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Standard-Free Quantitation of Mixtures using Clusters Formed by Electrospray Mass Spectrometry

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Abstract

Ion abundances in electrospray ionization mass spectra depend on many factors, including molecular hydrophobicity, basicity, solution composition, and instrumental parameters. A recently introduced method that uses nonspecific cluster ion abundances to obtain solution-phase molar fractions of analytes directly from ESI mass spectra without using standards was evaluated using solutions containing 0.03% to 24% L-threonine, D-threonine, L-leucine, L-lysine, L-glutamic acid or diglycine with L-serine as a major component. Because of the propensity of serine clusters to exhibit "magic" numbers, which can be chirally selective, these experiments provide a rigorous test of this standard-free cluster quantitation method, which requires that clusters form statistically from analytes in solution. For each of these solutions, the compositions of clusters containing ≥ 32 molecules reflect the solution molar fractions of each component. From the abundances of these larger clusters, the solution molar fraction can be determined to better than 10% accuracy over nearly three orders of magnitude in concentration. In contrast, the ionization/detection efficiency of the individual amino acids differs by as much as a factor of 460 in these experiments. The protonated octamer incorporates some molecules statistically but efficiently excludes other molecules that have significantly different properties or chirality. This standard-free quantitation method may be most advantageous for rapidly characterizing mixtures, such as products of chemical synthesis, which contain unknown products or molecules for which suitable standards are not readily available.

Introduction

Mass spectrometry (MS) has many advantages for analyzing complex mixtures, including high sensitivity, specificity and speed.[1] In combination with electrospray ionization (ESI), nonvolatile and thermally labile molecules can be readily ionized directly from solution and mass analyzed, making ESI-MS a powerful detection method when coupled with liquid chromatography or electrophoresis.[1-3] Elemental composition can be obtained from exact mass measurements,[4] and unknown molecular ions can be identified and structurally characterized using tandem mass spectrometry in which a precursor of interest is isolated, typically dissociated, and the resulting fragments mass analyzed.[5, 6]

A limitation of ESI-MS for mixture analysis is the difficulty of obtaining quantitative information about how much of each component is present in a solution mixture directly from ion abundances in the mass spectrum. Ionization efficiencies of molecules in a mixture can differ significantly as a result of many factors, including their surface activities or hydrophobicities, molecular basicity and conformation.[7-22] The matrix or other solutes present in solution can either enhance or reduce the ion abundance of some analytes.[23-26] Instrumental parameters or mass-dependent ion transmission and detection can also affect relative ion abundances.[27-29] Because of these and other factors, quantitation with ESI-MS is typically

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done with internal standards. Molecules with similar physical properties to the analyte of interest as well as analyte molecules that have been isotopically labeled can be used as standards.[30-34] Quantitation using internal standards is common in small molecule analysis, such as in pharmaceutical chemistry, where characterization of therapeutics and related impurities from unreacted starting materials, synthetic intermediates, and degradation products is necessary to ensure the quality, efficacy and safety of drugs.[35, 36] Internal standards, such as isotope-coded affinity tags[30, 31] and stable isotope labeling with amino acids in cell cultures,[32, 33] are commonly used in proteomics to obtain information about relative gene expression.[30-33]

A new standard-free quantitation method to obtain solution molar fractions using the abundances of cluster ions formed by ESI was recently introduced.[37] The composition of clusters formed from peptide-containing solutions approached that in the bulk solution with increasing cluster sizes. From the abundances of clusters containing ~15 or more peptide molecules, the solution composition could be determined to within ~20% or better even in cases where the ionization/detection efficiency of the individual molecules differed by over an order of magnitude.[37] This method has the advantages that reasonably accurate quantitative information can be obtained directly from an ESI mass spectrum without using either an internal or external standard, the components do not need to be identified, and effects of instrument or detector mass bias are significantly reduced. This method can greatly reduce the time and effort necessary to obtain quantitative information from mixtures.

A critical requirement to obtain quantitative information using this method is that clusters must form statistically, which appears to occur for large peptide clusters.[37] Clusters of small molecules often exhibit “magic” numbers, such as $H(H_2O)_{21}^+$,[38] which can either be due to the special stability of the specific cluster or instability of adjacent clusters. Protonated serine shows a strong propensity to form magic numbers and the protonated octamer is especially stable.[12, 39-59] Protonated serine octamer is often the most abundant ion observed in a mass spectrum of serine and is readily formed by ESI,[12, 39-47] sonic spray[48-50] and even by thermal sublimation.[51-53] The protonated octamer also has a strong homochiral preference, which has led some to suggest that serine may have played a role in the origin of homochirality in living systems.[39-42, 53] The structure of the protonated octamer has been investigated by H/D exchange,[54-56] ion mobility,[42, 43, 48] infrared photodissociation spectroscopy,[57] and quantum chemical calculations,[39, 41, 43] from which evidence for at least two different forms of the octamer have been deduced.

Although protonated serine octamer is homochirally selective, doubly protonated octamer and clusters with 9 - 10 serine molecules show a heterochiral preference.[44, 58] Higher-order octameric clusters, e.g., $[16Ser + 2H]^{2+}$, $[24Ser + 3H]^{3+}$, have also been reported to have slightly enhanced abundances and it has been suggested that the serine octamer is a building block in their assembly.[39, 42] Results from ion mobility experiments indicate that large clusters with as many as 500+ serine molecules form tightly packed spherical structures.[48]

Because of the strong preference of serine to form “magic” numbers and clusters that show strong chiral preferences, serine-containing solutions provide an excellent test of whether our standard-free cluster quantitation method is generally applicable because our method requires that the cluster composition is statistical and therefore representative of the solution-phase analyte concentrations. Here, we demonstrate that large serine clusters formed by ESI from solutions containing serine as a major component incorporate aliphatic, basic, and acidic amino acids statistically. Although the protonated octamer effectively excludes a dipeptide and threonine of the opposite chirality, these molecules are incorporated into larger clusters statistically. From the abundances of the larger clusters, the solution-phase percent molar

fractions can be determined with better than 10% accuracy. These results indicate that our method should be broadly applicable to a wide range of analytes.

