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Resolving Isomeric Peptide Mixtures: A Combined HPLC/Ion Mobility-TOFMS Analysis of a 4000-Component Combinatorial Library

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A reversed-phase high-performance liquid chromatography (HPLC) separation approach has been combined with ion mobility/time-of-flight (TOF) mass spectrometry in order to characterize a combinatorial peptide library designed to contain 4000 peptides of the general form NH_2-Xxx-Xxx-Xxx-CO_2H, NH_2-Ala-Xxx-Xxx-Xxx-CO_2H, NH_2-Ser-Ala-Xxx-Xxx-Xxx-CO_2H and NH_2-Leu-Ser-Ala-Xxx-Xxx-Xxx-CO_2H (where Xxx represents a randomization over 10 different amino acids: Ala, Arg, Asp, Glu, Gly, Leu, Lys, Phe, Ser, and Val). Addition of the gas-phase mobility separation between the HPLC separation and TOF measurement dimensions makes it possible to resolve many peptide isomers that have identical retention times (and masses).

Recently, a number of groups have used electrospray ionization (ESI)[1] coupled with mass spectrometry (MS) to directly assess the purity of combinatorial library mixtures.[2] This approach can provide important information about the reliability of synthetic protocols and, for small libraries containing only a few hundred components, is useful for screening assessment.[3,4] A general limitation in the use of MS for the analysis of complex mixtures is the inability of the mass measurement to discriminate isomers. Collision-induced dissociation would appear promising as a method for delineating and assigning mixtures of isomers; however, MS/MS data are often ambiguous when multiple components are dissociated simultaneously. It is often useful to couple a condensed-phase separation strategy [e.g., liquid chromatography (LC)] with MS in order to resolve isomeric species.[5]

We have recently developed an alternative strategy for analyzing mixtures of isomers.[6] In this approach, the library mixture is electrosprayed into a drift tube containing an inert buffer gas, and individual isomers are separated on the basis of differences in mobility prior to MS analysis. For a combinatorial library of tripeptides (expected to contain 676 components), we have demonstrated that it is possible to resolve many types of sequence-, structural- and stereoisomers.[7] The technique has several attractive features. For example, in favorable cases, it is possible to compare the experimentally measured mobilities with values that are calculated[8] for different isomers. The comparison of calculated and experimental mobilities for isobaric ions can be used to assign isomer structures to peaks in the mobility distributions.[9] Additionally, because of the relatively low density of the buffer gas (compared with condensed-phase separations), mobilities of ions in the gas phase are high; thus, separation can be carried out on millisecond time scales, making it possible to incorporate the gas-phase separation between condensed-phase separation methods and MS detection.

In this paper, we demonstrate the first high-performance LC/ion mobility/time-of flight (HPLC/IMS-TOFMS) analysis of a combinatorial library. The mixture is expected to contain ∼4000 different three-, four-, five-, and six-residue peptides (1000 each) and was designed to incorporate a large number of different sequence and structural isomers. The analysis was carried out by recording ion mobility/time-of-flight datasets (which we refer to as "nested" drift(flight) time datasets) in 1-min time intervals during a much longer (170 min) reversed-phase chromatographic separation. It is possible to assess the advantages of the additional dimension of mobility separation in some detail by analysis of these data. In the present system, we find many cases in which different peptide isomers have identical LC retention times,

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making them impossible to resolve by LC/MS alone. Using the HPLC/IMS-TOF approach, it is often possible to distinguish these components on the basis of their separation as ions in the gas phase.

The present work is similar to a number of approaches that are currently being developed as high-throughput screening strategies[10] for the characterization and screening of spatially addressable libraries.[11] For the most part, high-throughput approaches have focused on the analysis of single components within library arrays and have not addressed combinatorial library mixtures generated by mix-and-split synthesis.[12] The HPLC/IMS-TOF approach described here is well-suited for the analysis of complex synthetic mixtures containing many isomeric components. The advantages and current limitations of the technique are discussed.

EXPERIMENTAL SECTION

Library Synthesis. The synthesis of the combinatorial library mixture was designed to generate 4000 unique peptide sequences of varying length. A core tripeptide library sequence of the form NH_2-Xxx-Xxx-Xxx-CO_2H (where Xxx = Ala, Arg, Asp, Glu, Gly, Leu, Lys, Phe, Ser, and Val) was used as the scaffold for synthesis of the four-, five-, and six-residue peptides. The remaining library peptides were generated from the tripeptide sequences by N-terminal addition of Ala, Ser, and Leu residues to give sequences of the general form NH_2-Ala-Xxx-Xxx-Xxx-CO_2H, NH_2-Ser-Ala-Xxx-Xxx-Xxx-CO_2H and NH_2-Leu-Ser-Ala-Xxx-Xxx-Xxx-CO_2H.

The synthesis was carried out using a nitrogen-agitated manifold and standard mix-and-split synthetic protocols[13] with Fmoc (fluorenylmethoxycarbonyl) peptide chemistry.[14] The C-terminal peptide position was randomized by combining equimolar amounts of Fmoc-Xxx-Wang resins (Novabiochem): Fmoc-Ala, Fmoc-Arg(Pmc), Fmoc-Asp(OtBu), Fmoc-Glu(OtBu), Fmoc-Gly, Fmoc-Leu, Fmoc-Lys(Boc), Fmoc-Phe, Fmoc-Ser(tBu), and Fmoc-Val. Incorporation of amino acids at subsequent positions in the sequence was performed using a two-step procedure. The N-terminal Fmoc protecting group was removed via treatment with 20% piperidine in dimethylformamide (DMF). Activated OBt amino acid esters were then added to the deprotected resin. Amino acid OBt esters were generated by reaction of individual Fmoc amino acids (4.0 equiv) with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 3.9 equiv) and 0.4 M *N*-methylmorpholine in DMF (4.0 equiv). The coupling reactions were monitored using the ninhydrin reaction for quantitative measurement of free amine groups.[15]

Following completion of the synthesis of the tripeptide library sequences, the resin was divided into two fractions, a 1:3 split. The smaller of these fractions was reserved for subsequent cleavage to yield the tripeptides. The larger fraction was used to synthesize the tetrapeptides. This was accomplished by removing the Fmoc protecting group followed by N-terminal addition of an Ala residue. The five- and six-residue sequences were synthesized from the four- and five-residue peptides in an analogous fashion by the addition of Ser and Leu residues, respectively.

