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Quantitative Measurements of Small Molecule Mixtures Using Laser Electrospray Mass Spectrometry

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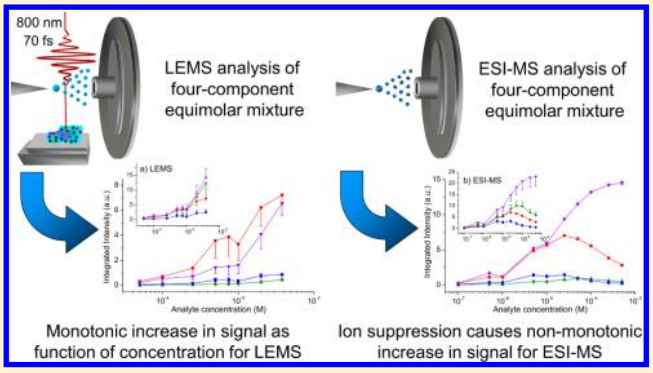
Supporting Information

ABSTRACT: Quantitative measurements of atenolol, tioconazole, tetraethylammonium bromide, and tetrabutylammonium iodide using laser electrospray mass spectrometry (LEMS) reveal monotonic signal response as a function of concentration for single analytes, two- and four-component equimolar mixtures, and two-component variable molarity mixtures. LEMS analyses of single analytes as a function of concentration were linear over ~2.5 orders of magnitude for all four analytes and displayed no sign of saturation. Corresponding electrospray ionization (ESI) measurements displayed a nonmonotonic increase as saturation occurred at higher concentrations. In contrast to the LEMS experiments, the intensity ratios from control experiments using conventional ESI-MS deviated from expected values for the equimolar mixture measurements due to ion suppression of less surface active analytes, particularly in the analysis of the four-component mixture. In the analyses of two-component nonequimolar mixtures, both techniques were able to determine the concentration ratios after adjustment with response factors although conventional ESI-MS was subject to a greater degree of saturation and ion suppression at higher analyte concentrations.



Electrospray ionization-mass spectrometry (ESI-MS)[1,2] can transfer large, nonvolatile analytes into the gas phase for mass analysis and is widely used in the analysis of a variety of biomolecules. The correlation of mass spectral response with analyte concentration is important for quantitative analysis but is challenging in the analysis of complex samples. Even quantification with widely used stable-isotope-labeled internal standards, which are chemical and structural analogues to the analyte of interest but differ in molecular mass, can result in suppression of the analyte signal and incorrect quantitation,[3,4] particularly when there are large matrix effects.[5−7] Ion suppression by other species in solution affects the mass spectral response of the analyte of interest[8,9] due to the reduction in the amount of analyte that reaches the detector.[10,11] Ion suppression can limit the linear response range of an analyte in ESI-MS analysis, typically spanning up to five or more orders of magnitude[11] with an upper concentration limit of ~10^{-5} M before the analyte signal saturates.[9,12−19]

The mechanism of ion suppression can be understood in the context of ion formation. ESI produces highly charged droplets that become smaller due to solvent evaporation. As the charge density increases and reaches the Rayleigh limit, Coulomb explosion produces smaller, offspring droplets. The continuous evaporation and fission events eventually produce gas phase analyte ions. Smaller analytes are assumed to follow the ion evaporation model (IEM)[20,21] whereas larger analytes, such as proteins, follow the charged residue model (CRM).[22,23] A recent model combines aspects of the IEM and CRM where the charge state of a macromolecule is determined by the emission of small charge carriers from the droplet during solvent evaporation.[24,25] This theory explains the shift in charge state distributions of native state proteins with varying electrolytes in the ESI-MS solution.

Models based on the ion evaporation theory have been proposed to explain the mass spectral response of singly charged analytes as a function of concentration. Quaternary ammonium ions with longer alkyl chains were observed to have higher mass spectral response than those with shorter alkyl chains.[26] The proposed mechanism, according to IEM,[20] is that surface-active analytes with a higher degree of nonpolar character reside at the liquid−air interface of the droplets and evaporate preferentially in comparison with the more polar analytes in the droplet interior. A theory based on the ion evaporation rates was developed to predict the mass spectral response.[9,12,27] In the analysis of simple cations, solvation energies were inversely correlated with mass spectral response as less solvated ions presumably have higher evaporation rates. However, evaporation rates could not fully explain ESI response when more complex molecules were examined and surface activity had to be included to predict mass spectral response.[9]

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The equilibrium-partitioning model[13,28] considers the degree of nonpolarity on mass spectral response of analytes without ion evaporation rates. This model introduced the notion of excess charge, [Q], the amount of charge produced in the electrospray process that is available to produce gas phase ions. Excess charge is expressed as a concentration (mol/L) and is proportional to the ESI spray current and inversely proportional to the flow rate.[13] Analytes partition between the two phases of the ESI droplet, an electrically neutral interior where charge balance is reached through ion pairing and an outer surface where the excess charge resides. In this model, the concentration of the analyte on the surface is directly related to mass spectral response. When the sum of the available charge sites of the analytes in the solution, C_{QT}, is less than [Q], the system is in the charge surplus limit and mass spectral response is linear with concentration as the excess charge can in principle ionize all molecules in the droplet.[13,16,17,29,30] The charge deficiency scenario occurs when C_{QT} is greater than [Q], resulting in deviations from linearity. This deficiency causes analytes to compete for charge resulting in preferential ionization of analytes with high surface partitioning coefficients. There are many examples of the preferential instrumental response of surface-active analytes that have significant nonpolar regions.[10] For instance, peptides with more extensive nonpolar regions had higher mass spectral response than those with polar side chains.[14,31] Also, octadecylamine, an extremely surface-active surfactant, was shown to severely suppress the mass spectral response of a low surface-active analyte, a polar amino acid, under charge deficient conditions.[10] Although the original equilibrium-partitioning model could not accurately predict the effect of an electrolyte or additive on the mass spectral response,[8] a newer model with modified partition coefficients properly accounts for the effects of additives.[18]

