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Direct Standard-Free Quantitation of Tamiflu® and Other Pharmaceutical Tablets using Clustering Agents with Electrospray Ionization Mass Spectrometry

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Abstract

Accurate and rapid quantitation is advantageous to identify counterfeit and substandard pharmaceutical drugs. A standard-free electrospray ionization mass spectrometry method is used to directly determine the dosage in the prescription and over-the-counter drugs, Tamiflu®, Sudafed®, and Dramamine®. A tablet of each drug was dissolved in aqueous solution, filtered, and introduced into solutions containing a known concentration of either L-tryptophan, L-phenylalanine or prednisone as clustering agents. The active ingredient(s) incorporates statistically into large clusters of the clustering agent where effects of differential ionization/detection are substantially reduced. From the abundances of large clusters, the dosages of the active ingredients in each of the tablets were determined to typically better than 20% accuracy even when the ionization/detection efficiency of the individual components differed by over 100×. Although this unorthodox method for quantitation is not as accurate as using conventional standards, it has the advantages that it is fast, it can be applied to mixtures where the identities of the analytes are unknown, and it can be used when suitable standards may not be readily available, such as schedule I or II controlled substances or new designer drugs that have not previously been identified.

Accurate and rapid quantitation of small molecules in pharmaceutical mixtures is critical to the entire drug development process,[1, 2] and is important for identifying counterfeit or substandard pharmaceutical drugs.[3–7] The high demand, cost and limited availability of many pharmaceutical drugs used to treat major diseases provide incentives to produce counterfeit drugs and motivates the low-quality production of the active ingredient once a drug is no longer under patent protection.[3–7] The individuals most at risk of exposure to counterfeit or substandard drugs are from impoverished countries.[3–7] It is estimated that between 6–15% of all medicines are counterfeit,[7, 8] and it is thought counterfeit medicines can exceed 50% in Asia and Africa.[7] Substances used to adulterate pharmaceutical drugs can include highly toxic substances, leading to unnecessary mortality which lowers public confidence in legitimate medicines.[3–7] Counterfeit drugs can also lead to drug resistance due to extended exposure to low dosages of active ingredients without physical improvement; this is particularly harmful for patients who require therapy programs for AIDS and malaria.[3] Currently, Tamiflu® is in short-supply as countries gather stockpiles of the drug in fear of the H1N1 flu pandemic, which could lead to an increase in counterfeit tablets.[9] Counterfeit drugs can potentially be identified either by visual inspection or by the absence of an active ingredient. However, counterfeit drugs that pass visual inspection but contain the active ingredient at substantially lower levels can be more challenging to identify if suitable standards are not available. Therefore, new analytical

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Supplementary Information: ESI mass spectra of dissolved Tamiflu®, Sudafed®, and Dramamine® and discussion of experimental design.

methods that increase the speed and accessibility with which quantitative information can be obtained to meet increasing analytical demands are important.

Mass spectrometry (MS) has several advantages for analyzing pharmaceutical components in complex mixtures, including high sensitivity, selectivity, and speed.[2] With electrospray ionization (ESI), a wide range of thermally labile molecules can be ionized and information about elemental composition and structure can be obtained from exact mass and tandem MS measurements.[10, 11] The abundances of ions in ESI mass spectra depend on many factors. Molecules that are more basic, or that have high surface activities or hydrophobicities are more readily ionized,[12–16] and the ionization efficiency depends on analyte conformation, concentration, and the solution composition or matrix as well.[17–20] Mass-dependent ion transmission and detection also affect the measured ion abundances.[16] Because of these and other factors, obtaining quantitative information about concentrations of analytes in solution directly from the corresponding ion abundances in the mass spectrum is challenging without the use of standards, such as isotopically labeled molecules or molecules that have similar structures or properties to the analytes of interest.[21]