Equal amounts of the four-resin fractions were combined to yield the 4000-component mixture. The Fmoc protecting group was removed, and the library peptides were cleaved from the resin with simultaneous removal of acid-labile side-chain protecting groups using a trifluoroacetic acid (TFA):phenol:water:thioanisole:ethanedithiol solution (82.5:5:5:5:2.5 by volume). Resin peptides were precipitated in ether, washed several times with ether, and vacuum-dried. The resulting library peptides were dissolved in 0.1% aqueous trifluoroacetic acid (TFA) and lyophilized.

Preparation of Nanospray Solutions. Solutions for direct nanospray analysis of the library (no HPLC) were prepared at a concentration of 2.7 mg mL^{-1} (total peptides) by dissolving the peptide library mixture in 49:49:2 water:acetonitrile:acetic acid.

HPLC Separation. Reversed-phase HPLC separation of the library mixture was performed using a Waters 600 series HPLC system and a C_{18} column (Waters, Xterra MS C_{18}, 3.5 μm, 4.6 × 150 mm). A solvent mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN) was used as the chromatographic mobile phase with the following gradient sequence: 0−140 min (5−35% ACN), 140−150 min (35% ACN), 150−155 min (35−100% ACN), 155−165 min (100% ACN). The chromatogram was recorded using a detector wavelength of 214 nm, a mobile-phase flow rate of 0.25 mL min^{-1}, and a column temperature of 35 °C.

HPLC/IMS-TOF Analysis. A schematic representation of the Waters 600 series HPLC system coupled to the high-resolution IMS-TOF instrument is shown in Figure 1. Detailed descriptions of the IMS-TOF experiments[16−18] and the application of these techniques to the analysis of combinatorial library mixtures[6,7,19] have been given. Only a brief description is given here. The combination of HPLC, IMS, and TOF (demonstrated recently)[20] is feasible because of the time scales of these techniques (minutes, milliseconds, and microseconds, respectively). In the multidimensional experiment, library peptide ions are electrosprayed directly from the HPLC effluent into the entrance of a high-pressure drift tube that is coupled to a reflectron geometry time-of-flight mass spectrometer. The estimated ESI flow rate from the HPLC column, following a 200:1 flow split, is ∼1.2 μL min^{-1}.

The high-pressure drift tube used in these experiments has been described in detail previously.[21] The instrument was operated using an electric field of 170.97 V cm^{-1} and helium buffer gas pressures of ∼150−200 Torr. The mobility experiment is initiated by allowing a short pulse of ions (50−300 μs in duration) to enter the drift region from an ion gate at the front of the drift tube. As the ions travel the length of the drift region under the influence of the uniform electric field, they are separated on the basis of differences in their gas-phase mobilities. These mobility differences result from differences in the overall charge state of the ions and variations in their collision cross sections. For a

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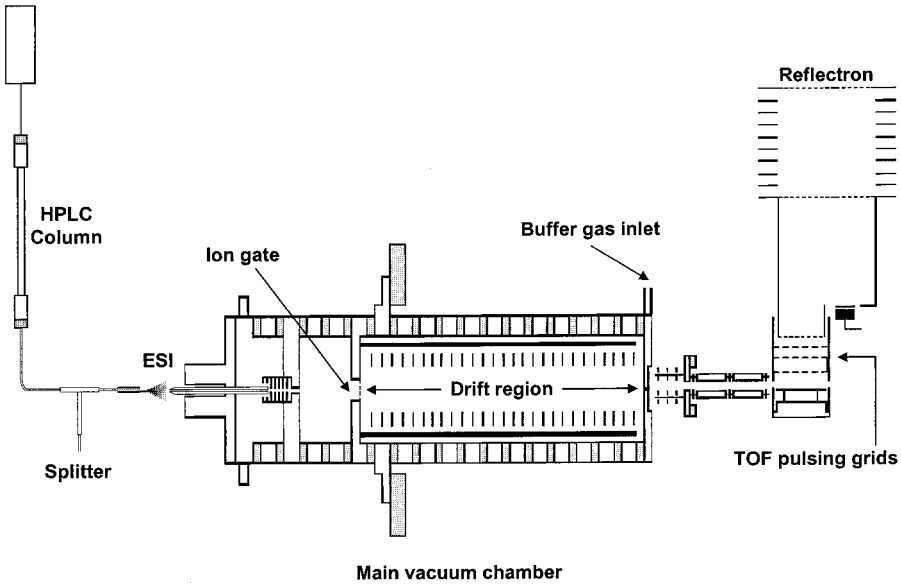

Figure 1. Schematic representation of the Waters 600 series HPLC coupled to the high-resolution ion mobility/time-of-flight (IMS-TOF) instrument.

distribution of ions with similar cross sections, high-charge-state ions experience a greater drift force than lower-charge-state ions; thus, the former will have higher mobilities (or shorter drift times). Ions of the same charge state are often separated because of variations in collision cross section. More-elongated structures undergo more collisions with the helium buffer gas and have longer experimental drift times than compact ions with the same m/z ratio.

As ions exit the drift tube, they are focused into the source region of an orthogonal geometry reflectron TOF mass spectrometer. Here, high-voltage pulses, synchronous with the mobility pulse, are used to introduce ions into the flight tube. The arrival time of each ion at the detector is referenced to both the high-frequency TOF pulse (which in these experiments was 2.5×10^4 Hz) and the lower-frequency mobility pulse (33 Hz). We refer to the IMS-TOF experiment as a nested experiment, because for each ion, we obtain both a drift time (t_D) and flight time (t_f). Positions of peaks in the nested drift (flight) time data are reported using the $t_D(t_f)$ nomenclature described previously;[16] unless otherwise noted, values of t_D and t_f are given in ms and μs, respectively. The nomenclature is easily extended to include additional dimensions. In this case, the two-dimensional $t_D(t_f)$ dataset becomes nested within a retention time window. We denote this as $t_r[t_D(t_f)]$. In this system, values of t_r are reported as the average retention time over which the $t_D(t_f)$ dataset was acquired. Flight times are converted to m/z ratios using a multipoint calibration method. Typically, the resolving power of the TOF measurement ranges from ~1500 to 2500 [$m/\Delta m$ definition, where Δm is the full width at half-maximum (fwhm) of a peak]. The resolving power of the high-pressure drift tube (defined as $t/\Delta t$, where Δt is the fwhm) is typically ~100−150.