There have been many studies on the quantitative ability for analyzing complex mixtures using ESI-MS; however, there are a limited number of studies on quantitative mass spectrometry where laser vaporization is coupled with electrospray capture and postionization. While electrospray-assisted laser desorption ionization (ELDI)[32] and matrix-assisted laser desorption electrospray ionization (MALDESI)[33] have been used to analyze protein mixtures, the quantitative aspects have not been addressed yet. Laser ablation electrospray ionization (LAESI) showed a 4 orders of magnitude linear dynamic range for verapamil and no ion suppression effects in the analysis of a multicomponent small analyte mixture.[34] However, subsequent LAESI investigations of tissues revealed significant matrix and suppression effects and required mathematical modeling to provide quantitative measurement.[35,36]

Few quantitative studies have been performed on complex mixtures using laser electrospray mass spectrometry (LEMS). LEMS combines a nonresonant, femtosecond (fs) laser for vaporization (10^{13} W/cm^2) and an ESI source for postionization for ambient mass spectrometric analysis. The LEMS signal as a function of concentration was determined to be linear for a mixture of two lipids in an anticorrelated concentration study.[37] Three orders of magnitude linear range was demonstrated using ammonium and nitrate ions in the analysis of a mixture of anions and cations vaporized from inorganic improvised explosive signatures using complexation agents in the electrospray plume to enable mass spectral detection.[38] LEMS has also enabled mass spectral analysis of small biomolecules,[39] proteins,[40,41] lipids,[37] explosives,[38,42] smokeless powders,[43] narcotics,[44] pharmaceuticals,[44] and tissues.[45,46]

In this paper, a comprehensive study is performed on the quantitative ability of LEMS in the analysis of small, singly charged ions and compared with ESI-MS. The mass spectral responses as a function of concentration in different experimental scenarios, including analyses of two- and four-component equimolar mixtures and multiple two-component nonequimolar mixtures, are measured to directly compare ion suppression effects for ESI-MS and LEMS.

EXPERIMENTAL SECTION

Materials. The solid samples of atenolol (Spectrum Chemical, New Brunswick, NJ), tioconazole (Sigma Aldrich, St. Louis, MO), tetraethylammonium bromide (TEABr) (Sigma Aldrich), and tetrabutylammonium iodide (TBAI) (Sigma Aldrich) were used as obtained. The structures of the analytes are shown in Figure S1 (Supporting Information). For conventional ESI-MS analyses, analyte stock solutions were prepared in plastic containers at a concentration of 5×10^{-2} M using HPLC grade methanol (EMD Chemicals, Gibbstown, NJ), and for LEMS analyses, analyte stock solutions were prepared in plastic containers at a concentration of 1.5×10^{-2} M using HPLC grade acetonitrile (Fisher Scientific, Fair Lawn, NJ).

Sample Preparation. The analytes were diluted from the stock solutions using serial dilutions with methanol, HPLC grade water (Fisher Scientific), and acetic acid (Fisher Scientific) to yield a 1:1 (v/v) water−methanol, 1% acetic acid solution for ESI-MS analyses or diluted with acetonitrile for LEMS analyses. Solutions for single analyte and equimolar mixture analyses, including two- and four-component equimolar mixtures, were prepared using atenolol, tioconazole, TEABr, and TBAI. Atenolol and tioconazole were used in the analysis of two-component nonequimolar mixtures. In the first nonequimolar mixture analysis, one analyte has a five times greater concentration than the other (abbreviated 5× Z, where Z is the analyte having higher concentration). In the second nonequimolar mixture experiment, the concentration of one analyte is kept constant as the concentration of the other analyte is varied (abbreviated CYVZ where Y is the analyte kept at a constant concentration and Z is the analyte with variable concentration). Table S1 in the Supporting Information lists the concentration ranges used for all experiments in this study.

Samples for LEMS analyses were prepared by spotting 15 μL aliquots on 7 mm × 7 mm stainless steel sample plates, with each solution spotted on multiple sample slides. After allowing the sample slides to dry, the procedure was repeated, resulting in 30 μL of each solution deposited onto multiple sample slides. The sample slides were placed into the LEMS source chamber on a metal plate attached to a three-dimensional translational stage, which allowed raster scanning in order for new sample to be analyzed with every laser pulse.

Laser Vaporization. The instrumentation for laser vaporization, electrospray postionization, and mass spectral detection has been previously described in detail.[37−42,44−46] The femtosecond laser system used for vaporization consists of a Ti:sapphire oscillator (KM Laboratories, Inc., Boulder, CO, USA) and a regenerative amplifier (Coherent, Inc., Santa Clara, CA, USA) to create 70 fs, 1.5 mJ pulses (reduced from 2.5 mJ using a neutral density filter) centered at 800 nm. The laser pulse, operated at 10 Hz to enable analysis of fresh sample and synchronization with the hexapole operating in the trapping mode,[39,46] was focused using a 16.9 cm focal length lens with an incident angle of 45° with respect to the sample to a spot

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size of ~350 μm in diameter, resulting in an intensity of ~2 × 10^{13} W/cm^2 at the area sampled.