A new standard-free quantitation method to obtain the relative solution-phase concentrations of components in a mixture using the abundances of clusters formed by ESI was recently introduced.[22, 23] The composition of large, nonspecific clusters can reflect the solution-phase composition, and differences in ionization/detection and transmission efficiencies are significantly reduced. Using this method, solution-phase mole fractions can be obtained with better than 10–20% accuracy even in cases where the ionization/detection efficiency of the individual components differed by up to a factor of 460.[23] This quantitation method has several important advantages, including that no conventional reference standard is required, the components in the mixtures do not need to be identified, and effects of instrument and detector mass bias are significantly reduced. Although not as accurate as methods that use standards, this method provides reasonable quantitative information about mixtures with very little time and effort. Here, we demonstrate this standard-free quantitation method for the analysis of pharmaceutical over-the-counter and prescription drug formulations, Tamiflu®, Sudafed® and Dramamine®, directly without conventional standards or chromatographic separations.

Experiments were performed on a 9.4 T Fourier-transform ion cyclotron resonance mass spectrometer described elsewhere.[24] Tamiflu®, Sudafed® Sinus and Allergy, and Dramamine® (generic) tablets were dissolved in water to a final concentration of 10 mM for at least one of their active ingredients, sonicated for 30–60 min. and filtered (0.45 μm). The filtrate was spiked into solutions containing 10 mM of L-phenylalanine, L-tryptophan, L-serine, or prednisone to a calculated % mole fraction for the active ingredient between 0.2 to 2.3%. A low % mole fraction was used to reduce effects of sodium from the tablets on cluster adduction which unnecessarily congests the mass spectral data. These clustering agents were chosen because they are inexpensive, readily available, and the three amino acids are known to form clusters.[25] Multiple ESI mass spectra of each of these solutions were obtained using conditions described previously (see also Supporting Information).[22, 23]

ESI Mass Spectra of Dissolved Tablets

Active ingredients in pharmaceutical tablets are expected to be water soluble, and ESI mass spectra of filtered aqueous solutions in which tablets of Tamiflu®, Sudafed®, and Dramamine® were individually dissolved have ions corresponding to their active ingredients (Supporting Information, Figure S1). Protonated oseltamivir, the only active ingredient in Tamiflu® (30 mg), is the predominant ion in the mass spectrum. Both protonated phenylephrine and chlorpheniramine, the two active ingredients in Sudafed® at 10 mg and 4 mg, respectively, are formed, but the abundances of these ions do not reflect the relative dosages of these

molecules in the tablet. The mole ratio of these active ingredients is ~12:1 (phenylephrine:chlorpheniramine) in the tablet but the relative ion abundance is ~1:4. For Dramamine®, which contains 50 mg of dimenhydrinate, a salt of diphenhydramine and 8-chlorotheophylline, the mass spectrum contains sodiated diphenhydramine, but no 8-chlorotheophylline is observed. Although the active ingredients in each of these tablets can be readily identified by ESI-MS, except in the case of Dramamine® for which only one salt component forms a positive ion, the concentration of these ingredients cannot be deduced from these mass spectral data alone.

Tablet Dosage from Cluster Ion Abundances

The concentration of the active ingredient in each of these tablets can be obtained by adding a known concentration of a clustering agent to these solutions. If clusters formed with this added agent incorporate the drug molecules nonspecifically and ionization/detection efficiencies do not depend significantly on cluster composition, then the concentration of the drug in the solution can be obtained from the abundances and composition of the clusters using a weighted average (eq. 1):

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

where A is the abundance of each observed cluster consisting of n total molecules with h molecules of the pharmaceutical analyte.[22, 23]