Experimental

Sample Preparation

L-serine, L-lysine, L-leucine, L-threonine, L-glutamic acid, D-threonine, and diglycine were purchased from Sigma-Aldrich, Co. (St. Louis, MO). Stock solutions for each analyte were prepared at 12 mM in water and all mixed analyte solutions were prepared to a final total concentration of 3 mM in water using these stock solutions. The minor component was incorporated into L-serine solutions at a % mole fraction ranging from 0.03% to 24%.

Mass Spectrometry

Experiments were performed on a 9.4 T Fourier-transform ion cyclotron resonance mass spectrometer with an external ESI source that is described elsewhere.[60] Ions are formed by ESI from borosilicate capillaries that are pulled so that the tips have a ~2 μm inner diameter (model P-87 capillary puller, Sutter Instruments, Novato, CA). The capillary is loaded with a small volume (~2-10 μL) of analyte solution, and a platinum wire is inserted into the solution and grounded. A new capillary was used for each solution to avoid sample contamination. The borosilicate capillary is positioned ~2 mm away from the source inlet capillary and a potential of -800 to -1200 V is applied to this inlet capillary. Ions are accumulated in an external hexapole ion trap for 1.5 s and subsequently are injected into the cell. Nitrogen gas introduced through a piezoelectric valve to a cell pressure of ~1×10^{-6} Torr is used to enhance ion trapping, and three hexapole injections are used prior to detection. Three mass spectra of 50 coadded scans were acquired at three different DC offset values between 4.5-6.3 V for each analyte to take into account the affect of this parameter on relative ion abundances in the mass spectrum. Error bars for data obtained from the protonated molecular ions, protonated octamer, and larger cluster abundances ($n \geq 32$) correspond to the standard deviation from the average value obtained from the mass spectra at three DC offset voltages.

Results and Discussion

Solute Concentration from Cluster Ion Abundances

For clusters that are formed statistically from the molecules in solution and for which the ionization efficiency is not influenced by their composition, the solution-phase concentration of various analytes that are incorporated into these clusters can be determined from the cluster abundances. The percent molar fraction of a minor component, F_m%, can be obtained by using a weighted average (eq.1):

$$F_m\% = \frac{\sum_h I_h \frac{h}{n}}{\sum_h I_h} \times 100 \quad (1)$$

where I is the abundance of each observed cluster consisting of n total molecules with h molecules of the minor fraction component. For example, if two clusters consisting of 40 molecules are formed, one a homogenous cluster containing 40 molecules of component **A** and the other a heterogeneous cluster containing 39 molecules of **A** and one molecule of **B**, and the normalized abundances of these clusters are 100 and 15, respectively, then the percent molar fraction (F_m%) of **B** is ((15 × (1/40))/(15 + 100.0)) × 100 = 0.33%. Solute concentration can also be obtained from cluster abundances using a binomial expansion, but the weighted

average method is more accurate for larger cluster sizes when the signal-to-noise ratio is low.[37]

In these experiments, relative ion abundances depend on a number of instrumental parameters. For example, the DC potential applied to the external hexapole used to store ions prior to injecting them into the cell can be varied to preferentially introduce higher *m*/*z* ions. To take this effect into account, mass spectra were acquired at three different DC offset voltages and the percent molar fractions determined from the ion abundances were averaged for these three spectra. The significantly higher error bars in the protonated molecular ion data compared to those for the large cluster ($n \geq 32$) data reflect the larger effect this parameter has at lower *m*/*z*.

L-Threonine in L-Serine

The side chain of threonine has an additional methylene group compared to that of serine, but this minor structural difference results in dramatically different ionization efficiencies for these two amino acids with ESI. A representative ESI mass spectrum of a solution containing 1% L-threonine with 99% L-serine at a total amino acid concentration of 3 mM is shown in Figure 1. The abundance of protonated L-threonine is 3.9% that of L-serine. Thus, the relative abundance of protonated L-threonine is nearly 4 times greater than its corresponding solution concentration. Preferential ionization of threonine over serine has been reported previously[43] and could be due to increased surface activity or hydrophobicity owing to the extra methylene group or a slightly higher proton affinity.[61] Other factors, including solution concentration, instrumental parameters, ion transmission and detection efficiency, can also play a significant role in the relative abundances of ions observed in ESI mass spectra.[7-11, 13-29] Differences in ionization efficiency and contributions from these other factors make it difficult to obtain solution concentrations directly from molecular ion abundances without using standards.

In addition to the protonated molecular ions, both homogenous clusters of L-serine and heterogeneous clusters that have incorporated L-threonine are observed (Figure 1). Large clusters are often formed from concentrated solutions[40, 43, 62] and the abundances of these clusters can be increased by changing several instrumental parameters, including both the ion accumulation time and dc offset potential of the external hexapole that is used to accumulate ions prior to injection into the ion cell.[37] In these experiments, clusters consisting of as many as 91 amino acids were observed.