Ionization and Mass Spectrometry. After vaporization, the sample material is captured, ionized, and transferred into a mass spectrometer by an electrospray source (Analytica of Branford, Inc., Branford, CT). The acidified electrospray solvent, 1:1 (v/v) water−methanol with 1% acetic acid, was pumped through the ESI needle by a syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 3 μL/min. The electrospray plume was dried by counter current nitrogen gas at 180 °C before entering the inlet capillary. After trapping in the hexapole for 250 μs, the ions were transferred via a RF only hexapole into a pulsed orthogonal time-of-flight, where mass separation and detection occurred. An ESI solvent background mass spectrum was acquired before vaporization of each sample set to allow background subtraction of solvent-related peaks. Each LEMS experiment resulted from the averaging of 50 laser shots, which were averaged using a digital oscilloscope (LeCroy Wavesurfer 422, LeCroy Co., Chestnut Ridge, NY). At least 15 separate measurements were performed at each concentration for LEMS analyses using multiple sample slides. Five mass spectra, each consisting of averaging for 5 s, were collected for conventional ESI-MS analyses.

Calculation of Integrated Intensities. The raw mass spectral data files obtained from the digital oscilloscope were saved using a custom Labview 8.5 program (National Instruments, Austin, TX) and imported into the Cutter program[47] for integration of the peaks of interest. Prior to integration, baseline subtraction and spectral realignment were performed to ensure consistent integration. Integrated intensities were determined for the four analytes of interest by integration of their major mass spectral features, $[TEA]^+$ at m/z 130.0, $[TBA]^+$ at 242.5, $[atenolol + H^+]^+$ at 267.0, and $[tioconazole + H^+]^+$ at 389.0 for TEABr, TBAI, atenolol, and tioconazole, respectively. The integrated intensities of TBA included a fragment peak ($[TBA^+ - C_4H_9]^+$) at m/z 185.5 for LEMS analyses but not for ESI-MS analyses as it was only prevalent in the LEMS experiments. The integrated intensities were then plotted versus the analyte concentration with LEMS and ESI-MS to compare quantitative ability and suppression effects.

Note that the plots of the integrated intensities as a function of analyte concentration may not produce the best comparison between the two techniques as the analyte consumed per 5 s experiment is different for conventional ESI-MS and LEMS analyses at the same listed concentration. The amount of analyte consumed can be calculated from the concentration of the solution and the volume delivered during analysis (0.25 μL to acquire a 5 s averaged mass spectra) for ESI-MS and from the amount of analyte deposited on the sample plate (30 μL times the solution concentration) and the 50 laser shots applied to acquire ~8 mass spectra per sample slide for LEMS. The amount of analyte consumed is over 5 and 6 orders of magnitude smaller than the concentration listed for LEMS and ESI, respectively (e.g., 3.75 × 10^{-9} mol of analyte consumed per LEMS experiment for the listed concentration of 1 × 10^{-3} M).

Safety Considerations. Appropriate laser eye protection was worn by all lab personnel.

RESULTS AND DISCUSSION

Analysis of Single Analyte Solutions and Calculation of Response Factors. A series of experiments was performed to test for quantitative ability and potential ion suppression effects in the analysis of multicomponent small analyte mixtures using laser electrospray mass spectrometry, which were compared with corresponding ESI-MS control experiments. The four model analytes investigated include atenolol, tioconazole, tetraethylammonium bromide (TEABr), and tetrabutylammonium iodide (TBAI), each of which results in a singly charged ion. The structures are shown in Figure S1 in the Supporting Information. Atenolol and tioconazole are two pharmaceutical compounds requiring protonation to enable mass detection. TEABr and TBAI are symmetrical quaternary ammonium salts where the magnitude of hydrophobicity and instrumental response is determined by the length of their carbon chains.[9,12,13] Tetraalkylammonium salts have been extensively studied as counterions in anionic surfactant systems.[48−51] The TBA salt can self-aggregate to form a surfactant at high concentrations[50] and has been used in mixed cationic−anionic (catanionic) surfactant mixtures for cloud point extraction of pesticide residues from water and fruit juice samples.[51] Therefore, according to the equilibrium-partitioning model, TBA is expected to have a high instrumental response and potentially suppress other analyte response.[10]

The integrated intensities of the four analytes are shown in Figure 1 as a function of concentration in pure solution for both LEMS and ESI-MS. Both mass spectral methods resulted in tioconazole having the highest instrumental response at all concentrations. The next highest response occurred for TBA, followed by atenolol and TEA. We propose that the intensity trend follows the equilibrium-partitioning model,[13] wherein tioconazole and TBA have higher mass spectral responses than

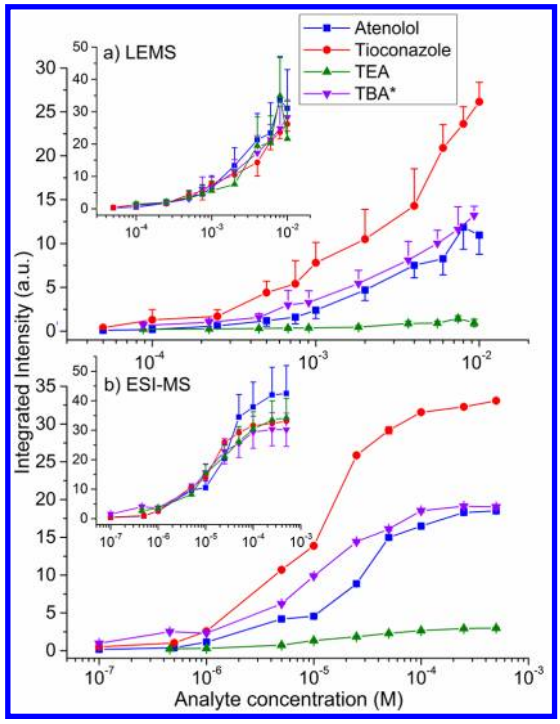

Figure 1. Integrated intensity plots for the analysis of single analytes including atenolol, tioconazole, tetraethylammonium bromide (TEA), and tetrabutylammonium iodide (TBA) using (a) LEMS and (b) ESI-MS. The insets in (a) and (b) are plots of the calibrated intensities after normalization to tioconazole. *The integrated intensity for the LEMS analysis of TBA includes the fragment at m/z 185.5, which corresponds to $[TBA^+ - C_4H_9]^+$.