Determination of Reduced Mobilities. The experimental measurement of mobility is highly reproducible; therefore, it is often useful to use this measurement in combination with m/z data to assign peaks. Reduced mobilities (or collision cross sections) are derived from the experimental data as described previously.[22]

Limitations in Ion Transmission. One of the current limitations of this approach is associated with the relatively low ion transmission through the drift tube. Currently, we circumvent this sensitivity issue by summing the $t_D(t_f)$ datasets for multiple LC runs. We find that virtually all components in complicated samples, such as the one described here, can be observed by summing five (or fewer) LC scans. We have recently improved the transmission and duty cycles on another instrument by more than an order of magnitude;[23] however, these modifications have not yet been adopted into the instrument used here.

Example $t_r[t_D(t_f)]$ Dataset. Before discussing the ~4000 component library, it is useful to illustrate the description of this approach using a relatively well-defined sample. Figure 2 shows a chromatogram for the HPLC separation of a peptide mixture generated by tryptic digestion of five different proteins [cytochrome *c* (horse), hemoglobin (pig and rabbit), albumin (horse), and β-casein]. The digest mixture is expected to contain 144 different tryptic peptide fragments. A sample nested $t_D(t_f)$ dataset acquired for a single 30-s retention time window (61.93−62.43 min) of the HPLC separation is also shown. The nested IMS-TOF dataset shown in the figure is one of 196 different 30-s frames acquired over the 120-min tryptic digest HPLC separation. Several $[M + 2H]^{2+}$ ions that correspond to tryptic peptides of four different proteins are observed in the $t_D(t_f)$ data for the 61.93−62.43-min window (e.g., EDLIAYLK [cyctochrome *c*, $t_D(t_f) = 19.4$-(24.4)], EAVLGLWGK [pig hemoglobin, 18.5(24.5)] ,and MFLG-FPTTK [pig hemoglobin/rabbit hemoglobin, 19.8(25.3)]). The digest mixture contains a number of peptides that have very similar m/z ratios [e.g., VGSR (horse albumin, $m/z = 418.24$) and TLGK (horse albumin, $m/z = 418.26$)]; however, there are typically no more than two different peptides expected at any given m/z ratio. Although HPLC/IMS-TOF analysis of the relatively simple digest mixture demonstrates the approach, analysis of the

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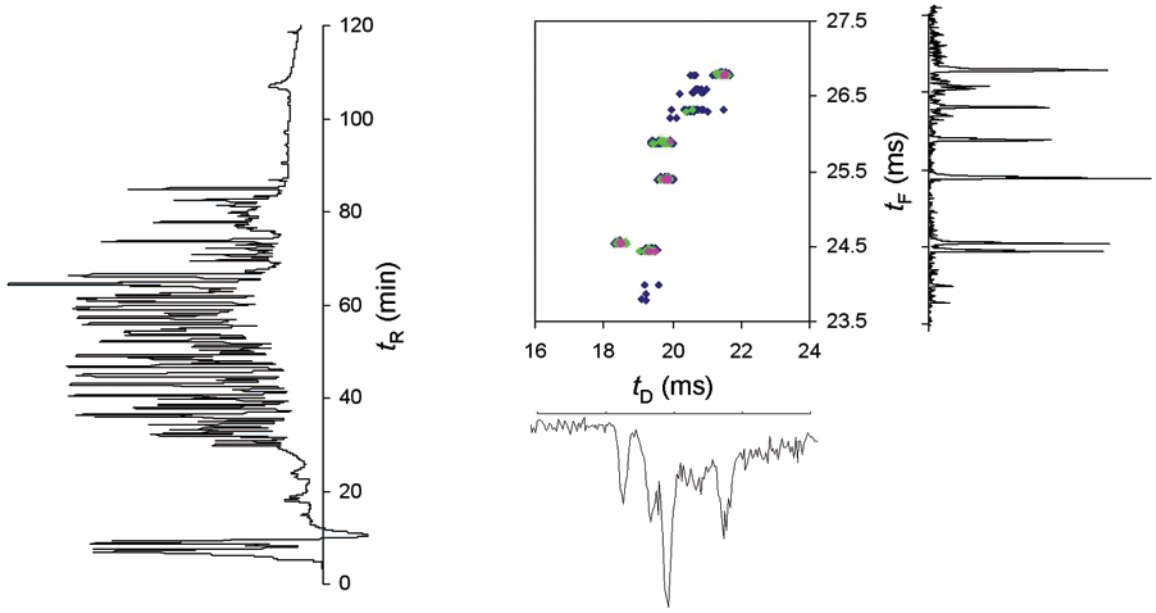

Figure 2. A sample $t_r[t_D(t_f)]$ dataset for a 30-s retention time window (t_r = 61.93−62.43) of the reversed-phase HPLC separation of a tryptic digest mixture generated from five proteins [cytochrome *c* (horse), hemoglobin (pig and rabbit), albumin (horse), and β-casein (bovine)]. These data were recorded using a helium buffer gas pressure of 161.3 Torr and a drift field of 170.97 V cm^{-1}. A TOF mass spectrum obtained for this t_r window by integration of the nested $t_D(t_f)$ dataset over all of the experimental drift times (right) is comparable to MS data obtained in a conventional LC/MS experiment. LC/IMS data can be obtained from the $t_D(t_f)$ data in an analogous fashion by integration over all of the experimental flight times (bottom). A standard UV chromatogram for the reversed-phase separation of the digest mixture is also shown (left). The separation was obtained using a water (0.1% formic acid):acetonitrile (0.1% formic acid) [ACN] gradient of the following sequence: 0−70 min (0−40% ACN), 70−85 min (40−60% ACN), 85−100 min (60−100% ACN), and 100−115 min (100% ACN). The chromatographic data was recorded using a detector wavelength of 214 nm, mobile-phase flow rate of 0.25 mL min^{-1}, and a column temperature of 35 °C.

