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Electrolyte-Induced Ionization Suppression and Microcystin Toxins: Ammonium Formate Suppresses Sodium Replacement Ions and Enhances Protiated and Ammoniated Ions for Improved Specificity in Quantitative LC-MS-MS

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This study examines the effects of electrolytes on microcystin (MC) electrospray ionization (ESI) mass spectrometry and quantitative LC-MS-MS. Sodium replacement ions (SRI) are prominent in MC ESI spectra in protic solvents such as HPLC grade methanol. In a methanol−water−0.006% acetic acid (v/v) gradient, envelopes with up to 11 SRI were apparent in both the +1 and +2 charge states with structures $[M + Na_x - (x-1)H]^+$ and $[M + Na_x - (x-2)H]^{+2}$. The *m/z* 135 product ion, $[Ø-CH_2-CH=O-CH_3]^+$, widely used in tandem LC-MS-MS determination of MC, is a low collision energy fragment of many doubly charged MC precursor ions (e.g., $[M+Na+H]^{+2}$, $[M+Na+NH_4]^{+2}$, $M+Na+H+CH_3OH]^{+2}$, $[M+2H]^{+2}$). These phenomena impair congener-specific LC-MS-MS detection of MC and degrade quantitative accuracy and precision. Pulse addition experiments established that ammonium formate (AF) strongly suppresses SRI in both +1 and +2 charge states and enhances MH^+ and MNH_4^+ adducts in neutral MC. Addition of the buffer either post-column or by incorporation in the mobile phase increases specificity for all of the MC which were determined as the $MNH_4^+ > MH^+$ and $MH^+ > [MH - 134]^+$ transitions for neutral MC (MCLA, MCLF, MCLW) and $[M+2H]^{+2} > 135^+$ and $[M+2H]^{+2} > [M+2H - 135]^+$ transitions for arginine-containing MC (MCLR, MCYR, MCRR). These findings shed light on mechanisms of electrolyte-induced ionization suppression, and demonstrate beneficial use of a buffer electrolyte for improved specificity and analytical ruggedness in quantitative LC-MS-MS.

Microcystins (MC), heptapeptides produced by blue-green algae (BGA),[1] are increasingly of interest in water supplies[2−4] because of their toxicity to wildlife, livestock, and humans.[5,6] MC are potent protein phosphatase enzyme inhibitors, hepatotoxins and liver tumor promotors.[6] Chronic exposure to MC has been implicated in liver damage,[3] tumorogenesis, and etiology of primarly liver cancer.[7] MC are cyclic peptides with a generalized structure of *cyclo*-(-D-Ala[1]-L-X[2]-D-isoMeAsp[3]-L-Y[4]-Adda[5]-D-isoGlu[6]-Mdha[7]) where the superscripts indicate the ring position (Figure 1). Both dextro (D) and levo (L) amino acids are present, and the principal congeners result from amino acid substitution in the "X" and "Y" positions leading to the shorthand notation for MC, for example, MCLR is substituted with leucine (L) and arginine (R). The unusual amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) is important for recognition of binding sites responsible for MC toxicity. Another unusual amino acid, *N*-methyldehydroalanine (Mdha), is responsible for covalent binding critical to MC-induced enzyme deactivation.

Screening water samples for MC is widely accomplished by enzyme linked immunosorbent assays which respond to MC as a class of compounds.[8] While not as sensitive, congener-specific determination by electrospray ionization (ESI) LC-MS-MS is preferred for MC speciation and confirmation. Congener-specific data is needed for monitoring water quality standards such as the World Health Organization (WHO) drinking water guideline (1 μg MCLR/L).[6]

Anecdotal evidence suggests that current LC-MS methods for MC are not very reliable or robust, sometimes causing discrepancies between environmental testing laboratories (unpublished results, 2007). Determination of MC is challenging because of a lack of commercially available toxin standards, poor recoveries from cleanup steps, interfering matrix compounds, and inconclu-

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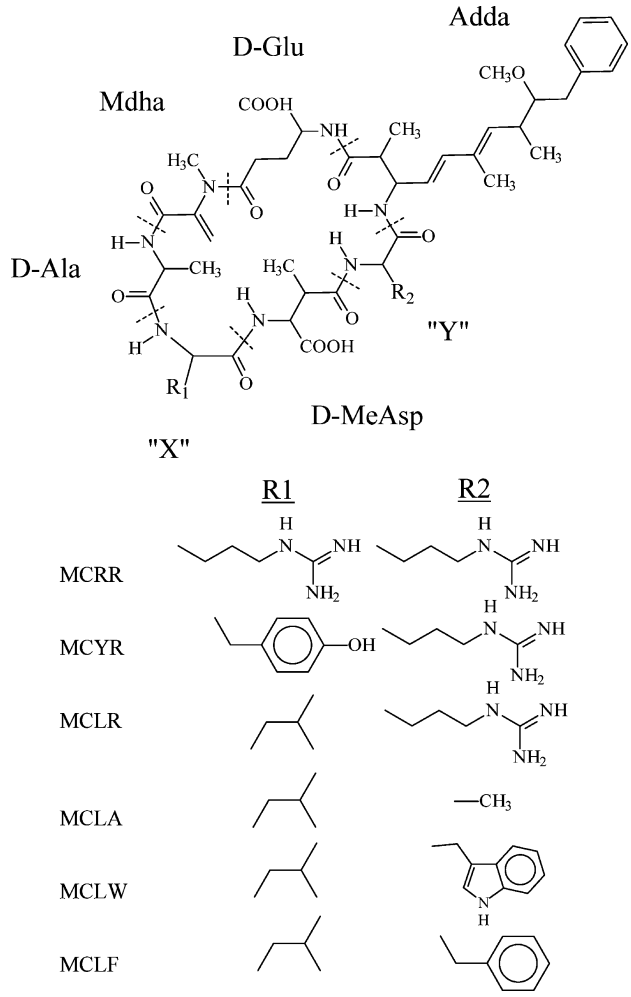