Tamiflu®

An ESI mass spectrum of a solution containing Tamiflu® and L-tryptophan in which the calculated % mole fraction of the active ingredient, oseltamivir, is 1.3% is shown in Figure 1a. In this spectrum, the relative abundance of protonated oseltamivir is ~1.6× greater than protonated L-tryptophan, indicating that oseltamivir has a 160× greater ionization/detection efficiency under these conditions. In addition to the protonated molecules, homogenous L-tryptophan clusters and heterogeneous clusters containing both L-tryptophan and oseltamivir are observed. From the abundances of these clusters, the % mole fraction of oseltamivir can be determined assuming that this molecule incorporates statistically into L-tryptophan clusters. These data as a function of cluster size are shown in Figure 1b. For clusters containing fewer than 10 molecules, the values are higher than the solution-phase value, which indicate that either the smaller clusters containing oseltamivir are more readily ionized, or that oseltamivir is preferentially incorporated into these smaller clusters. Both the significantly higher ionization efficiency of oseltamivir and the trend in these data with increasing cluster size suggest that this is an effect of ionization efficiency. The average % mole fraction determined from the larger clusters ($n \geq 20$) is 1.4 ± 0.6% from which a dosage in the original tablet of 32 ± 13 mg of oseltamivir is obtained. This value is 7% higher than the label value of 30 mg in the Tamiflu® tablet.

Similar results were obtained using L-phenylalanine and prednisone as clustering agents. Measured % mole fractions of 1.2 ± 0.4% and 1.3 ± 0.2% were obtained from clusters with $n \geq 20$ and $n \geq 13$ for L-phenylalanine and prednisone, respectively, (Supporting Information, Figure S2) from which a oseltamivir dosage in the Tamiflu® tablet of 28 ± 9 mg and 35 ± 6 mg was obtained. L-serine was also evaluated as a clustering agent,[23] but remarkably, no

higher-order clusters were observed. This is likely the result of the much higher ionization efficiency of the minor constituent oseltamivir compared to L-serine.

Sudafed®

An ESI mass spectrum of a solution containing Sudafed® and L-tryptophan is shown in Figure 2a. The abundances of the protonated molecules of the two active ingredients, phenylephrine and chlorpheniramine, are 52% and 44% of protonated L-tryptophan, despite the ~100× higher concentration of L-tryptophan indicating that both active ingredients are preferentially ionized/detected. Clusters consisting of up to 39 molecules are also observed. Although protonated chlorpheniramine is formed by ESI, the intact salt, chlorpheniramine maleate, incorporates into the L-tryptophan clusters. The % mole fraction determined from the cluster abundances as a function of cluster size are shown in Figure 2b and 2c for phenylephrine and chlorpheniramine maleate, respectively. The values for the smallest clusters are too high, likely a result of preferential ionization of clusters that contain the more readily ionized molecules at small sizes. However, the values obtained from cluster ion abundances approach the solution-phase values at larger size, indicating that incorporation into these larger clusters is statistical and that differences in ionization efficiencies are greatly reduced. A % mole fraction calculated from the higher-order clusters ($n \geq 21$) results in values of 1.1 ± 0.4% and 0.45 ± 0.15% for these respective active ingredients. From these values, the measured dosages obtained from the Sudafed® tablet that contains 10 mg of phenylephrine HCl and 4 mg of chlorpheniramine maleate are 8.7 ± 2.9 mg and 7.0 ± 2.3 mg, respectively.

Similar results were obtained using L-phenylalanine and prednisone as clustering agents. For L-phenyalanine, dosages of 7.5 ± 1.9 mg and 6.3 ± 1.6 mg were obtained for phenylephrine HCl and chlorpheniramine maleate, respectively, from clusters with $n \geq 11$. From prednisone clusters with 10 or more molecules, values of 12.4 ± 3.4 mg and 7.2 ± 1.2 mg were obtained for these respective ingredients. For all three clustering agents, the value obtained for chlorpheniramine maleate is slightly higher than the label value and these data are remarkably self-consistent. These results indicate that some preferential incorporation of this active ingredient into all of these clusters may occur, or the higher ionization efficiency of chlorpheniramine has a similar effect on these clusters irrespective of the clustering agent. No higher-order clusters were observed with L-serine as a clustering agent, likely due to the significantly lower ionization efficiency of L-serine compared to the two active ingredients.