more complex combinatorial library described below emphasizes the application of the combined HPLC/mobility dimensions for separation of up to ∼20−40 isomers at each m/z ratio. The mix-and-split synthesis results in many peptide isomers of the following form: (1) peptides of identical composition with amino acid sequence inversions (sequence isomers) and (2) various individual amino acids or combinations of amino acids that have identical or very similar masses (structural isomers). Examples of amino acid combinations that give rise to these structural isomers at m/z = 547.7 are Gly-Ala and Lys (mass = 128.1 and 128.2), Gly-Leu and Val-Ala (mass = 170.2 and 170.2), and Gly-Ser-Leu, Val-Ser-Leu, and Ala-Asp-Ala (mass = 257.3, 257.3, and 257.3).

For analysis of the 4000-component library, individual nested $t_D(t_f)$ datasets were obtained for 147 different 1-min retention time windows during a 170-min chromatographic run. About 9 s was required to save each $t_D(t_f)$ retention time window. No data are acquired while distributions are saved.

RESULTS AND DISCUSSION

Overview. In the discussion that follows, we summarize the results obtained for 147 different $t_D(t_f)$ datasets acquired during the library separation. This discussion is applicable to all of the datasets that we have recorded for this sample. We begin by considering a nested two-dimensional $t_D(t_f)$ distribution recorded for the direct ESI of the library. For less complex mixtures, the analysis of such two-dimensional datasets has provided detailed insight about the number of different isomers that are present; however, the complexity of the present system is sufficiently high that only a limited number of isomers are resolved. The analysis of the three-dimensional $t_r[t_D(t_f)]$ separation begins with a presentation of what is effectively an LC/MS analysis; that is, we have integrated across the ion-mobility dimension. This is followed by a discussion of the full $t_r[t_D(t_f)]$ dataset. The presentation is intended to illustrate the IMS separation as a complement to LC/MS for resolving isomers that have identical LC retention times.

Two-Dimensional $t_D(t_f)$ Distribution for the 4000-Component Library. Figure 3 shows a nested $t_D(t_f)$ dataset for direct ESI analysis of the 4000-component peptide library. Peaks in this distribution fall into three general families of ions. The spectrum is dominated by a series of peaks from m/z = 190 to 750 that correspond to relatively low-mobility ions. From previous work[24] and from examination of the isotopic spacing of peaks, we assign this abundant series as a family of $[M + H]^+$ ions. A family of higher-mobility ions is observed from m/z ∼280 to 400. These peaks can be assigned to $[M + 2H]^{2+}$ peptides and are discussed more below. Finally, a lower-intensity series of peaks that corresponds to ions with relatively high mobilities is observed from m/z ∼350 to 750. This family can be assigned to multiply charged multimers of the general form $[2M + 2H]^{2+}$. These $[2M + 2H]^{2+}$ peaks appear at m/z ratios that are identical to many of the $[M + H]^+$ peptide ions; however, it is possible to distinguish them on the basis of differences in their experimental drift times.

In some regions of the distribution, isobaric ions appear to be resolved on the basis of differences in their mobilities through the buffer gas; however, most regions show that the ion mobility distributions (for a group of isomers at a single m/z value) are broad with unresolved features. A typical mobility distribution is

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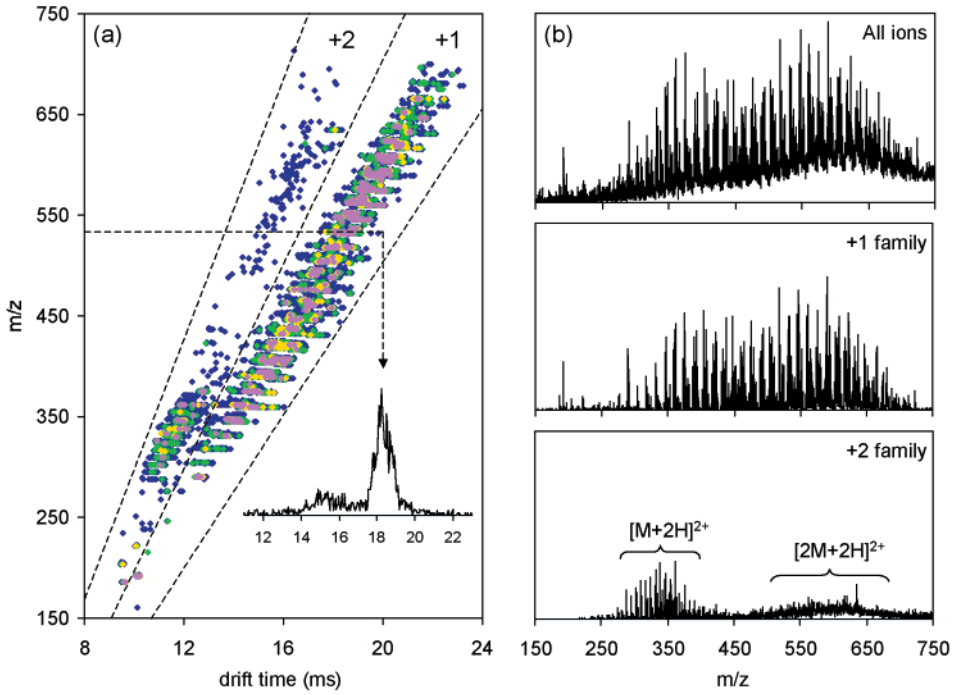