Figure 1. Microcystin structural variants showing L-amino acid substitution in the "X" and "Y" ring positions. The unusual D-amino acids are *N*-methyldehydroalanine (Mdha), 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) and methylaspartic acid (MeAsp).

sive identification of unknowns.[9] Chromatographic separation of the naturally occurring MC congeners is limited on reversed phase HPLC columns because of small differences in polarity. Our hypothesis is that ionization suppression is a major contributing factor to poor data quality in this endeavor.

Ionization suppression is widely reported[10−14] and is associated with poor accuracy and precision and increased detection limits.[14−17] Ionization suppression is often encountered in tandem MS because analysts may use shorter separations to take advantage of the technique's high specificity.[14]

Ionization suppression is a matrix effect where sample components alter the analyte signal. Ionization suppression is quantified by comparing the analyte response in standard mixtures to that in the matrix of interest:[10,15,18] the matrix effect (ME%) is >100% in cases of enhancement and <100% where suppression occurs. In this paper we refer collectively to ionization suppression, ionization enhancement, and related matrix effects as suppression. Ionization suppression is a problem in trace analysis because the proportion of the analyte to the bulk sample is minute. Overloading the analytical system with sample to attain lower method detection limits aggravates ionization suppression.[12] Mobile phase additives can cause ionization suppression including buffers,[19] ion pairing agents,[20] and surfactants.[21] High electrolyte concentrations (e.g., $>10^{-3}$ M)[22] cause substantial loss in signal. Co-eluting analytes and isotope-labeled standards suppress ionization of each other [23,24] as previously found in particle beam LC-MS.[25]

The mechanisms underlying ESI suppression are not completely understood.[14] Changes in droplet solution properties (e.g., surface tension, ionic strength/conductivity) associated with nonvolatile components are important while gas phase processes apparently are not.[11] Signal suppression at high salt concentrations is explained in relation to the electrical double layer in desolvating electrospray particles[22] and competition for charged surface sites.[24] Ionization suppression may involve coprecipitation of analytes and reduced vaporization from droplets.[11] It is not clear whether ionization suppression is primarily a physical or chemical phenomenon.[26]

Methods to control ionization suppression are of interest as they provide improved analytical accuracy, precision, and method robustness. The techniques which have proven effective include sample cleanup including solid phase extraction[16] and liquid−liquid extraction, improved HPLC separation, two-dimensional LC,[27] use of labeled internal standards and use of mobile phase additives (e.g., buffers, weak acids and bases, and surface active agents like isopropanol and methoxyethoxyethanol).[15,28] Ionization suppression is minimized only when the buffer concentration is high enough to suppress the analyte signal.[12] Buffers such as ammonium acetate are reported to counter ionization suppression by precipitating Na^+ and Cl^- ions within evaporating electrospray droplets.[28]

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This paper examines effects of ESI suppression in quantitative analysis of MC by LC-MS-MS and addresses two aspects: the influence of electrolytes on spectra and the specificity of MS-MS transitions. While the overarching goal of this work is development of more robust methods for MC, the findings also have relevance to the analysis of other polypeptides and proteins.

EXPERIMENTAL SECTION

Chemicals. MC (Figure 1) were obtained from two sources: Sigma-Aldrich (Milwaukee, WI, U.S.A.) for MCYR, MCLR, and MCLA and Calbiochem (LaJolla, CA, U.S.A.) for MCRR, MCLW, and MCLF. All of the microcystins were purified from *Microcystis aeruginosa* cultures. Ammonium formate (AF) was purchased from Fluka (>99%, Fluka Chemie AG, Switzerland), and methanol was purchased from Burdick & Jackson (for HPLC, GC, pesticide residue and spectroscopy, Burdick & Jackson, Muskegan, MI). Laboratory reagent water and water for HPLC mobile phases was produced with a Millipore Milli-Q Gradient A10 water purification system (Billerica, MA, U.S.A.).

Caution! Microcystins are toxic substances that should be handled with appropriate safety equipment including gloves, protective clothing, respiratory protection, and/or a high draft hood, especially when neat materials or concentrated solutions are in use.

Instruments. A Thermo-Finnigan TSQ Quantum triple-stage quadrupole atmospheric pressure ionization (API) mass spectrometer (San Jose, CA, U.S.A.) with an Agilent 1100 capillary binary HPLC pump, microautosampler and degasser (Agilent Instruments, Wilmington, DE, U.S.A.) was used. The capillary HPLC was equipped with a 100 μL/min flow controller. Xcalibur version 1.3 software was used for MS and HPLC control and data acquisition/processing. Nitrogen was supplied by a Parker Balston N2-35 nitrogen generator (Parker-Hannifin, Haverhill, MA, U.S.A.). In limited comparative studies an Applied Biosystems API 4000 triple-stage quadrupole API mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) also was used.

Mass Spectrometer Operation. The ESI source was operated as follows: typical source voltage, 4.3 kV; source gas pressure, 25 (arbitrary units); auxiliary gas pressure, 24 (arbitrary units); transfer capillary temp., 270 °C; argon collision gas pressure, 1.5 mTorr. For MS-MS operation precursor ions were isolated with a scan width of 0.7 amu, the Q3 peak width was 1.5 amu and dwell times were 0.2 ms.