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atenolol and TEA because they have higher surface activities due to decreased polarity, which was verified using reversed phase HPLC-ESI-MS (Figure S2 in the Supporting Information). Under ideal conditions, the analytes should have similar instrumental responses until the excess charge limit ([Q]) is approached at higher concentrations. However, the analytes have different instrumental responses at low concentrations since the electrolyte concentration, C_E, or in this case, the additive (acetic acid) concentration, C_{Add}, does not equal [Q].[13] Note that the additive has a higher effective concentration than the electrolytes from solvent impurities,[9] ~1.7 × 10^{-4} M vs ~10^{-5} M, respectively.

The average response factors are shown in Table 1 in which each ion's intensity was normalized to tioconazole. Response factors were calculated using the single analyte measurements by averaging the analyte/tioconazole integrated intensity ratios throughout the broad concentration ranges of 5 × 10^{-5} M to 1 × 10^{-2} M and 1 × 10^{-7} M to 5 × 10^{-4} M for LEMS and ESI-MS, respectively. Assuming that the equilibrium-partitioning model applies to these analyses, the response factor of analyte I, f_I, is directly related to the partition coefficient, K_I, as the instrumental response varies based on K_I/K_E, where K_E is the partition coefficient of the electrolyte, when C_E is greater than [Q].[13] The normalized response factors, $f_I/f_{tioconazole}$, which are the normalized partition coefficients according to the Enke model,[13] $K_I/K_{tioconazole}$, could be used to correct for low mass spectral intensities when attempting quantitative analysis. The insets in Figure 1 provide the normalized integrated intensities for the four molecules in pure analyses using LEMS and ESI-MS. The normalized intensity ratios remain approximately at one throughout the concentration range, as shown in Figure S3a,b (Supporting Information). Since the analytes have the same concentration, the normalized measurements demonstrate the ability to perform quantitative analysis of single analyte solutions with LEMS and conventional ESI-MS.

Table 1. Average Calculated Response Factors for LEMS and ESI-MS Analyses Normalized to Tioconazole Intensity

	calculated response factors, f_I	
analyte	LEMS	ESI-MS
atenolol	0.35 ± 0.12	0.44 ± 0.10
tioconazole	1	1
TEA	0.04 ± 0.01	0.09 ± 0.02
TBA	0.46 ± 0.07	0.63 ± 0.12

With ESI-MS analyses, the analyte signal response saturates at higher concentrations, which is presumably due to the excess charge limit being approached.[13] In the LEMS measurements, the analyte signal responses are essentially linear throughout the whole concentration range. Table S2 in the Supporting Information lists the linearity regions for all experimental analyses using conventional ESI-MS and LEMS. An approximate value of [Q] can be determined by finding the concentration where deviation from linearity (saturation) occurs for the analyte with the highest response.[13,14] The saturation concentration is approximately 5 × 10^{-5} M for tioconazole in ESI-MS analysis. The estimated excess charge concentration of the initial electrospray droplet can also be calculated with the equation[29,30,52,53]

$$[Q] = \frac{4.2}{F}\left(\frac{K\gamma}{V_f \kappa_e}\right)^{1/2}$$

where F is the Faraday constant, K is the conductivity of the solution, γ is the surface tension, κ_e is the dielectric constant, and V_f is the flow rate of the electrospray solution. For a 1:1 (v/v) water−methanol solution with 1% acetic acid, using values

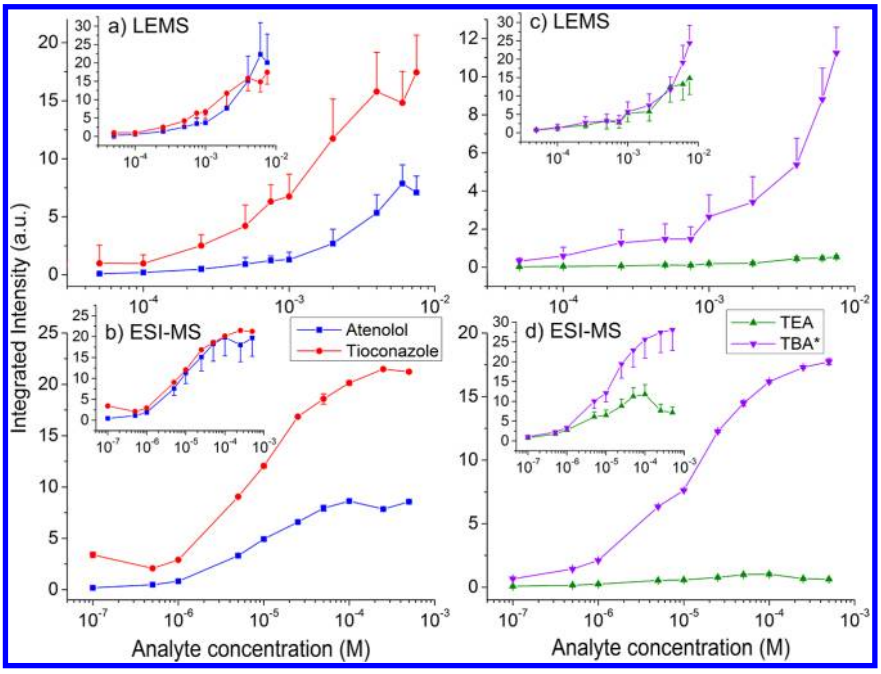

Figure 2. Integrated intensity plots for the analysis of two-component equimolar mixtures of atenolol and tioconazole using (a) LEMS and (b) ESI-MS and of tetraethylammonium bromide (TEA) and tetrabutylammonium iodide (TBA) using (c) LEMS and (d) ESI-MS. The insets are plots of the calibrated intensities after normalization to tioconazole. *The integrated intensity for the LEMS analysis of TBA includes the fragment at m/z 185.5, which corresponds to $[TBA^+ - C_4H_9]^+$.