Figure 3. Nested $t_D(t_i)$ data obtained for direct ESI of the 4000-component peptide library mixture is shown in part a. These data were recorded using a helium buffer gas pressure of 154.2 Torr and a drift field of 170.97 V cm^{-1}. Diagonal dashed lines indicate regions of the dataset corresponding to low mobility, singly charged and higher-mobility, doubly charged peptide ions. A sample ion mobility distribution obtained by integrating the $t_D(t_i)$ data across all drift times is also shown in part a for $m/z = 533.6$. Drift times for library isomer ions at this m/z ratio vary from ~17.5 to 19.2 ms. The TOF mass spectra shown in part b were obtained by integration over the diagonal drift(flight) time regions indicated on the $t_D(t_i)$ plot for the +1 and +2 ion families. Integration over all drift times yields an all-ion mass spectrum that is similar to spectra obtained in conventional ESI-MS experiments (top). Integration over the specified regions of the $t_D(t_i)$ data indicated in the figure gives TOF mass spectra for the singly charged ion family at longer drift times (middle) and the higher-mobility doubly charged ion family (bottom).

shown in Figure 3 for $m/z = 533.6$. From $m/z = 533.5$ to 533.7, it is expected that 39 different peptides will be present. Experimental drift times for the $[M + H]^+$ ions at $m/z = 533.6$ vary from ~17.5 to 19.2 ms, a range of ~10%. The broad feature suggests that many peptides with similar mobilities are present but are not resolved in the ion mobility separation.

It is often useful to examine mass spectral slices across different regions of the $t_D(t_i)$ distribution. Figure 3 shows several mass spectral slices across the dataset. Integration across all drift times yields the mass spectrum for all ions. In this plot, peaks for the $[M + H]^+$ ions are superimposed on the lower intensity $[2M + 2H]^{2+}$ peaks, making it difficult to resolve individual ions. In addition, many $[M + 2H]^{2+}$ peaks are obscured by more intense features associated with $[M + H]^+$ ions. Contributions to the mass spectrum from the doubly charged $[M + 2H]^{2+}$ and $[2M + 2H]^{2+}$ ions can be removed by integrating over regions of the nested dataset that correspond to only the lower-mobility $[M + H]^+$ ions. In this type of plot, virtually all of the $[M + H]^+$ peaks are resolved to near baseline. An analogous procedure can be used to generate a mass spectrum for the doubly charged ions. Many of the peaks that are apparent in this slice are not observed in the mass spectrum for all ions, because the peak intensities are less than the relatively large baseline in the all-ion mass spectrum.

Analysis of data integrated over these narrow ranges makes it possible to assign amino acid composition to many $[M + 2H]^{2+}$ peaks. Most of these peaks correspond to five- and six-residue peptides with at least one basic Arg or Lys residue. For example, the peak at $m/z = 366.5$ is consistent with the formation of $[M + 2H]^{2+}$ ions of the NH_2-Leu-Ser-Ala-Xxx-Xxx-Xxx-CO_2H sequences (where Xxx = Arg, Glu, Lys, and Phe). Similarly, the peak at $m/z = 287.9$ corresponds to $[M + 2H]^{2+}$ ions of the six-residue sequences NH_2-Leu-Ser-Ala-Xxx-Xxx-Xxx-CO_2H (where Xxx = Ala, Arg, Gly, and Val) and the five-residue NH_2-Ser-Ala-Xxx-Xxx-Xxx-CO_2H (where Xxx = Arg, Leu and Lys) sequences. It appears that in such small peptides, two relatively basic sites (the amino terminus and a basic Arg or Lys residue) are required to stabilize the doubly protonated forms. There is no evidence in the spectrum for the formation of three-residue $[M + 2H]^{2+}$ peptide ions, suggesting that these peptides cannot stabilize the repulsive force between the two protons.

Eyler, Benner, and their collaborators have shown that some information about the number of sequence isomers that is present can be obtained by comparing the relative intensities of different peaks in the mass spectrum.[25] Figure 4 shows a comparison of the integrated mass spectrum for the $[M + H]^+$ family with a theoretical mass spectrum that is calculated by assuming that all expected components are present in equal abundance and have identical ionization efficiencies.[26] Relative intensities of peaks in the calculated spectrum are determined from the total number of expected library peptides at each m/z value (0.1 m/z unit bin size). Peaks are represented by Gaussian distributions with fwhm's that are consistent with a TOF resolution of 2000 ($m/\Delta m$ = fwhm

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(26) In this calculation, contributions from natural abundances of heavy-atom isotopes are small and have not been considered.