Percent molar fractions are obtained from the cluster abundances as a function of cluster size assuming statistical incorporation of the amino acids. Date from this mass spectrum (and mass spectra at two additional DC offset potentials) are shown in Figure 2a. For clusters with $n < 10$, the molar fraction of L-threonine obtained from the gas-phase cluster data is higher than that in solution (dashed line in Figure 2a). This could be due to the higher ionization efficiency of L-threonine, which has a larger effect on smaller clusters or it could be due to preferential incorporation of L-threonine at small cluster sizes. The general trend with cluster size suggests that the higher ionization efficiency of L-threonine may be more important for these clusters. For the larger clusters ($n \geq 32$), the calculated molar fraction of L-threonine does not change significantly with cluster size. An average molar fraction of L-threonine determined from the $n = 32 - 89$ cluster abundances is 0.88% ± 0.10%, which is close to the solution-phase value of 1.0%. This indicates that any differences in ionization/detection efficiency or effects of preferential incorporation of L-threonine into these larger clusters are minor. The abundances of these larger clusters gradually decrease with increasing size and no “magic” numbers are observed, consistent with nonspecific aggregation. The weighting of these data either by their respective abundances or cluster sizes results in a negligible change in the percent molar fraction determined. The molar fraction of L-threonine determined just from octameric clusters

is 1.6% ± 0.5%, indicating that the octamer can readily incorporate L-threonine as previously reported.[43, 45, 53]

These data were obtained as a function of the % molar fraction of L-threonine ranging from 0.05% to 20%. The L-threonine molar fraction determined from the abundances of clusters with $n \geq 32$ is shown in Figure 2b. These data are linear over nearly three orders of magnitude change in solution molar fraction and have a slope of 1.02 ($r^2 = 0.999$). This slope is close to the ideal slope of 1.00 indicating that the relative molar fractions can be determined from the cluster abundances with about 2% accuracy. In contrast, the slope for the molar fraction calculated from the abundances of the protonated monomer is 3.75 ($r^2 = 0.993$) indicating that preferential ionization of protonated L-threonine occurs consistently over this range of solution concentration. Data for the octamer has a slope of 1.41 ($r^2 = 0.994$), consistent with either preferential ionization of the heterogeneous octamer or preferential incorporation of L-threonine into the L-serine octamer. These data suggest that L-threonine does not significantly disrupt the structure of the octamer.

D-Threonine in L-Serine

Because of the strong propensity of serine octamers to form homochiral clusters, the effects of the chirality of an impurity molecule on incorporation into serine clusters, including the octamer, were investigated. Partial ESI mass spectra of 5.0% L-threonine and D-threonine in L-serine are shown in Figure 3a and 3b, respectively. Protonated homochiral serine octamer is most abundant, but protonated octamers that contain 1-3 L-threonine molecules but only 1-2 D-threonine octamers are also observed. The abundances of the heterogeneous octamer clusters containing L-threonine are significantly higher than those containing D-threonine indicating that the L-serine octamer has a clear preference for incorporating threonine that has the same chirality.

The measured % molar fraction of D-threonine as a function of cluster size for the 5.0% solution is shown in Figure 4a. As with L-threonine, the % molar fraction of D-threonine calculated from the very smallest clusters is higher than the solution value. In contrast, data for the octamer clearly show that D-threonine is excluded. For the larger clusters ($n \geq 32$), no chiral preference is apparent and the measured molar fraction calculated for these clusters is 5.5% ± 1.0%, consistent with the solution-phase molar fraction of 5.0%. Previously, it has been suggested that the octamer may be the building blocks for $[16Ser+2H]^{2+}$ and $[24Ser+3H]^{3+}$ due to their enhanced abundance compared to neighboring clusters of the same charge state, but this effect is significantly lower than for the octamer.[39, 42] Interestingly, no significant chiral preference is observed for higher-order clusters with integer multiples of the octamer (n = 16, 24, 32, 40, etc.), indicating that octamers are not simply the building blocks for these higher-order clusters.

The measured % mole fraction of D-threonine in L-serine obtained from the protonated molecular ions, the octamer cluster, and clusters with $n \geq 32$ as a function of solution molar fraction from 0.05% to 20% are shown in Figure 4b. These data are linear and have slopes of 3.01 ($r^2 = 0.957$), 0.38 ($r^2 = 0.992$), and 1.05 ($r^2 = 0.999$), respectively. Protonated D-threonine is preferentially ionized, but the low value for the octamer clearly indicates that it is significantly excluded. However, D-threonine is incorporated statistically into the larger clusters.

L-Leucine in L-Serine

To determine the effect of incorporation of an amino acid with an aliphatic side chain, ESI mass spectra were obtained from solutions consisting of 0.03% to 18% L-leucine in L-serine and the measured % molar fraction obtained from these data are shown in Figure 5. L-leucine is preferentially ionized at all concentrations, consistent with its higher surface activity or

hydrophobicity, but this effect is greatest for a solution containing 0.17% L-leucine. At this concentration, the abundance of protonated L-leucine is 10.8% that of protonated L-serine indicating that protonated L-leucine is preferentially ionized/detected by factor of 54. The strong dependence of protonated L-leucine abundance on the solution composition illustrates the challenges of relating the abundance of protonated ions to their corresponding concentrations in solution.

In contrast, the measured % molar fraction for both the octamer and clusters with $n \geq 32$ are linear over this range of concentration with slopes of 0.90 ($r^2 = 0.993$) and 1.03 ($r^2 = 0.997$), respectively. This indicates that there is a slight propensity for the octamer to exclude L-leucine, but incorporation of L-leucine into the larger clusters is statistical.

L-Lysine in L-Serine

To determine the effect of incorporation of a basic amino acid, ESI mass spectra were obtained from solutions consisting of 0.04% to 18% L-lysine in L-serine and the measured % molar fraction obtained as a function of cluster size for the 4.3% solution molar fraction is shown in Figure 6a. Protonated L-lysine is 21-fold more abundant than protonated L-serine despite its much lower solution-phase concentration, likely owing to its significantly higher basicity. The difference in ionization efficiency depends on the relative molar fraction (Figure 6b) but L-lysine is more readily ionized and detected by as much as a factor of 460 over L-serine. However, incorporation of even a single L-lysine molecule into small serine clusters can greatly reduce this difference in ionization efficiency for the clusters (Figure 6a).