Dramamine®

The % mole fraction obtained as a function of cluster size for dimenhydrinate using L-tryptophan and L-phenylalanine as clustering agents are shown in Figure 3a and 3b, respectively. In these solutions, the % mole fraction of dimenhydrinate is 1.1% and 1.0%, respectively. Dimenhydrinate is preferentially ionized over both of these clustering agents, and the smaller clusters appear to reflect this preferential ionization efficiency. However, the % mole fraction obtained for clusters with 10 or more molecules are remarkably constant indicating that this effect is negligible for these larger clusters. Using data obtained from larger clusters ($n \geq 20$), % mole fractions of 1.2 ± 0.3% and 1.2 ± 0.4% are obtained with L-tryptophan and L-phenylalanine, respectively. From these respective values, a dosage of 56 ± 14 mg and 59 ± 18 mg is obtained. These values are slightly higher than the label value of 50 mg of dimenhydrinate. As was the case for the solutions containing the other drugs, no clusters were observed with L-serine.

Conclusions

The dosage of active ingredients in several pharmaceutical tablets can be obtained without conventional standards or separation of the active ingredients from salts and other inactive

ingredients by adding a clustering agent to filtered solutions containing dissolved tablets and obtaining the % mole fraction from the abundance and composition of large clusters. Internal calibrants are often considered to be the “gold standard” of quantitation, but analysis with external standards is routinely used even though in some cases they might not be as accurate. There are trade-offs between ease-of-use and accuracy. This method is not as accurate or precise as methods that use conventional standards. This standard-free quantitation method has the advantages that it is fast, simple, and applicable to solutions containing unknown compounds or to solutions containing compounds where suitable standards may not be readily available, such as schedule I or II controlled substances or new designer drugs that have not been previously identified. This method does require enough resolution to separate clusters with different chemical composition, including salt adducts, and the resolution required will depend on the