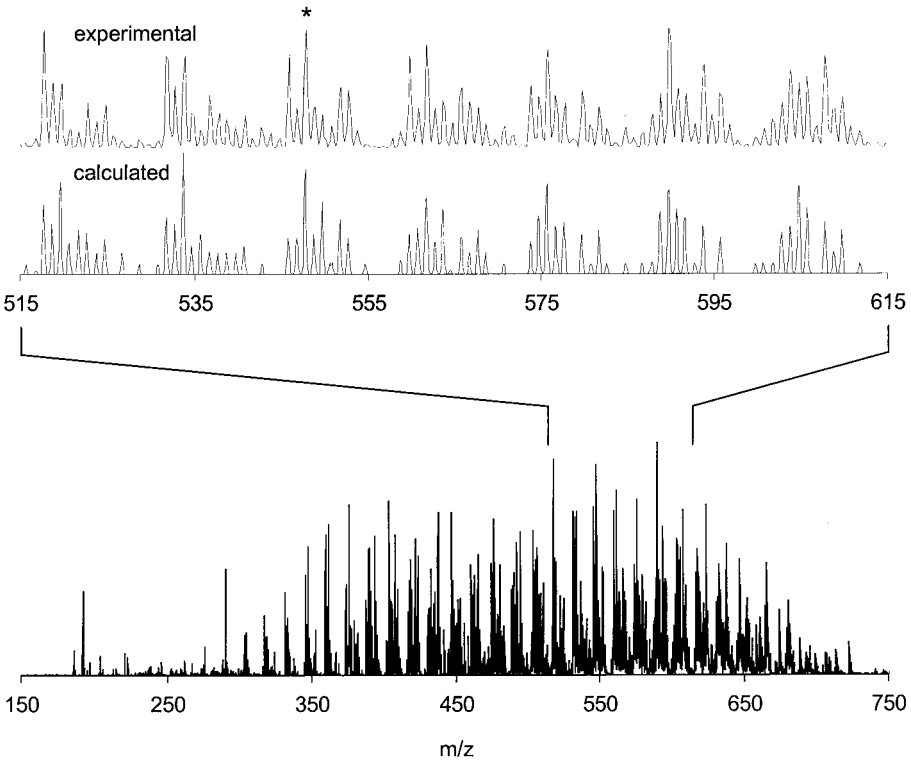

Figure 4. Integrated TOF mass spectrum for the $[M + H]^+$ peptide ions in the nested $t_D(t_f)$ data obtained by direct ESI of the library mixture (bottom). A comparison of the experimental mass spectrum with a theoretical spectrum calculated by adding the number of expected peptides at each *m*/*z* ratio is also shown (top). Theoretical peptide *m*/*z* values for the calculated spectrum were determined using the monoisotopic masses of the component amino acid residues. Contributions from the natural abundances of heavy atom isotopes for these sequences are small and were not included. The peak at *m*/*z* = 547.7 (indicated with the asterisk) corresponds to the following expected library sequences (*m*/*z* = 547.6−547.7): Ser-Ala-Arg-Asp-Val, Ser-Ala-Arg-Val-Asp, Ser-Ala-Asp-Arg-Val, Ser-Ala-Asp-Val-Arg, Ser-Ala-Val-Arg-Asp, Ser-Ala-Val-Asp-Arg, Leu-Ser-Ala-Ala-Glu-Gly, Leu-Ser-Ala-Ala-Gly-Glu, Leu-Ser-Ala-Glu-Ala-Gly, Leu-Ser-Ala-Glu-Gly-Ala, Ala-Leu-Ser-Ala-Gly-Ala-Glu, Leu-Ser-Ala-Gly-Glu-Ala, Ser-Ala-Glu-Leu-Lys, Ser-Ala-Glu-Lys-Leu, Ser-Ala-Leu-Glu-Lys, Ser-Ala-Leu-Lys-Glu, Ser-Ala-Lys-Glu-Leu, Ser-Ala-Lys-Leu-Glu, Leu-Ser-Ala-Ala-Ala-Asp, Leu-Ser-Ala-Ala-Asp-Ala, Leu-Ser-Ala-Ala-Ser-Val, Leu-Ser-Ala-Ala-Val-Ser, Leu-Ser-Ala-Asp-Ala-Ala, Leu-Ser-Ala-Gly-Leu-Ser, Leu-Ser-Ala-Gly-Ser-Leu, Leu-Ser-Ala-Leu-Gly-Ser, Leu-Ser-Ala-Leu-Ser-Gly, Leu-Ser-Ala-Ser-Ala-Val, Leu-Ser-Ala-Ser-Gly-Leu, Leu-Ser-Ala-Ser-Leu-Gly, Leu-Ser-Ala-Ser-Val-Ala, Leu-Ser-Ala-Val-Ala-Ser, and Leu-Ser-Ala-Val-Ser-Ala.

definition), near the experimental resolution over this m/z range. An inspection of the experimental and calculated distributions across the entire dataset shows that essentially all of the calculated peaks are present in the experimental distributions. Moreover, there is a reasonable correlation of expected and experimental peak intensities.

The similarities of the experimental and calculated mass spectra suggest that many aspects of the synthesis were completed as expected; however, for such a large library containing many sequence and structural isomers, only a gross characterization can be obtained from analyzing the relative intensities of mass spectral peaks. Consider, for example, the peaks at $m/z = 547.7$, 549.6, and 551.6. The synthesis is expected to generate 33, 21, and 16 different $[M + H]^+$ peptides at these m/z values. Thus, we would expect the ratios of the intensities of these respective peaks to be 1.00:0.64:0.48. The measured relative ratios are 1.00: 0.27:0.51, suggesting that some sequences at $m/z = 549.6$ are in low abundance (or perhaps missing entirely). Our experience with this type of comparison suggests that an analysis of library integrity that is based on the relative intensities of peaks should detect when large changes in the composition of peaks occur (>20% intensity variations); however, we would not expect to be sensitive to a case for which only a few (out of many) isomers are missing entirely. Additionally, this analysis cannot differentiate between the complete absence of some peptides and a case in which isomers are present but are in low concentrations.

It is important to note that an analysis of library integrity based on the comparison of relative peak intensities may be influenced by variations in ionization efficiency for library peptides of different length or amino acid sequence. Below, we describe an analysis that involves an assessment of the number of peptides that have unique combinations of experimental retention time, drift time, and m/z ratio, which is essentially independent of small variations in ionization efficiency.

Analysis of a $t_r[t_D(t_f)]$ Dataset: UV and Ion Chromatograms. Figure 5 shows a comparison of the UV and ion chromatograms that are obtained upon analysis of this system. The UV chromatogram shows an intense broad feature that peaks at ~15 min. This feature rapidly decays, and a complex series of unresolved features is observed (from ~20 to 100 min). The appearance of the ion chromatogram is substantially different. Ion signal intensities remain relatively low until ~30 min. From 30 to 80 min., a broad peak with many relatively sharp features is observed. The intensity of ion chromatogram features gradually decreases at t_r > 80 min. The broad peak at ~15 min may result from coelution of multiple peptide components in the LC separation. Small peptides that contain high fractions of polar amino acid residues are expected to have very short retention times because

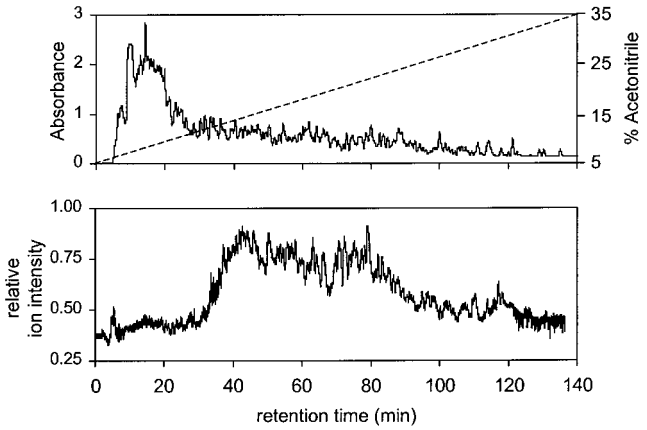

Figure 5. (top) Chromatogram for the reversed-phase HPLC separation of the 4000-component peptide library acquired using a Waters 2487 detector and a wavelength of 214 nm. The dashed line shows the solvent gradient employed. (bottom) Ion chromatogram obtained by integrating the total ion intensity over all of the retention time windows for the $t_r[t_D(t_f)]$ library data.

of their low affinity for the C_{18} stationary phase. It is also likely that the absorbance measurement at ~15 min in the UV dataset is high as a result of residual TFA that was used to cleave the peptides from the solid-phase support. Because it has a strong absorption band at 214 nm, very low concentrations of TFA can be detected in the UV chromatogram. The discrepancy between the UV and ion chromatograms suggests that changes in mobile-phase composition during the gradient elution affect the ionization efficiencies of library peptides. There is no significant ion intensity observed in the ion chromatogram at retention times <30 min. At these low retention times, the mobile phase composition is <10% acetonitrile; these solvent compositions may lead to inefficient ESI of components.