Results for both the octamer and higher-order clusters ($n \geq 32$) are shown in Figure 6b. These data can be fit with lines with slopes of 0.11 ($r^2 = 0.997$) and 0.93 ($r^2 = 1.00$), respectively. Thus, the L-serine octamer efficiently excludes L-lysine from these clusters whereas L-lysine is incorporated statistically into the larger clusters (within 7%) and effects of this incorporation on the ionization/detection efficiency of these clusters are small.

L-Glutamic Acid in L-Serine

As is the case for L-leucine and L-lysine, protonated L-glutamic acid is preferentially ionized and detected in ESI mass spectra when the molar fraction of L-glutamic acid is between 0.05% and 24%, and the relative ionization efficiency depends strongly on concentration. The proton affinity of L-glutamic acid is higher than that of L-serine[61] owing to the ability of the side-chain oxygen atoms to solvate the charge in glutamic acid,[63] and the higher proton affinity of L-glutamic acid is the likely origin of the significantly higher ionization efficiency. This effect, as well as any effects of specific clustering, becomes negligible at larger cluster size (Figure 7a).

Data for the octamer and larger clusters ($n \geq 32$) as a function of solution molar fraction can be fit to lines with slopes of 0.40 ($r^2 = 0.991$) and 0.90 ($r^2 = 0.999$) (Figure 7b). As was the case for L-lysine, the octamer preferentially excludes L-glutamic acid but incorporation of this amino acid into the larger clusters approaches the statistical value within 10%.

GlyGly in Serine

To determine the effects of incorporation of a small peptide into serine clusters, ESI mass spectra of diglycine at % molar fractions between 0.05% and 5.0% were obtained and these data are summarized in Figure 8. Protonated diglycine is preferentially ionized over protonated L-serine at all concentrations, consistent with its higher surface activity or hydrophobicity, but the ionization efficiency depends on the % molar fraction. At 0.05% molar fraction, diglycine is preferentially ionized/detected by a factor of 93 over L-serine. In contrast, the protonated octamer and larger cluster ($n \geq 32$) data can be fit to lines with slopes of 0.01 ($r^2 = 0.898$), and

1.01 (r^2 = 0.999), respectively. The much higher discrimination of the octamer for the dipeptide vs. the amino acids investigated indicates that this dipeptide causes a greater disruption of the very stable octamer structure. In striking contrast, the larger serine clusters can incorporate this dipeptide readily and the % molar fraction determined from the larger cluster data is within 1% of the solution-phase % molar fraction.

Conclusions

Quantifying the relative concentrations of components in solution using ESI mass spectrometry is challenging owing to many different factors that affect relative ionization/detection efficiencies, including molecular structure, matrix effects, instrumental parameters, etc. A newly introduced standard-free quantitation method, which uses the abundances of larger molecular clusters formed by ESI to obtain relative solution-phase molar fractions,[37] was investigated using L-serine as a major component. Serine has a strong propensity to form "magic" number clusters that show either homo- or heterochiral preferences and is a rigorous test of this method, which requires that impurity molecules are incorporated statistically and do not influence the ionization/detection efficiency of larger clusters. Incorporation of aliphatic, basic and acidic amino acids, and a dipeptide, into larger serine clusters is statistical and the abundances of these clusters reflect the solution-phase molar fractions with better than 10% accuracy over nearly three orders of magnitude in concentration. By comparison, some of the protonated molecular ions in these mixtures were ionized/detected up to a factor of 460 more efficiently than protonated serine under these experimental conditions. The octamer effectively included some amino acids but excluded others. Although the octamer is chirally selective, higher-order serine clusters incorporated threonine molecules of the opposite chirality statistically. The results obtained from amino acids that have significantly different physical properties using serine that has by far the highest propensity to form "magic" number clusters of any of the amino acids suggest that this standard-free quantitation method should be generally applicable to a broad range of analytes.

Although not as accurate or as generally applicable as methods that employ internal standards, this standard-free quantitation method has the advantages that reasonably accurate quantitative information can rapidly be obtained directly from an ESI mass spectrum with no prior knowledge of the composition or the identity of the impurity molecules necessary, making it applicable to a wide range of analytical problems. This method also significantly reduces effects of instrument or detector mass bias, but does require high resolution in order to identify the charge states and compositions of larger clusters. This standard-free cluster quantitation method may be particularly useful in molecular synthesis because unreacted starting materials, intermediates, degraded or modified catalysts or unintended products of side reactions could be quantified directly from an ESI mass spectrum. This method would be especially advantageous when some of the reaction products are unknown or when appropriate standards are not readily available. Absolute concentrations of each individual component could be obtained by spiking the solutions with a known concentration of a clustering agent, such as serine, at relatively high concentrations like those used here. From the relative molar fractions of each component determined from the abundances of larger clusters, the absolute concentration of each component could be obtained. The sensitivity and accuracy of this method could be improved on trapping mass spectrometers by selectively introducing higher m/z ions into the ion cell. The m/z of clusters generally increases with cluster size[62] making it possible to selectively introduce larger clusters that should have compositions more representative of analyte concentrations in solution. The abundances of larger clusters could also be increased by using other ionization methods, such as sonic spray.[48] It is likely that other molecules that have an even greater propensity to form large clusters could be identified and different agents with physical properties matching those of molecules suspected to be present in mixtures of unknowns could be used.

The statistical incorporation of analytes into the larger clusters suggests that these clusters are formed by solvent evaporation from larger droplets whose composition reflects that of the original bulk solution, i.e., a charge residue mechanism.[7-12] Solvent evaporation from a droplet formed by ESI would increase the concentration of the analytes and ultimately, the resulting charged clusters formed from the nonvolatile analytes should reflect the original solution-phase composition.