complexity of the sample. Sufficient resolution for these analyses could also be obtained with orbitrap or high-resolution time-of-flight mass spectrometers. The relatively poor precision in these initial studies can be attributed to the relatively low S/N for the larger clusters owing to the low concentration of the active ingredient used. Higher concentrations were not used because of potential mass interferences owing to salt adduction from the inactive ingredients, which complicate the mass spectra. If these salts were removed prior to analysis, higher concentrations could be used, which should greatly improve the precision of these measurements. A separation was not used here because oseltamivir phosphate, the active ingredient in Tamiflu®, is a prescription drug and is not readily available in pure form, but the label claim is expected to be sufficiently accurate to demonstrate the potential of this standard-free quantitation method. Tryptophan, phenylalanine, and prednisone were found to be suitable clustering agents, although serine and small peptides were ineffective, perhaps because of their significantly different physical properties, including lower ionization efficiency or larger differences in molecular weight compared to the drugs of interest. Further investigations into molecular properties that enhance formation of large, nonspecific clusters could improve the accuracy and would be advantageous to making this standard-free quantitation method a rapid and reasonably accurate method to obtain quantitative information directly from a wide range of analytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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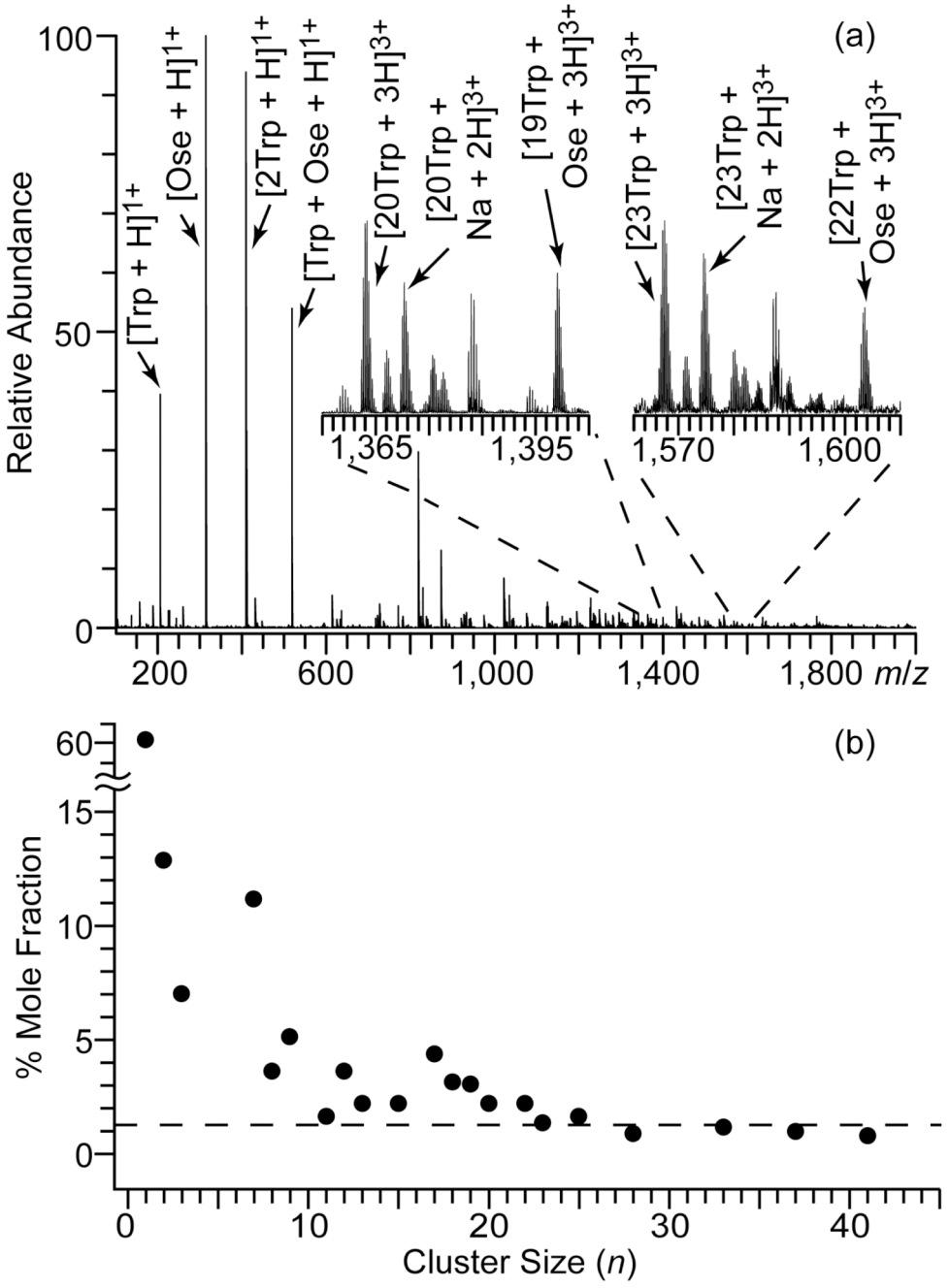

Figure 1.

(a) ESI mass spectrum of a solution containing 1.3% mole fraction of oseltamivir (Ose) from a Tamiflu® tablet preparation in L-tryptophan (10 mM total concentration) with some regions of the spectra with large molecular clusters inset. (b) Percent mole fractions calculated from protonated molecule and cluster ion abundances obtained from three ESI mass spectra of a 1.3% mole fraction of oseltamivir from a Tamiflu® tablet preparation as a function of cluster size, *n*. The dashed line corresponds to the % mole fraction in solution.