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from the literature[30,53] of K = 0.4 S/m, κ_e = 56, and γ = 0.047 N/m, the calculated value of $[Q]$ at a flow rate of 3 μL/min is approximately 1.1 × 10^{-4} M. The calculated $[Q]$ is in good agreement with the ESI-MS measurement as nonlinear response typically starts to occur as the total charge of the analytes in solution approaches the excess charge limit.

Analysis of Two-Component Equimolar Mixtures. Analyses of equimolar mixtures were performed to determine the effect of ion suppression, and therefore $[Q]$, on the quantitative ability of LEMS in comparison with ESI-MS. The mass spectral responses are plotted as a function of concentration for the two-component equimolar mixtures of atenolol and tioconazole in Figure 2a,b, and for TEABr and TBAI in Figure 2c,d, using LEMS and ESI-MS, respectively. The mass spectral responses for the equimolar mixtures are similar to the analyses of the single analytes (Figure 1), except for a slight decrease in the overall intensities. As seen in single analyte analyses, tioconazole and TBA have higher mass spectral responses presumably due to higher partitioning coefficients than atenolol and TEA. Without the response factors, determination of the concentration would be impossible, especially in the case of TEA and TBA (Figure 2c,d), where the intensity of TBA reaches a ~33× and ~20× greater intensity than TEA at higher concentrations using ESI-MS and LEMS, respectively. Only after correction of the intensities with the instrumental response factors, as seen in insets of Figure 2, do we find that these are equimolar mixtures (as seen in Figure S3a,b in the Supporting Information), although the intensity ratio of TEA/TBA is much lower than one for ESI-MS due to the ion suppression. Performing quantitative analysis is impossible without the response factors in the analysis of mixtures using either ESI-MS or LEMS, particularly when $[Q]$ does not equal C_E.

ESI-MS provides a linear response for equimolar mixtures of atenolol and tioconazole and of TEA and TBA over 2 orders of magnitude in concentration (from 1 × 10^{-7} M to 1 × 10^{-5} M for atenolol and tioconazole, to 5 × 10^{-5} M for TEA, and to 2.5 × 10^{-5} M for TBA). Near the excess charge limit, TBA saturates and suppresses the instrumental response of TEA at higher concentrations, as seen in the inset in Figure 2d. Tioconazole does not suppress atenolol to the same extent as seen in the ratio of their experimentally derived response factors or partition coefficients assuming that the equilibrium-partitioning model is valid in these analyses (f_A/f_T or K_A/K_T = 0.44 vs f_{TEA}/f_{TBA} or K_{TEA}/K_{TBA} = 0.14 for ESI-MS). Saturation and suppression occurs in ESI-MS analyses at higher concentrations, which is presumably due to surpassing the excess charge limit according to the equilibrium-partitioning model, and this suppression restricts the quantitative capability of ESI-MS.

LEMS provides a linear response over the entire concentration range investigated, from 5 × 10^{-5} M to 7.5 × 10^{-3} M, for all four analytes in the two-component equimolar mixtures. Two-component analyses with LEMS provides little to no ion suppression in either equimolar mixture analysis and provides calibrated intensities for the pair of mixtures (insets in Figure 2a,c) with an intensity ratio close to one over the concentration range investigated (Figure S3c, Supporting Information). This suggests that LEMS can be used in the quantitative analysis of two-component equimolar mixtures with the use of response factors.

Analysis of Four-Component Equimolar Mixture. An equimolar mixture containing all four model analytes was investigated as a function of concentration using LEMS and ESI-MS. The integrated intensity plots are seen in Figure 3a,b

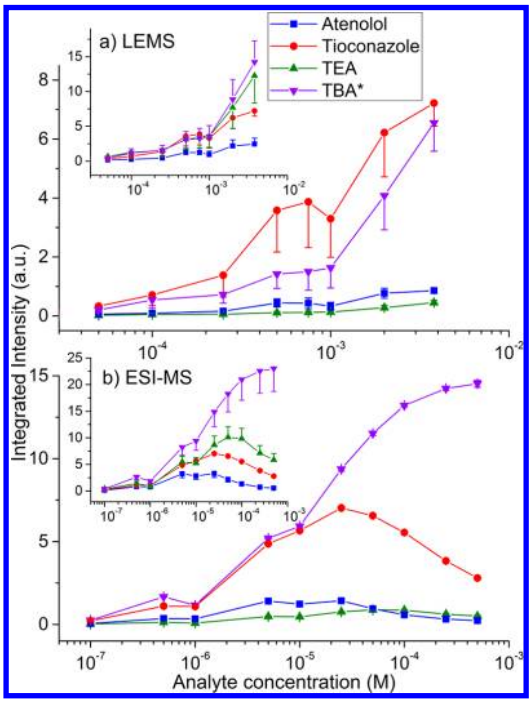

Figure 3. Integrated intensity plots for the analysis of four-component equimolar mixtures of atenolol, tioconazole, tetraethylammonium bromide (TEA), and tetrabutylammonium iodide (TBA) using (a) LEMS and (b) ESI-MS. The insets in (a) and (b) are plots of the calibrated intensities after normalization to tioconazole. *The integrated intensity for the LEMS analysis of TBA includes the fragment at m/z 185.5, which corresponds to $[TBA^+ - C_4H_9]^+$.

for LEMS and ESI-MS, respectively. In the analysis using LEMS, the mass spectral responses of the four analytes in the complex mixture are linear with concentration and are in agreement with the calculated response factors derived from single analyte analysis. Once again, tioconazole has the highest instrumental response, followed by TBA, atenolol, and TEA in descending order. The corrected instrumental responses using LEMS, the inset in Figure 3a, for tioconazole, TBA, and TEA are similar in magnitude, resulting in analyte/tioconazole intensity ratios of approximately one (Figure S3c, Supporting Information). This is expected for equimolar mixtures. However, the atenolol corrected intensity was lower than the other three analytes, resulting in an atenolol:tioconazole ratio of ~0.5. The intensity of the four analytes was fairly linear over the whole concentration range investigated (5 × 10^{-5} M to 3.8 × 10^{-3} M) with LEMS. There may be slight ion suppression at higher concentrations as TBA and TEA have higher adjusted mass spectral intensities than tioconazole.