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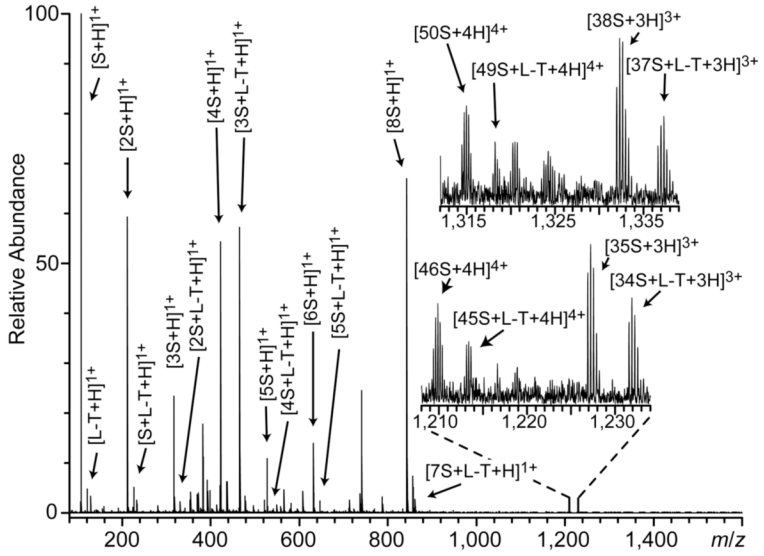

Figure 1.

ESI mass spectrum of a solution containing 1.0% molar fraction of L-threonine in L-serine (3.0 mM total peptide concentration) with some regions of the spectra with large molecular clusters inset.

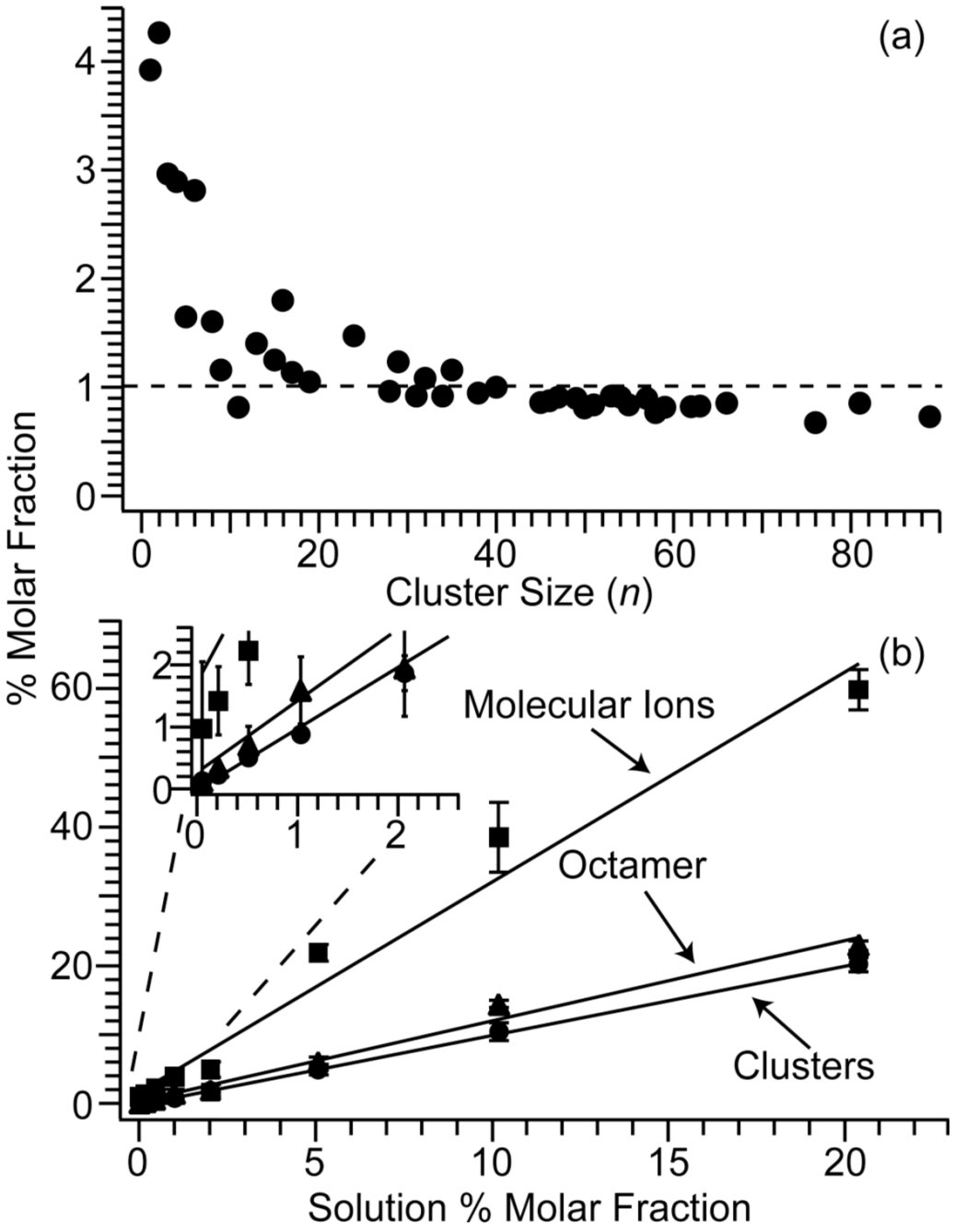

Figure 2.