Table 1. Summary of Expected Peptides at m/z = 423.4, 452.5, and 517.6

m/z[a]		
423.4−423.5	452.5−452.6	517.6−517.7
Ala-Glu-Gly-Phe[b,c]	Ser-Ala-Ala-Gly-Phe	Leu-Ser-Ala-Ala-Gly-Val
Ala-Glu-Phe-Gly	Ser-Ala-Ala-Phe-Gly	Leu-Ser-Ala-Ala-Val-Gly
Ala-Gly-Glu-Phe	Ser-Ala-Gly-Ala-Phe	Leu-Ser-Ala-Gly-Ala-Val
Ala-Gly-Phe-Glu	Ser-Ala-Gly-Phe-Ala	Leu-Ser-Ala-Gly-Val-Ala
Ala-Phe-Glu-Gly	Ser-Ala-Phe-Ala-Gly	Leu-Ser-Ala-Val-Ala-Gly
Ala-Phe-Gly-Glu	Ser-Ala-Phe-Gly-Ala	Leu-Ser-Ala-Val-Gly-Ala
Ala-Ala-Asp-Phe	Ala-Lys-Phe-Ser	Leu-Ser-Ala-Gly-Gly-Leu
Ala-Ala-Phe-Asp	Ala-Lys-Ser-Phe	Leu-Ser-Ala-Gly-Leu-Gly
Ala-Asp-Ala-Phe	Ala-Phe-Lys-Ser	Leu-Ser-Ala-Leu-Gly-Gly
Ala-Asp-Phe-Ala	Ala-Phe-Ser-Lys	
Ala-Phe-Ala-Asp	Ala-Ser-Lys-Phe	Ala-Arg-Arg-Asp
Ala-Phe-Asp-Ala	Ala-Ser-Phe-Lys	Ala-Arg-Asp-Arg
		Ala-Asp-Arg-Arg
Ala-Phe-Ser-Val		
Ala-Phe-Val-Ser		Ser-Ala-Ala-Arg-Leu
Ala-Ser-Phe-Val		Ser-Ala-Ala-Leu-Arg
Ala-Ser-Val-Phe		Ser-Ala-Arg-Ala-Leu
Ala-Val-Phe-Ser		Ser-Ala-Arg-Leu-Ala
Ala-Val-Ser-Phe		Ser-Ala-Leu-Ala-Arg
		Ser-Ala-Leu-Arg-Ala
Gly-Lys-Phe		
Glu-Phe-Lys		Ser-Ala-Leu-Lys-Val
Lys-Glu-Phe		Ser-Ala-Leu-Val-Lys
Lys-Phe-Glu		Ser-Ala-Lys-Leu-Val
Phe-Glu-Lys		Ser-Ala-Lys-Val-Leu
Phe-Lys-Glu		Ser-Ala-Val-Leu-Lys
		Ser-Ala-Val-Lys-Leu

[a] Calculated m/z values for expected peptides. [b] Sequences are written from N-(NH_2-) to C-terminus (-CO_2H). [c] Spaces in each column are used to delimit groups of peptides that have the same amino acid composition.

Consideration of LC/MS Data from a $t_r[t_D(t_f)]$ Dataset. The nested $t_r[t_D(t_f)]$ dataset can be represented in terms of an LC/MS analysis. Figure 6 shows several typical mass spectra obtained

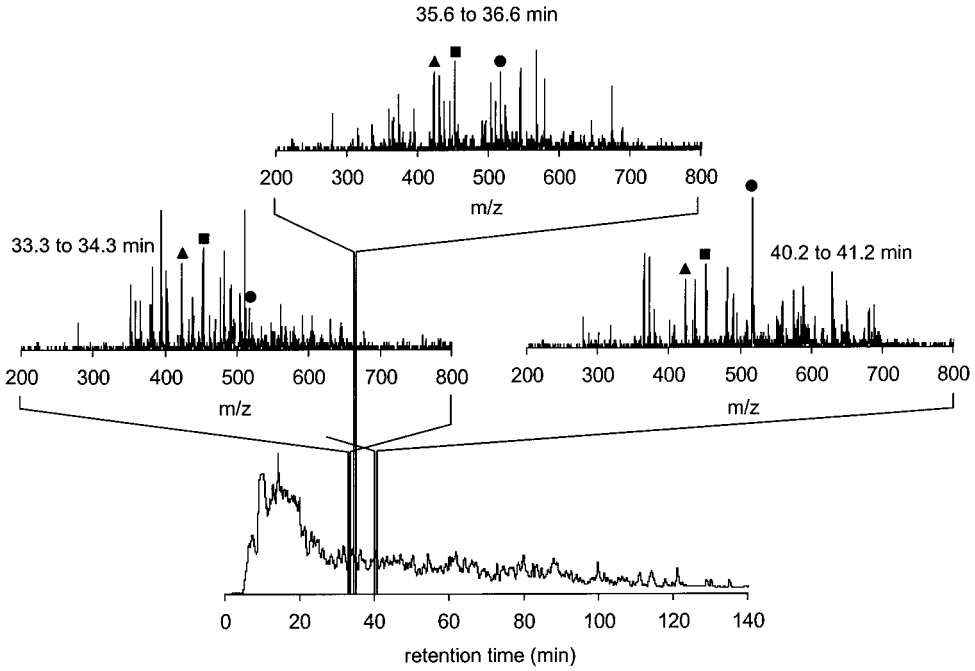

Figure 6. LC/MS data for three different 1-min retention time windows of the reversed-phase HPLC separation of the 4000-component library. The mass spectra were obtained by integration of the $t_D(t_f)$ data for the following retention time windows: t_r = 33.3−33.4 min (left), t_r = 35.6−36.6 min (center), and t_r = 40.2−41.2 min (right). Peaks at 423.4 (▲), 452.5 (■), and 517.6 (●) are indicated on the plots. The location of these t_r windows with respect to the full retention time range is indicated on the UV chromatogram.