Chromatographic Separation. Varian Pursuit C_8 or C_{18} columns (5 μm, 1 × 100 mm; Lake Forest, CA, U.S.A.) with guard columns were eluted with a methanol−water gradient and a uniform flow rate of 60 μL/min in "micro" pump mode. The columns had comparable performance, but the C8 column was used predominantly. For LC-MS-MS gradient elution both water (solvent A) and methanol (solvent B) contained 0.006% acetic acid (v/v).[29] In LC-MS operation AF was added either post-column (5 μL/min of 200 mM AF) or by addition of 15 mM AF to both A and B solvent reservoirs. The optimal gradient for sharp peaks and MC separation was 40% B, 2 min; 40−100% B, 2−16 min; 100% B, 16−17 min; 100−40% B, 17−19 min (column regeneration); 40% B, 5 min (equilibration). The injection volume was 8 μL, and a wash vial was used to minimize carryover.

Syringe Pumps and Switching Valve for Buffer Addition. Experimental buffers were introduced using one or two supplemental syringe pumps configured according to Figure 2. The syringe pump infusing the candidate analyte was integral to the mass spectrometer. B and C are shown with cross fittings, but in LC-MS(-MS) operation a tee fitting was used to avoid mixing in dead zones. For post-column addition experiments 200 mM AF was introduced at 5 μL/min. The chromatographic mobile phase in infusion experiments was 60 μL/min methanol−water (3:1, v/v) with 0.006% acetic acid.

RESULTS AND DISCUSSION

A number of publications have described reversed phase HPLC separation of MC with detection by either UV absorbance or ESI mass spectrometry. Gradient elution with acetonitrile−water or methanol−water mixtures has been reported and various additives have been used including trifluoroacetic acid[30,9,31] and formic acid/ammonium formate.[3] All of the more than 80 known MC elute between MCRR and MCLF[3] resulting in frequent co-elutions[30,32] and placing strong demands on the detection system for specificity. Particularly important is the observation that traces of acetic acid in the mobile phase improve the ESI signal.[29] In the current study, for example, the LC-MS-MS response for the MCLR $509^{+2} > 135^{+}$ transition was increased 500-fold by acetic acid, and for this reason an acetic acid containing methanol−water gradient was selected for this study.

Electrospray Spectral Features. MC spectra are strongly influenced by substitution with the basic amino acid, arginine, which determines the charge state.[33] The neutral MC are dominated by the +1 charge state with prominent MH^+ and MNa^+ adduct ions—the sodium adduct was usually the base peak. MC with a single arginine form both singly and doubly charged ions such as $[M+2H]^{+2}$ and $[M+Na+H]^{+2}$ while MCRR with two arginines has the simplest spectrum with only the $[M+2H]^{+2}$ ion, m/z 520.[29,30]

Sodium replacement ions (SRI) are a pervasive feature of MC spectra as seen in the MCLR spectrum (Figure 3). In this spectrum the +1 SRI envelope appears as four ions with 22 Da spacing. The +2 charge state envelope appears as three intense ions with 11 Da spacing. As a result the MCLR spectrum is unusually complex with over 18 resolved ions. MC SRI have been reported previously[9] and are widely encountered in mass spectrometry of peptides and proteins[35] where they can be used to assign charge state.[34]

The neutral MC principally exhibit the +1 charge state SRI envelope, for example, close inspection of the MCLF spectrum revealed 11 sodiated species. The MCRR SRI envelope contained 8 or more intense, doubly charged ions, and with sufficient

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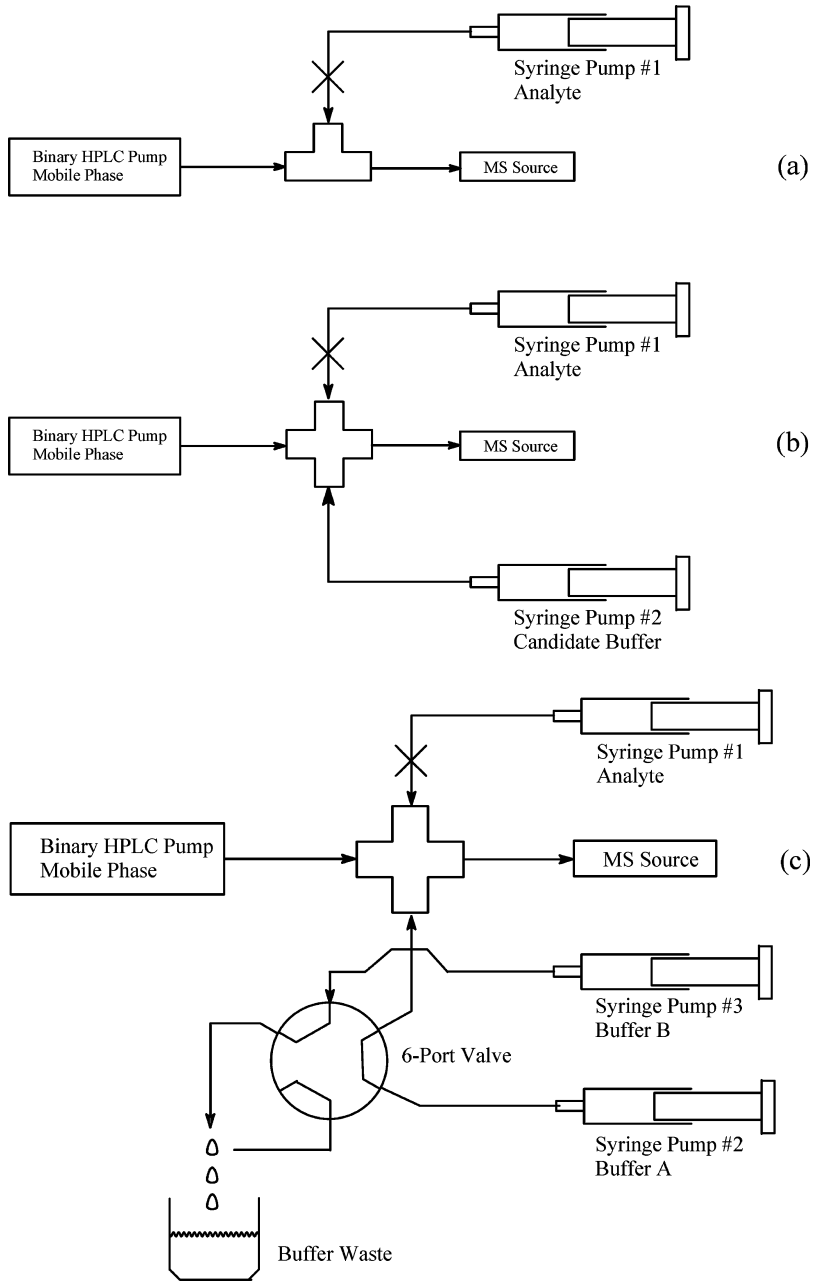