(a) Percent molar fractions calculated from the cluster ion abundances assuming statistical incorporation and identical ionization efficiencies obtained from ESI mass spectra of a 1.0% molar fraction of L-threonine in L-serine (3.0 mM total peptide) as a function of cluster size, *n*. The dashed line corresponds to the solution % molar fraction. (b) Percent molar fractions of L-threonine in L-serine obtained from the protonated molecular ions (squares), from protonated octamer (triangles), and from cluster abundances for $n \geq 32$ (circles) as a function of the solution % molar fraction. These data are linear with slopes of 3.75, 1.41, and 1.02 for the protonated molecular ions, protonated octamer, and clusters with $n \geq 32$, respectively. Error bars

correspond to a standard deviation of three spectra acquired at dc offset voltages of 4.5, 4.8, and 5.2 V.

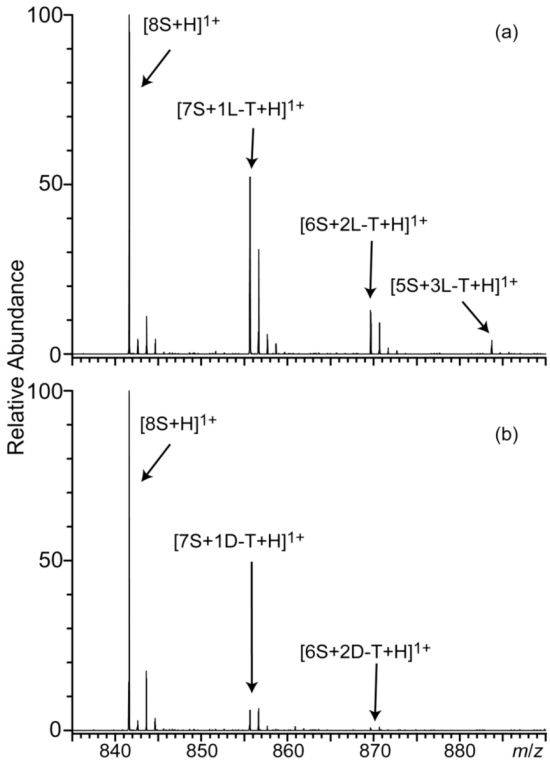

Figure 3.

Partial ESI mass spectra of solutions containing 5.0% molar fraction of L-threonine (top) and D-threonine (bottom) in L-serine (3.0 mM total peptide concentration) showing homogeneous and heterogenous clusters of protonated octamer.

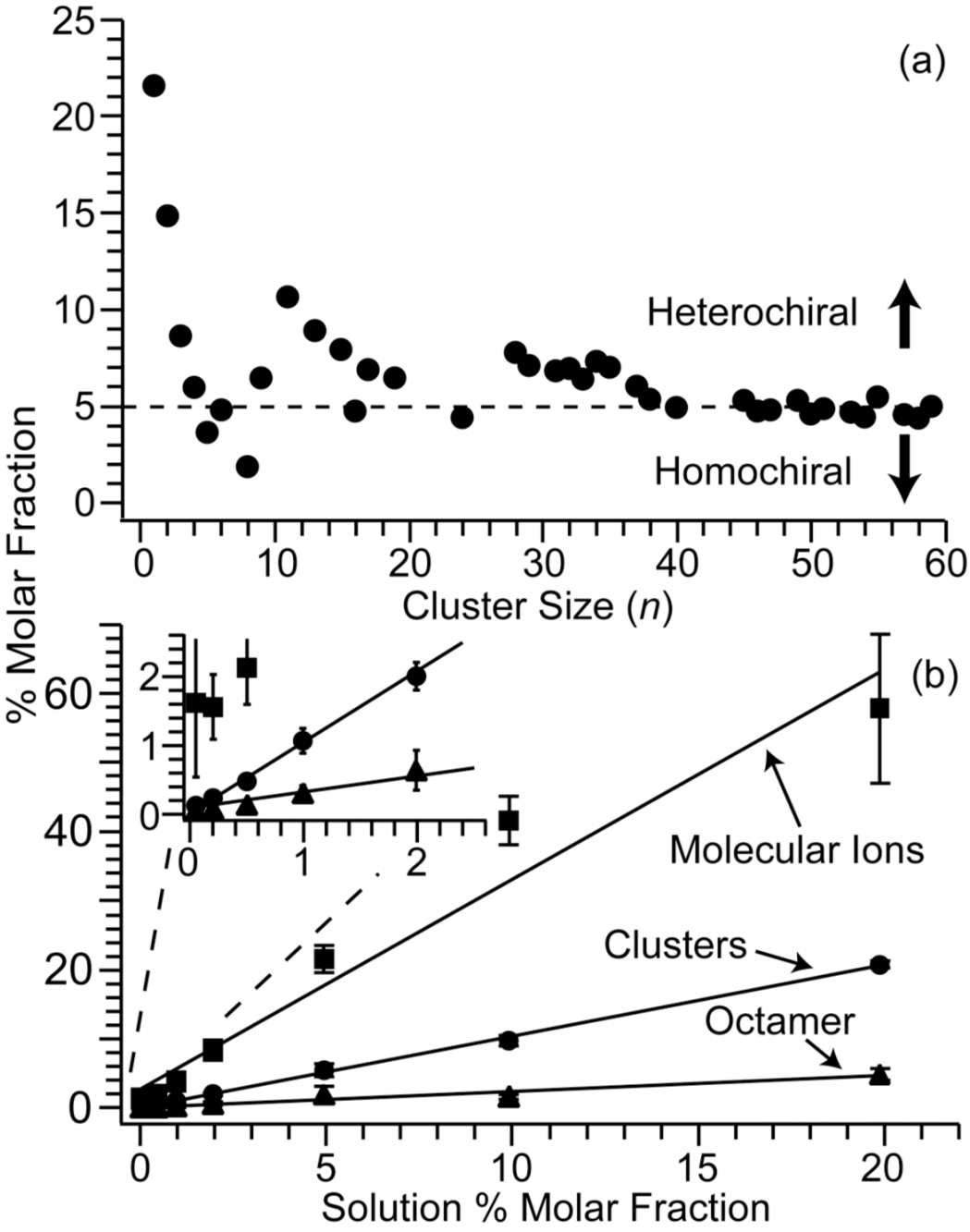

Figure 4.