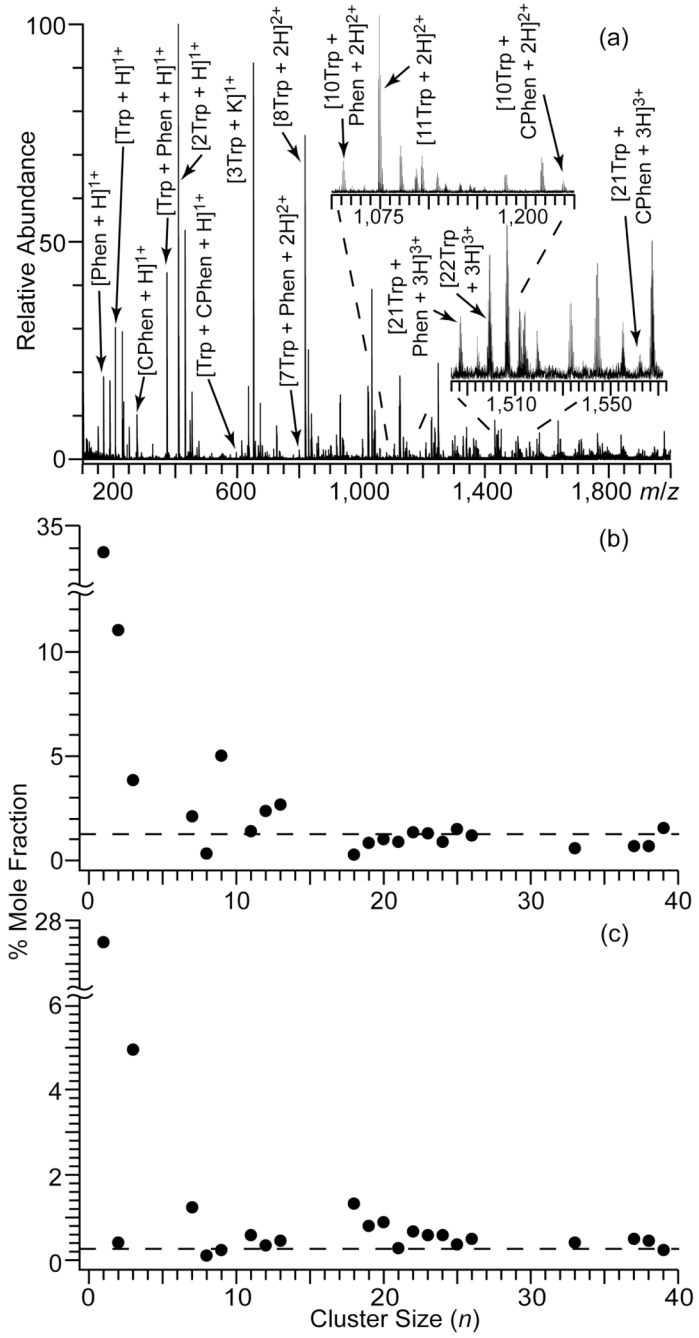

Figure 2.

(a) ESI mass spectrum of a solution containing 1.3% mole fraction of phenylephrine HCl (Phen) and 0.3% of chlorpheniramine maleate (CPhen) from a Sudafed® tablet preparation in L-tryptophan (10 mM total concentration) with some regions of the spectra with large molecular clusters inset. Percent mole fractions calculated from protonated molecule and cluster ion abundances obtained from three ESI mass spectra with L-tryptophan for (b) 1.3% Phen and (c) 0.3% CPhen as a function of cluster size, *n*. The dashed line corresponds to the % mole fraction in solution.