Figure 2. Syringe pump apparatus for supplemental addition of buffers in LC-MS(-MS) method development: (a) conventional apparatus; (b) with ancillary buffer addition via a cross fitting; and (c) with ancillary addition of selected buffer(s) via a cross fitting and 6-port, electrically actuated valve.

resolving power a total of 16 ions and isotopes are found. Isotope peaks of the +2 charge state are 1/2 Da apart and cannot be resolved by all quadrupole mass spectrometers. SRI were observed in MC spectra produced by both of the mass spectrometers under a wide range of source operating conditions.

SRI and replacement ions of other alkali and alkaline earth salts create two significant problems in quantitative LC-MS: multiple adducts (1) reduce the analyte signal and (2) compromise specificity. The connection between electrolytes and specificity is an important aspect of ionization suppression which has received little attention, whereas the deleterious effects on signal are well established. Electrolyte impurities occur even in purified HPLC solvents, for example, reagent grade methanol has an alkali metal content of $\sim 10^{-5}$ M.[19] The variable nature of electrolyte contributions from the matrix creates inconsistent spectra and poor quantitative performance. Importantly, salt concentrations in typical eutrophic lake water average 500 to 700 mg/L.[2] Spectrum variation may be associated with changes in the alkali metal load of the HPLC solvents,[35] sample carryover, or previous use of buffers that modify the adventitious ion concentration in the system.

Fragmentation of MC Ions. In the $[M+2H]^{+2}$ ion of arginine-containing MC the charges reside preferentially on the quanidinium and the Adda methoxy moieties (Figure 1). This doubly charged ion fragments efficiently at low collision energy forming two singly charged product ions: $[Ø{-}CH_2{-}CH{=}O{-}CH_3]^+$ (m/z 135) and $[M + 2H - 135]^+$.[36] The side chain fragmentation results from a charge migration and bond cleavage mechanism.

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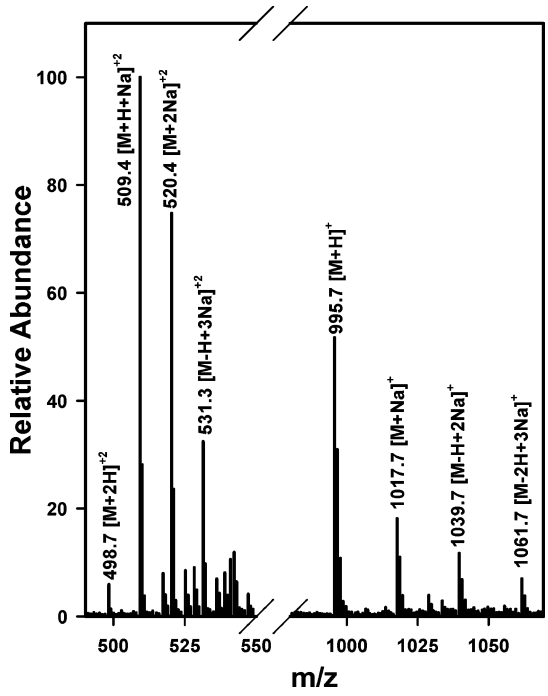

Figure 3. MCLR +ESI spectrum with +1 and +2 charge state envelopes shown.

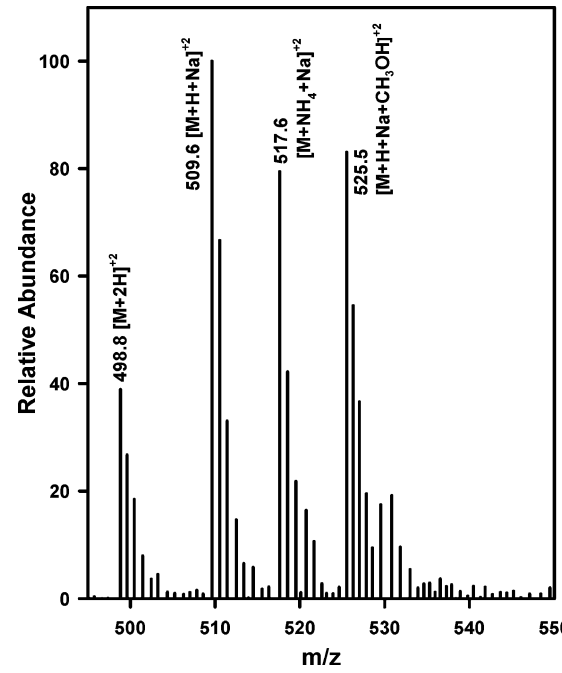

Figure 4. *M/z* 135 precursor ions from low energy fragmentation of MCLR. Operating conditions: sample introduced by infusion, 26 V collision energy, 15 V source CID, and 475 to1, 200 Da scan range. Precursors ions in the +1 charge state were negligible (<5%) at this low collision energy.