(a) Percent molar fractions calculated from cluster ion abundances obtained from ESI mass spectra of a 5.0% molar fraction of D-threonine with L-serine (3.0 mM total peptide concentration) as a function of cluster size, *n*. The dashed line corresponds to the % molar fraction in solution. (b) Percent molar fractions of D-threonine in L-serine calculated from the protonated molecular ions (squares), from protonated octamers (triangles), and from cluster abundances for $n \geq 32$ as a function of the solution % molar fraction. These data are linear with slopes of 3.01, 0.38, and 1.05 for the protonated molecular ions, protonated octamers, and cluster abundances for $n \geq 32$, respectively.

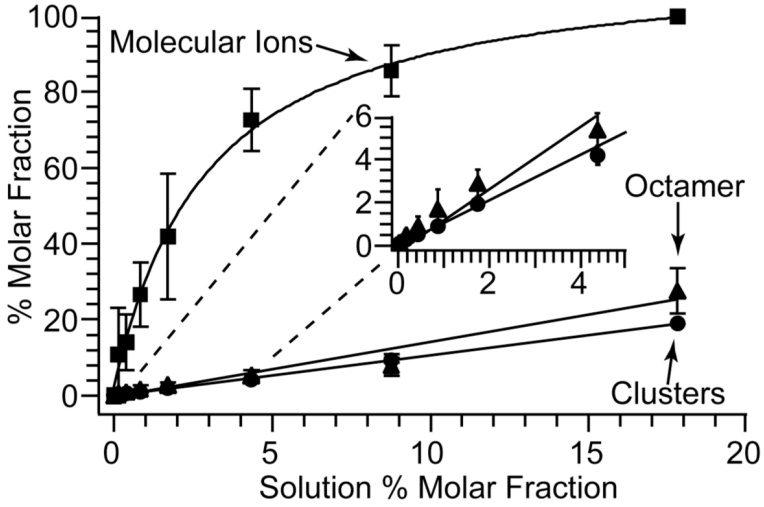

Figure 5.

Percent molar fractions of L-leucine in L-serine obtained from the protonated molecular ions (squares), from protonated octamers (triangles), and from cluster abundances for $n \geq 32$ (circles) as a function of the solution % molar fraction. The data from protonated octamers and cluster abundances for $n \geq 32$ are linear with slopes of 0.90 and 1.03, respectively. Data for the molecular ions are non-linear and at the 0.17% molar fraction, L-leucine is preferentially ionized/detected by a factor of 63 over L-serine.

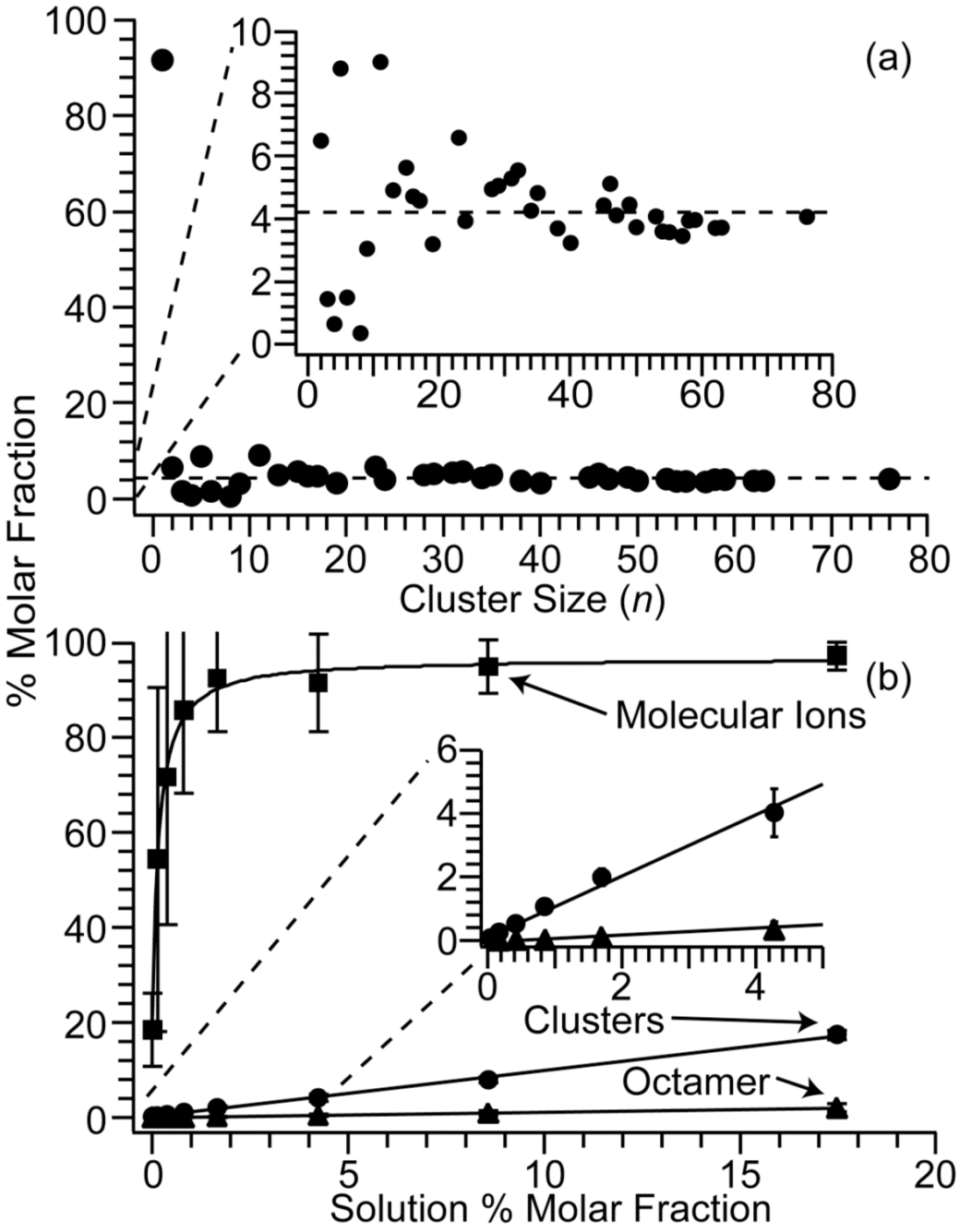

Figure 6.