The characteristics of the integrated intensities for the four-component equimolar mixture with ESI-MS were quite different than those obtained with LEMS. The mass spectral responses of the complex mixture with ESI-MS (Figure 3b) resulted in TBA and tioconazole having the highest intensities, followed by atenolol and TEA. Unlike the single analyte and LEMS four-component analyses, TBA had the highest intensity, resulting in significant suppression of the tioconazole, atenolol, and TEA instrumental responses at higher concentrations. This is not surprising since ionic surfactants, including TBA, have

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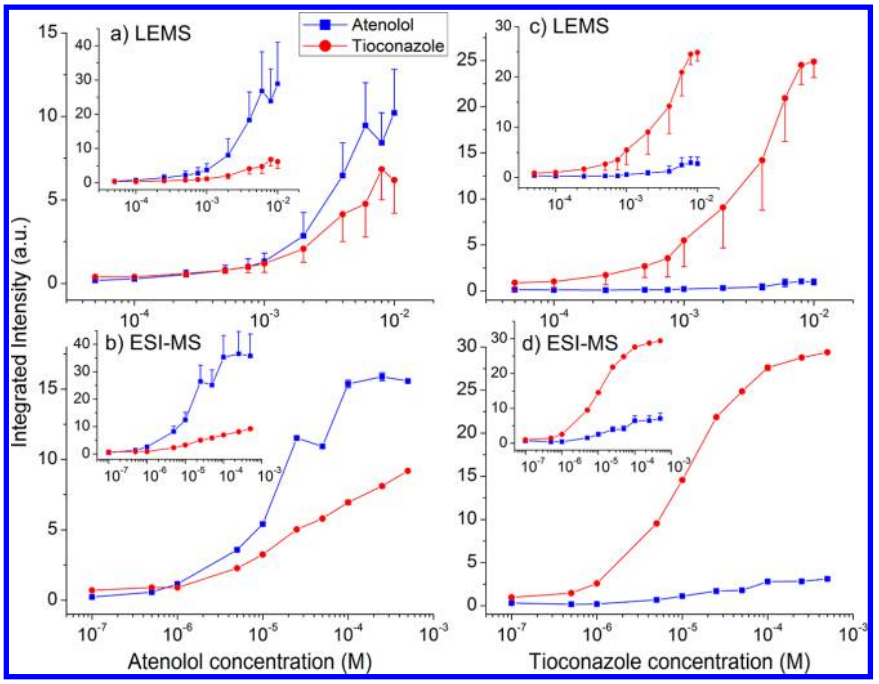

Figure 4. Integrated intensity plots for the analysis of nonequimolar mixtures of atenolol and tioconazole, where one analyte had a five times greater concentration than the other analyte. (a and b) the integrated intensity plots for the 5× atenolol mixtures and (c and d) the integrated intensity plots for the 5× tioconazole mixtures using LEMS and ESI-MS, respectively. The insets are the calibrated intensities after normalization to tioconazole.

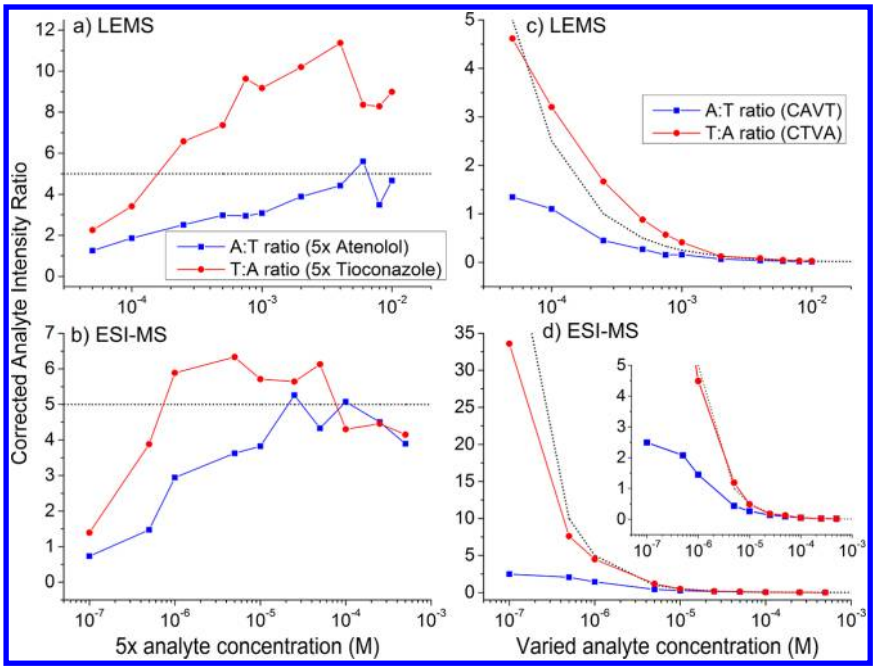

Figure 5. Calibrated analyte intensity ratio plots in the analysis of nonequimolar mixtures of atenolol (A) and tioconazole (T), where one analyte had a five times greater concentration than the other analyte in (a and b), and where one analyte has a constant concentration as the other analyte's concentration is varied in (c and d), using LEMS and ESI-MS, respectively. The dotted black line represents the theoretical intensity ratio for each nonequimolar mixture.