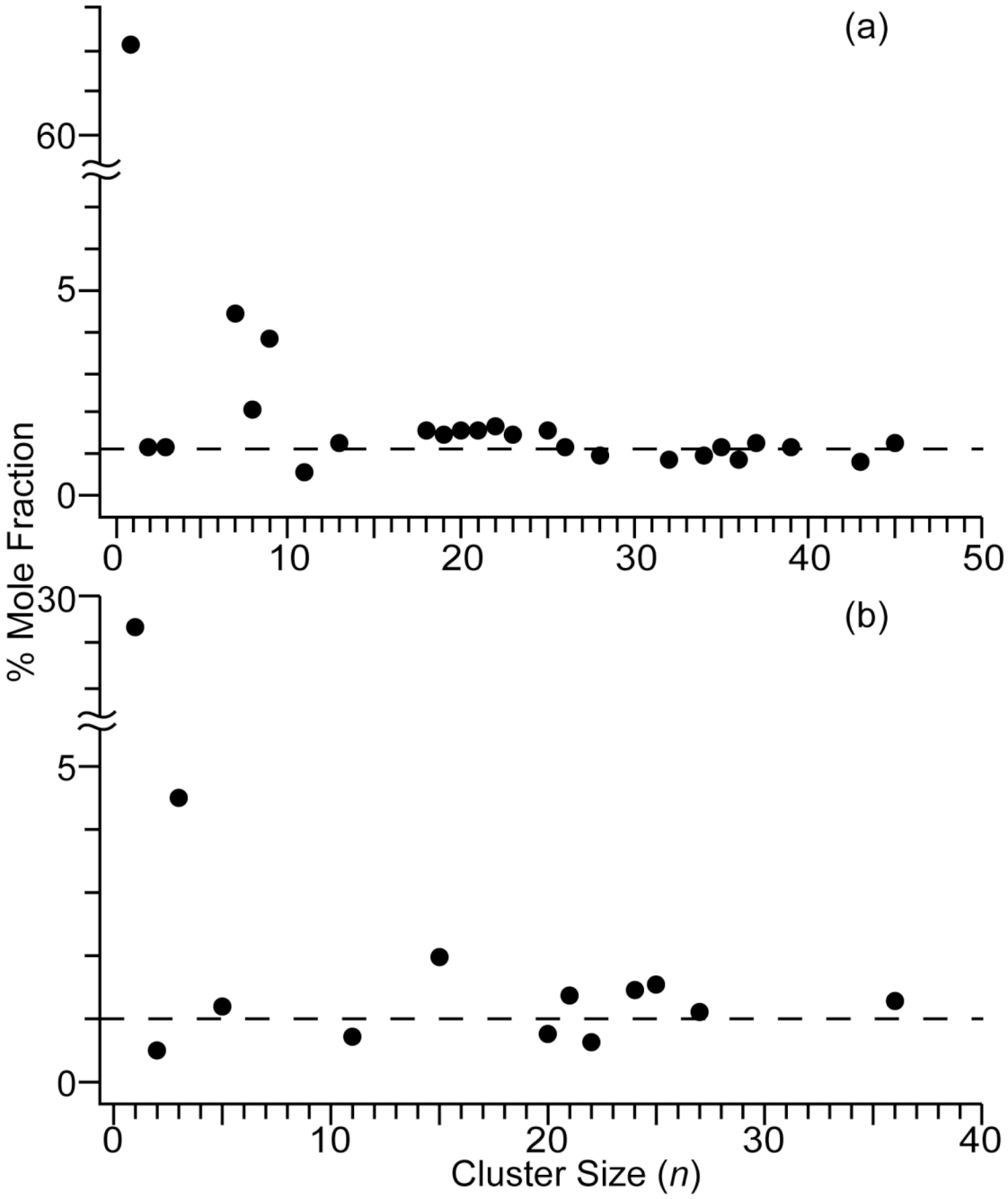

Figure 3.
Percent mole fractions calculated from protonated molecule and cluster ion abundances obtained from three ESI mass spectra of 1.1% and 1.0% dimenhydrinate from a Dramamine® tablet preparation in (a) L-tryptophan and (b) L-phenylalanine, respectively, as a function of cluster size, *n*. The dashed line corresponds to the % mole fraction in solution.