(a) Percent molar fractions calculated from cluster ion abundances obtained from ESI mass spectra of a 4.3% molar fraction of L-lysine with L-serine (3.0 mM total peptide concentration) as a function of cluster size, *n*. The dashed line corresponds to the % molar fraction in solution. (b) Percent molar fractions of L-lysine in L-serine calculated from the protonated molecular ions (squares), from protonated octamers (triangles), and from cluster ion abundances (circles) for $n \geq 32$ as a function of the % molar fraction in solution. Data for the protonated octamers and larger clusters ($n \geq 32$) are linear with slopes of 0.11 and 0.93, respectively. Data for the molecular ions are non-linear and at 0.04% molar fraction, L-lysine is preferentially ionized/detected by a factor of 460 over L-serine.

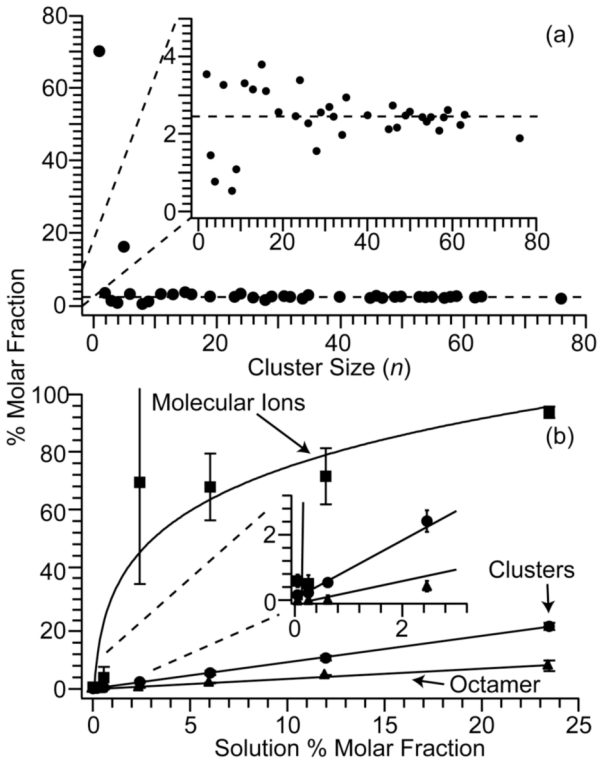

Figure 7.

(a) Percent molar fractions calculated from cluster ion abundances obtained from ESI mass spectra of a 2.5% molar fraction of L-glutamic acid with L-serine (3.0 mM total peptide concentration) as a function of cluster size, *n*. The dashed line corresponds to the % molar fraction in solution. (b) Percent molar fractions of L-glutamic acid in L-serine calculated from the protonated molecular ions (squares), from protonated octamers (triangles), and from cluster ion abundances (circles) for $n \geq 32$ as a function of the % molar fraction in solution. Data for the protonated octamers and larger clusters ($n \geq 32$) are linear with slopes of 0.40 and 0.90, respectively. Data for the molecular ions are non-linear and at 2.5% molar fraction, L-glutamic acid is preferentially ionized/detected by a factor of 29 over L-serine.

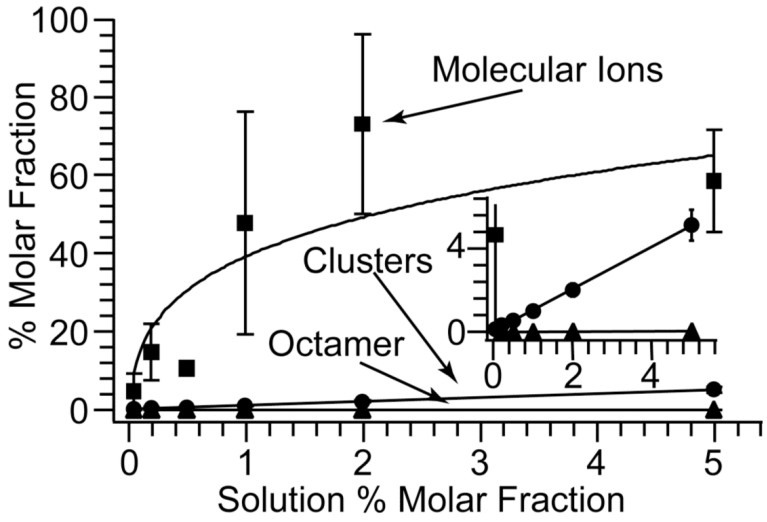

Figure 8.

Percent molar fractions of Gly-Gly in L-serine obtained from the protonated molecular ions (squares), from protonated octamers (triangles), and from cluster abundances for $n \geq 32$ as a function of the solution % molar fraction. The data from protonated octamers and cluster abundances $n \geq 32$ are linear with slopes of 0.01 and 1.01, respectively. Data for the molecular ions are non-linear and at 0.05% molar fraction, diglycine is preferentially ionized/detected by a factor of 93 over L-serine.