high surface activity and have been shown to suppress the mass spectral responses of other analytes in ESI-MS analysis.[10] However, we anticipated from the HPLC analysis (Figure S2, Supporting Information) that tioconazole would still have the highest signal in the four-component mixture. Suppression of the other three analytes by TBA shows that the response factors based on the pure solutions are not valid for the four component mixture analysis. Due to the ion suppression, the conventional ESI-MS linear dynamic ranges for the four-component equimolar mixtures were from 1×10^{-7} M to 2.5×10^{-5} M for TBA and TEA, to 1.0×10^{-5} M for tioconazole, and to 5.0×10^{-6} M for atenolol. As seen in Figure 3b, suppression of the atenolol signal occurred after 2 orders of magnitude and eventually dropped below the TEA signal. The calibrated intensity plot for ESI-MS (inset in Figure 3b) displays a similar intensity hierarchy (from TBA to atenolol) to the LEMS

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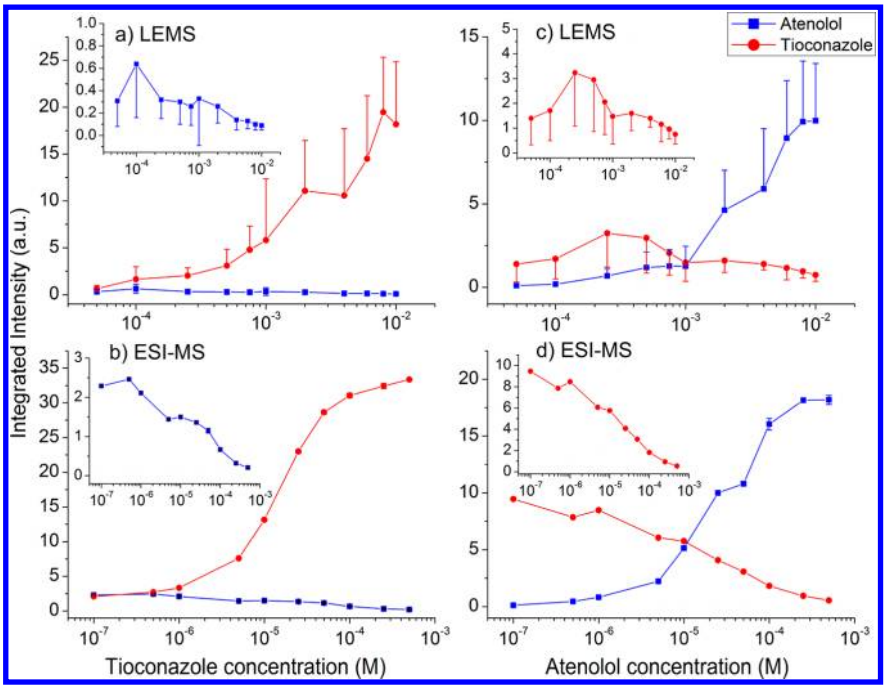

Figure 6. Integrated intensity plots for the analysis of nonequimolar mixtures of atenolol and tioconazole, where one analyte has a constant concentration as the other analyte's concentration is varied. (a and b) the integrated intensity plots for the CAVT mixtures and (c and d) the integrated intensity plots for the CTVA mixtures using LEMS and ESI-MS, respectively. The insets are magnified plots of the analyte that is held at constant concentration to show the effect of the concentration of varied analyte. The analyte with a nonvarying concentration was held constant at 2.5×10^{-4} M and 5×10^{-6} M for LEMS and ESI-MS, respectively.

analysis, but the discrepancy in the ESI-MS adjusted intensities are increased, resulting in nonuniform intensity ratios (Figure S3d, Supporting Information) that preclude quantitative measurement, even at lower concentrations.

Analysis of Two-Component Nonequimolar Mixtures. As LEMS analyses provided reasonable correlation between calibrated mass spectral responses and concentration over 2 orders of magnitude for equimolar mixtures, two experiments with nonequimolar mixtures were performed to test whether LEMS can be used as a quantitative tool for complex small molecule analyses. The first nonequimolar mixture consisted of one analyte having a five times greater concentration than the other. The mass spectral intensities for the 5:1 mixtures using LEMS and ESI-MS, Figure 4, are qualitatively analogous. Tioconazole had a much greater mass spectral response than atenolol for either method, resulting in a similar atenolol/tioconazole (A:T) intensity ratio for the 5:1 atenolol/tioconazole mixture (5× atenolol) and a large tioconazole/atenolol (T:A) intensity ratio for the 5:1 tioconazole/atenolol mixture (5× tioconazole). This slightly skewed the adjusted ratios from the theoretical ratio of 5:1, with a lower A:T ratio in the 5× atenolol mixture and a higher T:A ratio in the 5× tioconazole mixture, as seen in Figure 5a,b. The LEMS analyses deviated more from the expected ratio than the ESI-MS analyses due to the high relative standard deviation (RSD) values for the LEMS analyses, ~40% for LEMS as compared to ~4% for ESI. This was presumably caused by the inhomogeneity of the dried droplet method for preparing sample slides, which was seen before in previous LEMS[37,38] and MALDI[54] analyses. Little ion suppression occurred in the 5:1 mixtures with LEMS or ESI-MS, although the analyte response in ESI-MS analyses saturated above 2.5×10^{-5} M for atenolol and tioconazole in the analysis of both 5:1 mixtures. The analyte responses in LEMS analyses were fairly linear throughout the whole concentration range (from 5×10^{-5} M to 1×10^{-2} M for the more concentrated analyte) but may be approaching saturation at the highest concentration (1×10^{-2} M).