Because Adda occurs in all MC, the *m/z* 135 fragment is considered diagnostic for these BGA toxins. Additional diagnostic fragments have been reported including *m/z* 155, 163, 213, and 375.[32,36,37] Of these ions only $[M + 2H - 135]^+$ is variant specific. Thus, both $498^{+2} > 135^+$ and $498^{+2} > 861^+$ transitions detect MCLR, but the $498^{+2} > 135^+$ transition is far more susceptible to interference by other MC variants. While the doubly charged ions fragment at low collision energies (~20 V CE), at higher collision energies (50−70 V CE) MC MH^+ ions also fragment to the *m/z* 135 ion.[31,3] The nonspecific $498^{+2} > 135^+$ transition has been widely used in LC-MS-MS determination of MCLR,[32,38,29,39,3] and *m/z* 135 precursor scans are useful in screening for MC.[3]

Precursor ion experiments established that the *m/z* 135 ion is a product of most of the doubly charged MC ions, not just $[M+2H]^{+2}$, from any given MC. The *m/z* 135 precursor ion spectrum of MCLR (Figure 4), for example, has prominent *m/z* 498.8 ($[M+2H]^{+2}$), *m/z* 509.6 ($[M+Na+H]^{+2}$), *m/z* 517.6 ($[M+Na+NH_4]^{+2}$ or $[M+H+K]^{+2}$), and *m/z* 525.5 ($M+Na+H+CH_3OH]^{+2}$) ions. In other experiments the *m/z* 527.9 ($[M+K+Na]^{+2}$) ion also was present. In total this precursor spectrum has over 20 ions and isotope peaks, and at higher collision energies there are additional *m/z* 135 precursors. Thus, the *m/z* 135 product ion is highly generic originating from many precursor ions and all MC congeners. These findings from the study of MC standards in purified HPLC solvents and without electrolyte contributions from complex samples shed light on factors that impair specificity and degrade performance in quantitative LC-MS-MS.

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Pulsed Buffer Addition. AF buffer addition was found to markedly influence MC spectra. Using a cross fitting (Figure 2) allowed simultaneous infusion of mobile phase, the MC of interest, and an experimental buffer or additive. In this case the HPLC pump delivered the mobile phase, 60 μL/min of 0.006% acetic acid in methanol−water (3:1, v/v), syringe pump #1 introduced a one μg/mL solution of MCLW at 5 μL/min and syringe pump #2 delivered 5 μL/min of 200 mM AF. Pulsing the buffer provided information on the reproducibility of spectrum and signal changes. In data presented the buffer was delivered in 1 min pulses in a 2 min cycle, but in other experiments pulses as short as 10 s were investigated.

Initially AF did not appear to be beneficial because the total ion chromatogram signal (500−1120 Da scan) was reduced by about 60%. The MCLW spectrum, however, was altered by AF with a significant and reproducible suppression of the SRI in both the +1 and +2 charge states (Figure 5). Because SRI dominated the +2 charge state, the +2 charge state was reduced concomitant with increases in MH^+ and MNH_4^+ adduct ions. The mechanism of MH^+ enhancement may involve protonation by NH_4^+ or fragmentation of the ammonium adduct by in source CID—loss of NH_3 occurs efficiently a low Collision Induced Dissociation (CID) collision energies as described later.

None of the buffer-induced spectrum changes were stabilized during typical LC-MS scan times ruling out buffer injection in alternate scans with this apparatus. Spectrum changes occurred over tens of seconds to several minutes (Figure 6). Both MH^+ and MNH_4^+ signals dropped rapidly when the AF buffer flow was discontinued; in ~45 s their signals dropped 2.9- and 3.7-fold, respectively. When the buffer flow began there was a more rapid increase in signal, ~2.4- and 3.4-fold, respectively, for the protiated and ammoniated adducts occurring in ~20 s. While

