for the analysis of MCs.

CONCLUSIONS

The goal of this study was development of a rapid, simple method for quantitation of MCs from cyanobacterial cells using equipment and materials likely to be found in many laboratory settings. Four ISs were tested; NDN demonstrated the best R^2 value, lowest MDL and LOQ, and least uncertainty in the regression as demonstrated by small joint confidence ellipses. The overall method provided R^2 values >0.99 for mixed MC samples in a matrix containing cell components and similar to conditions expected for field samples. Extensive data regarding IE and suppression for typical MCs under various conditions are provided.

Although the presence of cell components does suppress MC signals to some degree, the polar variants exhibit sufficiently low detection limits (0.023−0.071 μM) to permit use with field samples when a typical particulate collection protocol is used. In our field work, 2−20 L of water is typically filtered to collect the cyanobacterial cells, which are then extracted in 10 mL of solvent to obtain the MCs. This provides a 200- to 2000-fold concentration factor, sufficient to detect MCs at levels as low as 0.012 μg L^{-1} and to quantify when [MC] is 0.055 μg L^{-1} or higher, well below the World Health Organization advisory level of 1.0 μg L^{-1} for MC-LR. For example, of the samples analyzed in our laboratory using PPIA, ELISA, and HPLC over the past several field seasons for the NOAA MERHAB monitoring program[41] (n = 3390), all but 26 of the samples fall within the linear range reported here.

The use of MALDI-TOF MS for qualitative identification of MC congeners is already well established. This method allows both quantitation and congener identification to be achieved much more rapidly than with other available techniques. Application of the method to field samples from several regions of the United States is underway.

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