In the second nonequimolar mixture experiment, the concentration of one analyte was kept constant as the concentration of the other analyte was varied. The calibrated atenolol/tioconazole intensity ratio plots for the CYVZ nonequimolar mixtures using LEMS and ESI-MS are shown in Figure 5c,d, respectively. Using the response factors, LEMS and ESI-MS analyses of the complex mixtures yielded results practically identical to the theoretical concentration ratio, which shows good quantitative potential for both techniques in this particular comparison. For both ESI-MS and LEMS, the T:A intensity ratios for the CTVA mixture followed the trend much closer than the A:T intensity ratios for the CAVT mixture. This may be the result of the use of a nonconcentration dependent response factor that was obtained from the average across the concentration range. Although the data appears quantitative, the raw mass spectral intensities plot of the CYVZ mixtures (Figure 6) illustrates significant ion suppression of the analyte held at a constant concentration as the concentration of the other analyte is increased. The decrease in the overall intensity of the constant analyte is much greater for ESI-MS than LEMS across 2 orders of magnitude, which is the quantitative concentration regime of the varying analyte. The normalized decrease of the integrated intensities for the analyte with the constant concentration can be defined as $((I_{V[low]} - I_{V[high]})/(I_{V[low]}))*100\%$, where $I_{V[low]}$ is the integrated intensity of the constant analyte at low concentration of the variable analyte and $I_{V[high]}$ is the integrated intensity of the constant analyte at high concentration. This value is roughly equivalent for both techniques with a decrease of 65.9% and 31.2% using LEMS for CAVT and CTVA experiments, respectively, and a decrease of

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40.5% and 56.9% using ESI-MS for CAVT and CTVA experiments, respectively.

Why is LEMS Quantitative? Although ion suppression was not a major factor for LEMS analyses, it was a limitation for ESI-MS experiments at higher concentrations. The lack of major ion suppression effects at high concentrations in the multicomponent mixtures with LEMS could be due to nonequilibrium partitioning, assuming that the equilibrium-partitioning model is valid in the conventional ESI-MS analyses of these analytes. A previous LEMS experiment showed nonequilibrium retention of protein structure in comparison with conventional ESI-MS due to the short interaction time with the electrospray plume.[41] To determine whether the lack of ion suppression is caused by nonequilibrium partitioning, the sum of the available charge sites from the analytes, C_{QT}, is compared with the theoretical excess charge, which was found to be $\sim 5.0 \times 10^{-5}$ M graphically or calculated to be 1.1×10^{-4} M. Nonequilibrium processes might occur in LEMS analyses in the event that excess charge is approached without any suppression effects occurring. The C_{QT} of each LEMS solution was found by summing the effective ESI concentrations for the highest intensities obtained in the LEMS experiments (Table S3 in the Supporting Information). Effective ESI concentrations were calculated for each LEMS experiment using the calibration curves from the corresponding ESI-MS experiment and from ESI-MS single analyte analysis. Only the C_{QT} of the LEMS four-component mixture, calculated by fitting to the calibration curves from the four-component ESI-MS experiment, was above the [Q] graphical estimate of 5×10^{-5} M at 7.3×10^{-5} M. However, none of the LEMS experiments reached the calculated excess charge limit (1.1×10^{-4} M), even at the highest concentrations investigated. Therefore, nonequilibrium partitioning effects are unlikely as the reason for the lack of ion suppression in the set of molecules investigated here. Small aqueous ions are able to diffuse to the surface of a typical electrospray droplet prior to the time required for fission events,[55] maintaining equilibrium (or near equilibrium) partitioning throughout the offspring droplets as suggested by the role of surface activity on mass spectral responses.

One explanation for the quantitative capability would be the lower amount of analyte delivered to the electrospray droplet in LEMS in comparison with conventional ESI-MS. Previous experiments showed that ESI-MS was 2 orders of magnitude more sensitive than LEMS[37,38] with atenolol neutral capture efficiencies of 0.25%.[44] The limit of detection (LOD) for the current LEMS experiments was approximately 3 orders of magnitude poorer than ESI-MS (Table S4 in the Supporting Information). The decrease in sensitivity was caused by the lower neutral capture efficiency of ~0.01−0.05%. The low neutral capture efficiency would cause a reduction in the concentration of analytes in electrospray droplets, which in turn produce C_{QT} values lower than [Q], resulting in little to no ion suppression for LEMS analyses.

CONCLUSION

The quantitative ability of LEMS in the analysis of small, singly charged molecules is similar to conventional ESI-MS for singly charged analyte analysis. The comparison of measured intensity ratios, calibrated with response factors, to the theoretical concentration proportions establish the quantitative capability of LEMS in the analysis of various multicomponent mixtures. The linear dynamic ranges were similar for LEMS and ESI-MS, approximately 2 to 2.5 orders of magnitude for any of the four analytes, although it may be higher for LEMS due to the limited experimental concentration range used in this study (~3.5 vs ~2.5 orders of magnitude for ESI-MS and LEMS, respectively). Experiments with highly concentrated solutions using conventional ESI-MS create a charge deficiency scenario resulting in ion suppression of the mass spectral responses of less surface active analytes. In contrast, LEMS analyses of highly concentrated sample showed limited ion suppression due to the low neutral capture efficiency resulting in the charge surplus regime. Within the context of the equilibrium-partitioning model, the small, singly charged analytes appear to partition between the inner and outer phases of the electrospray droplet in the LEMS experiment given the observation that the degree of nonpolarity of the molecule determines the surface activity which in turn determines the instrumental response.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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