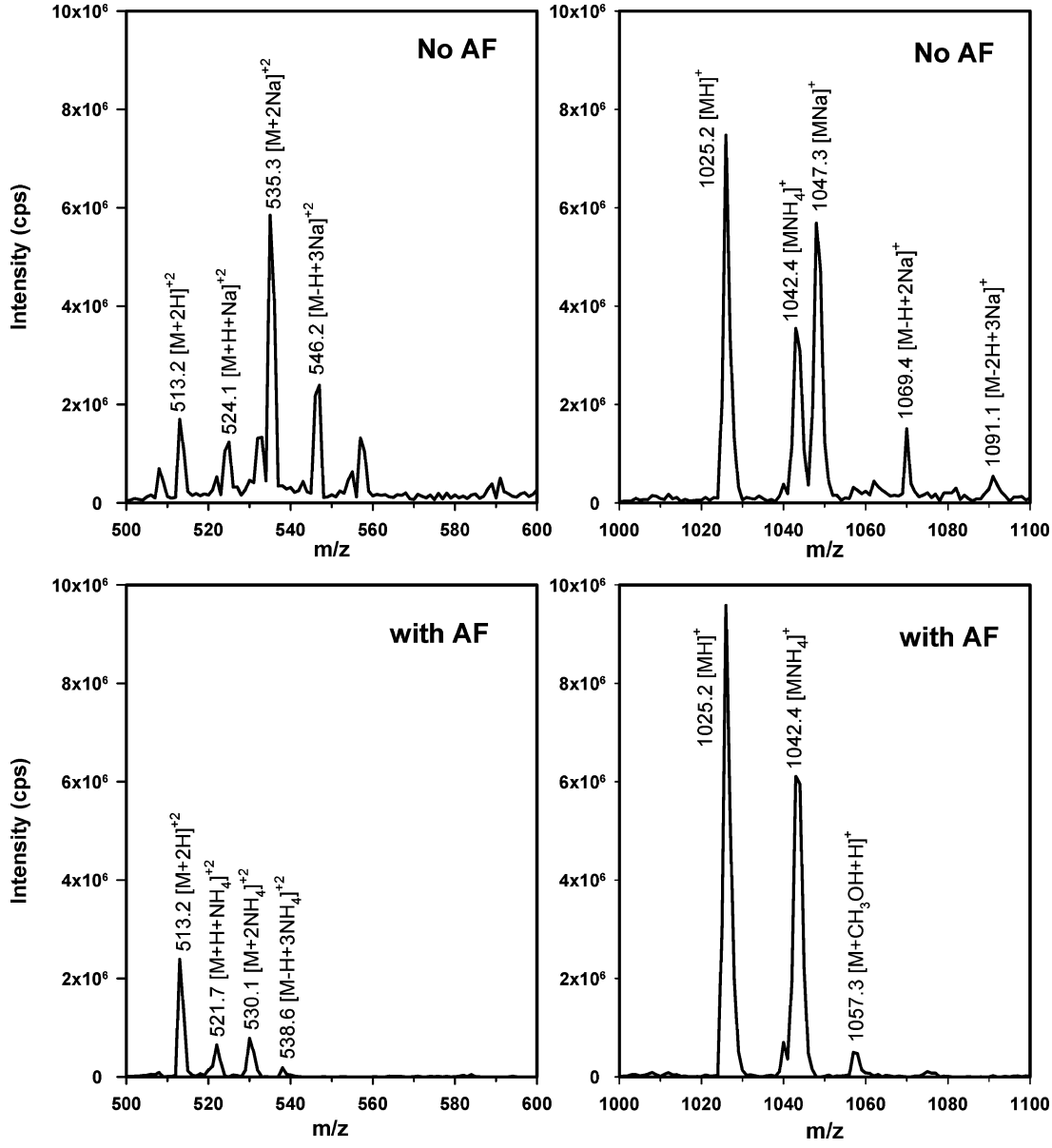

Figure 5. Spectral changes on addition of ammonium formate. For MCLW and other neutral microcystins under nominal conditions (upper) MH^+, MNa^+, and sodium substitution ions predominate.

there was some instability, both MH^+ and MNH_4^+ signals intensified with continued infusion of the buffer.

The SRI showed the opposite trend: stopping the AF flow increased the +1 SRI by 3.0-fold. When the buffer flow was started there was a 67% reduction in SRI signal. The SRI signal fades slightly, possibly because of depletion of the adventitious Na^+ ions in the system.

The spectral changes in the +2 charge state were similar with a marked drop in SRI as the buffer flow started; in ~10 s there was 75% SRI signal suppression. The reverse process, removal of buffer from the system, required 3 times as long. The $[M+2H]^{+2}$ signal, about 30% of either MH^+ or MNH_4^+ ions, was unstable and not clearly responsive to AF. For arginine-containing MC, AF addition also suppressed SRI and enhanced $[M+2H]^{+2}$.

The buffer effects were reproduced in LC-MS operation. In particular, the spectra of the neutral MC (MCLA, MCLW, MCLF) were simplified because of a reduction in the SRI. For each neutral MC a strong triplet of ions was observed (MNH_4^+ intensity > MH^+ > MNa^+), and the +2 charge state ions were diminished.

The influence of AF on the MCLR m/z 135 precursor spectrum was dramatic in LC-MS-MS. Without buffer there were three major precursors: m/z 517 ($[M+H+K]^{+2}$ and $[M+Na+NH_4]^{+2}$); m/z 509 ($[M+H+Na]^{+2}$); and m/z 528 ($[M+Na+K]^{+2}$). With added AF, two precursor ions were prominent: m/z 498 ($[M+2H]^{+2}$) and m/z 509. Not only was the spectrum shifted to lower masses, but the $498^{+2} > 135^+$ signal was enhanced 600-fold, and the $509^{+2} > 135^+$ signal was 20-fold greater (collision energy, 20 V; scan range, 450−600 Da). Therefore, AF simultaneously suppresses ($517^{+2} > 135^+$) and enhances ESI signals ($498^{+2} > 135^+$) depending on the transition. Similar increases in signal for the $[M+2H]^{+2} > 135^+$, $[M+Na+H]^{+2} > 135^+$, and $[M+NH_4+H]^{+2} > 135^+$ transitions were seen with the other arginine-containing MC (MCRR, MCYR), as well as MCLW.

The ammonium adducts efficiently fragmented at low collision energies (14−16 V) with loss of ammonia forming MH^+ ions.

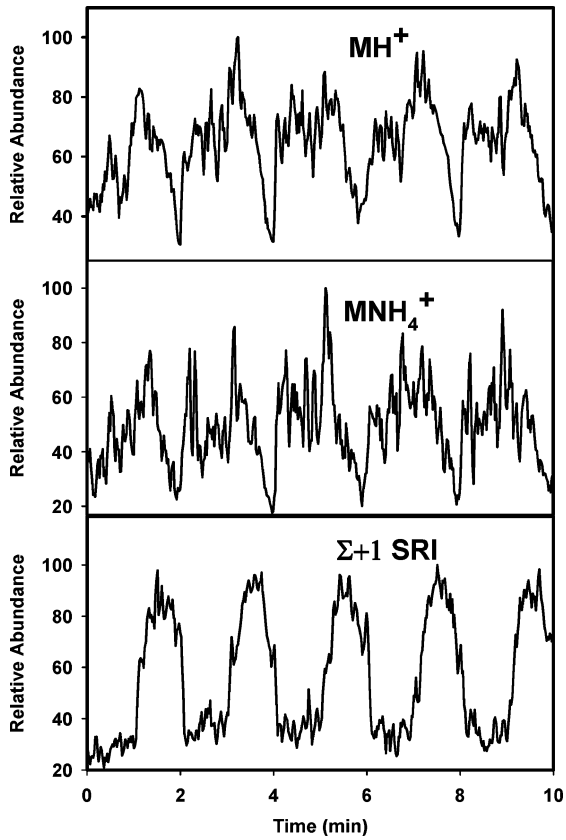

Figure 6. Pulsed addition of ammonium formate buffer to MCLW using the apparatus in Figure 2b. Reconstructed ion current is plotted for MH^+ (upper), MNH_4^+ (middle), and the sum of three +1 charge state SRI (Σ MNa^+, $[M+2Na-H]^+$, $[M+3Na-2H]^+$). The total ion current for the 500−1120 Da scan range was ~2.5 times higher with no AF, and the experiment begins with AF on for 60 s, then off for 60 s, etc.

With a collision energy of 20−22V the MH^+ ions lose Ø−CH=CH−O−CH_3 generating the corresponding [MH − 134]$^+$ ions and *m/z* 375 ([$C_{11}H_{25}O$ − Glu − Mdha]$^+$) fragment ion. Sodium adducts in contrast require much higher collision energies (48−60V) and produce predominantly the *m/z* 347 and *m/z* 419 product ions.

Congener-Specific Transitions in Analysis of Algal Bloom Samples. Use of the ammonium formate buffer modification has been adopted as the standard procedure for LC-MS-MS determination of MC in our laboratory. Similar results are achieved by AF addition post-column or by addition to the HPLC mobile phase reservoirs. Two transitions are monitored for each MC including the nonspecific transitions to *m/z* 135 and a congener-specific transition (Table 1). Excellent correspondence between these transitions was observed in bloom samples with MCLR concentrations varying over 2- to 3- orders of magnitude (Figure 7) and greater than 3 orders of magnitude with other MC variants. Were MC co-elutions and tandem MS interferences to occur, a disparity between the transitions would be apparent.

Mechanisms. The present study demonstrates that electrolyte-induced ionization suppression can be both detrimental (signal suppression, sodium adduction) and beneficial (signal enhancement, diminished sodium adduction) in ESI mass spectrometry. The deleterious effects relate to both adventitious and matrix electrolytes which vary in concentration leading to both poor spectrum reproducibility and reduced quantitative and qualitative method performance. In the extreme, high levels of sodium can totally suppress ionization.[34,28]

Controlled addition of electrolyte such as ammonium formate may create a more stable chemical environment. The introduced electrolyte resists composition fluctuations that exist between calibrators and complex samples moderating matrix effects and improving qualitative and quantitative method performance.

Electrolytes impact ESI by physical, electrochemical, and chemical mechanisms. Electrolytes influence the physical properties of droplets by changing surface tension, ionic strength, conductivity, droplet size, and surface area. Physical effects influence the partitioning of the analyte between the bulk liquid and the charged surface layer,[22] the analyte population on the charged droplet surface, and the rate of ion evaporation. Changes in the size of electrosprayed particles, especially in relation to particle surface area, may influence both the rate of desolvation and the transfer of analyte ions to the gas phase.

ESI is dependent upon the excess charge carried by droplets, and ionic strength/conductivity may influence the capacity to sustain this charge. Electrolytes also influence the repulsive forces leading to coloumbic explosion, and electrolytes (ionic liquids) are essential for ESI in non-polar solvents.[40] Chemical equilibria have received little attention in relation to electrolyte-induced ionization suppression mechanisms.

The chemical effects of ammonium formate electrolyte were manifested in distinct spectral changes. Principally, the dominanting SRI were replaced by MH^+ and MNH_4^+ adduct ions in neutral MC. For arginine-containing MC the SRI of both the +1 and +2 charge states were reduced relative to protiated ions such as $[M+2H]^{+2}$. These spectral changes can be explained by displacement of chemical equilibria as shown in eqs 1−7.

$$RNa^+ + NH_4^+ <> RNH_4^+ + Na^+ \quad (1)$$

$$RNH_4^+ <> RH^+ + NH_3 \quad (2)$$

$$R + NH_4^+ <> RH^+ + NH_3 \quad (3)$$

$$[R + Na + H]^{+2} + NH_4^+ <> [R + NH_4 + H]^{+2} + Na^+ \quad (4)$$

$$[R + NH_4 + H]^{+2} <> [R + 2H]^{+2} + NH_3 \quad (5)$$

$$RH^+ + NH_4^+ <> [R + 2H]^{+2} + NH_3 \quad (6)$$

$$[R + NH_4 + H]^{+2} <> RH^+ + NH_4^+ \quad (7)$$

Sodium ions are displaced in reactions 1 and 4. The ammonium adducts lose volatile ammonia via reactions 2 and 5. The ammonium ion also protonates MC (reaction 3) and singly charged MC (reaction 6). In reaction 7 the charge state is reduced with the loss of ammonium cation. The net effect of reactions 1 coupled

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Table 1. Microcystin Transitions Used in Ammonium Formate Modified ESI LCMSMS Method

microcystin variant	retention time (min)	transition	transition (mass)[d]	low standard concentration (μg/L)[e]	R^{2f}
MCRR_Q1[a]	8.9	$[M+2H]^{+2} > C_9H_{11}O^+$	$520.0^{+2} > 135^+$	2.0	0.98
MCRR_Q2[b]		$[M+2H]^{+2} > [M+2H - C_9H_{11}O - NH_3]^{+c}$	$520.0^{+2} > 887^+$	6.0	0.97
MCYR_Q1	8.8	$[M+2H]^{+2} > C_9H_{11}O^+$	$523.7^{+2} > 135^+$	2.0	0.99
MCYR_Q2		$[M+2H]^{+2} > [M+2H - C_9H_{11}O]^+$	$523.7^{+2} > 911.7^+$	3.0	0.99
MCLR_Q1	9.3	$[M+2H]^{+2} > C_9H_{11}O^+$	$498.7^{+2} > 135^+$	2.5	0.99
MCLR_Q2		$[M+2H]^{+2} > [M+2H - C_9H_{11}O]^+$	$498.7^{+2} > 861.6^+$	3.0	0.99
MCLA_Q1	10.6	$MNH_4^+ > MH^+$	$927.5^+ > 910.4^+$	2.0	0.99
MCLA_Q2		$MH^+ > [MH - C_9H_{10}O]^+$	$910.5^+ > 776.2^+$	2.5	0.98
MCLW_Q1	11.6	$MNH_4^+ > MH^+$	$1042.5^+ > 1025.2^+$	2.5	0.98
MCLW_Q2		$MH^+ > [MH - C_9H_{10}O]^+$	$1025.5^+ > 891.2^+$	4.0	0.99
MCLF_Q1	12.2	$MNH_4^+ > MH^+$	$1003.5^+ > 986.5^+$	2.5	0.99
MCLF_Q2		$MH^+ > [C_{11}H_{25}O - Glu - Mdha]^{+c}$	$986.5^+ > 375^+$	5	0.97

[a] Primary quantitation transition. [b] Alternate or confirming transition. [c] Fragment structures from ref 36. [d] The source CID voltage was 12V, and the collision energy was 20 V for all microcystins except MCLW where the collision energy was 22 V. [e] Lowest concentration producing a integrated peak, approximately the instrument detection limit. Note: the injection volume was limited to 8 μL with the capillary HPLC system used. [f] Linear correlation coefficient across calibration range - high calibrator concentration was 250 μg/L.

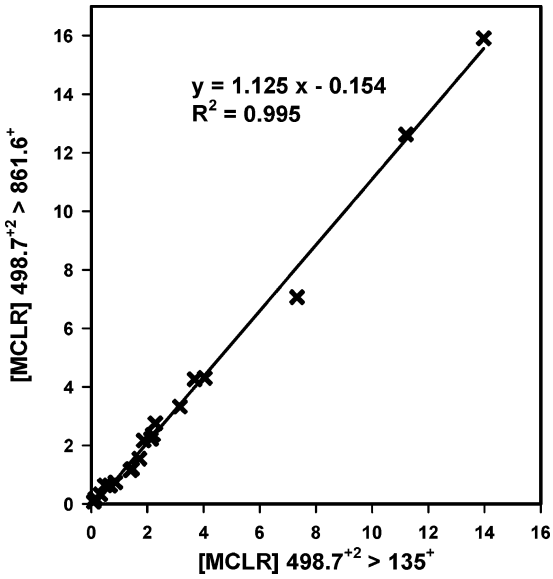

Figure 7. MCLR concentrations in algal bloom samples from the Klamath River of northern California (August, 2007). MCLR concentrations were based on both specific ($498.7^{+2} > 861.6^+$) and nonspecific ($498.7^{+2} > 135^+$) transitions. Cells were lysed to release MC, and lysates enriched by SPE before analysis.

with 2 and 4 coupled with 5 is substitution of Na^+ with H^+. A further possibility is that sodium ions displaced are precipitated within the ESI droplet.[28]

CONCLUSION

This paper has demonstrated the influence of electrolytes on LC-MS-MS determination of microcystin toxins. Specific MC detection is compromised by their strong tendency to form SRI evident as extensive ion envelopes in both the +1 and +2 charge states, especially in protic solvents. Certain MC transitions are particularly nonspecific, notably low energy fragmentation of doubly charged ions to the $[Ø—CH_2—CH═O—CH_3]^+$ product ion. *M/z* 135 precursor spectra may have 20 or more intense ions spaced at 1/2 Da. Ammonium formate is shown to improve specificity by substantially suppressing SRI resulting in simplified ESI spectra with prominent MH^+ and MNH_4^+ adducts. LC-MS-MS methods utilizing multiple and especially congener-specific transitions including $MNH_4^+ > MH^+$ and $[M + 2H]^{+2} > [M + 2H - 135]^+$ are more reliable for determination of MC in complex samples such as algal blooms and pond scum.

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