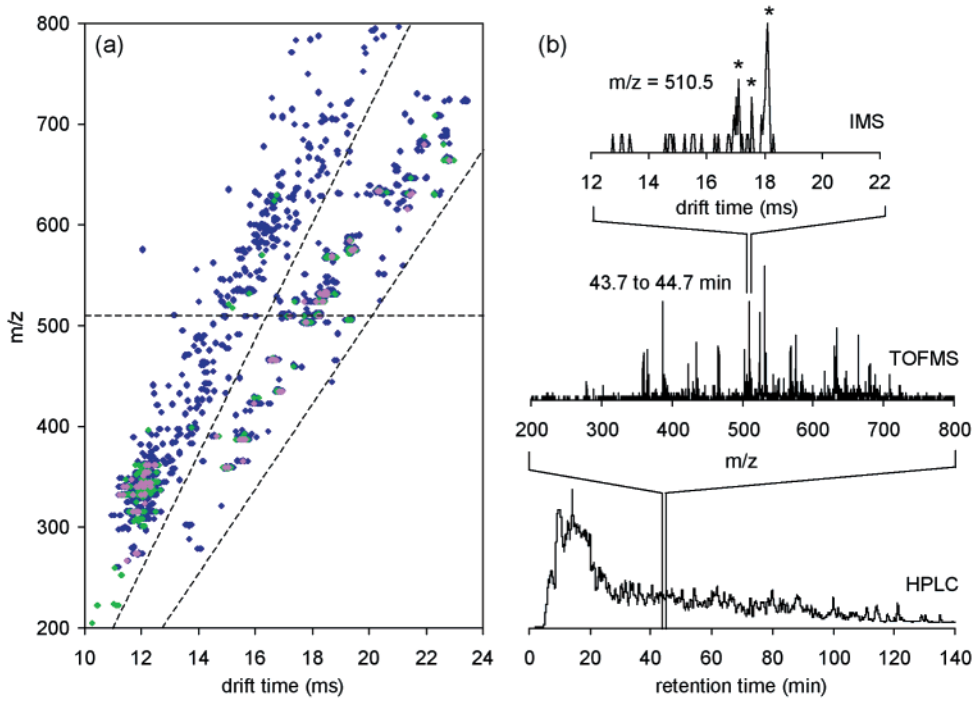

Figure 7. Nested $t_D(t_f)$ data for the LC/IMS-TOF analysis of the library mixture obtained for the t_r = 43.7−44.7-min retention time window is shown in part a. These $t_D(t_f)$ data were acquired using a drift field of 170.97 V cm^{-1} and a helium buffer gas pressure of 156.11 Torr. Integrated data obtained from the nested $t_D(t_f)$ dataset for the individual IMS and TOF dimensions are shown in part b. An integrated TOF mass spectrum for the $[M + H]^+$ ions is shown with respect to the t_r = 43.7−44.7-min retention time window of the chromatographic separation over which the $t_D(t_f)$ dataset was acquired (middle). In addition, an example drift-time distribution for m/z = 510.5 is shown for this retention time window to illustrate the mobility separation of isomeric peptide ions (top).

for $[M + H]^+$ ions (from integration of $t_D(t_f)$ datasets) for three different 1-min retention time windows. Although these data are analogous to those that would be acquired using a conventional LC/MS approach, we note that the integration over only the $[M + H]^+$ family simplifies the mass spectrum (and the interpretation). It is instructive to consider several peaks as examples. Peaks at m/z values of 423.4, 452.5, and 517.6 are observed in the mass spectra for all three of the retention time windows that are shown. Over m/z ranges of 423.4−423.5, 452.5−452.6, and 517.6−517.7, we calculate that there should be 24, 12, and 24 different peptides, respectively. Over the 147 LC frames that have been recorded, we observe an m/z = 423.4 ion at 10 different retention times. The m/z = 452.5 and 517.6 ions are observed at seven and eight different retention times, respectively.

The sequences of peptides expected for these narrow m/z ranges are summarized in Table 1. Examination of these sequences shows that several amino acid compositions are present within each narrow m/z range. For example, within the 423.4−423.5 range, there are four different compositions (each composed of six different sequences). There are two and five compositions for the m/z = 452.5−452.6 and 517.6−517.7 ranges, respectively. The observation that we resolve more peaks in the LC/MS dimensions than compositions (within a narrow m/z range) indicates that at least some of the sequence isomers must be separated in the HPLC; however, we note that fewer than 50% of the peptides over these narrow ranges are resolved. Assuming that the integrity of this synthesis is similar to others that we (and others) have observed,[7] the low number of LC/MS peaks indicates that some of the peaks must be due to sequences within the narrow m/z range that coelute in the LC experiment.

Although we can obtain estimates of the number of components that are present from the number of different LC/MS peaks for a specific m/z ion, these estimates are often not as reliable as estimates from MS data alone. Because the ESI efficiency changes for different solvent compositions, it is not possible to reliably compare peak intensities from LC/MS data. Below, we show that the additional gas-phase separation improves the analysis of the number of different isomers observed.

Analysis of an Example $t_D(t_f)$ Window within the $t_r[t_D(t_f)]$ Dataset. Figure 7 shows an example $t_D(t_f)$ distribution recorded from t_r = 43.7 to 44.7 min. This dataset falls in the broad flat region of the chromatogram and is typical of most other $t_D(t_f)$ distributions. The complementary nature of the mobility and m/z analysis can be seen by comparing an integrated mass spectrum for the $[M + H]^+$ peptides with the two-dimensional $t_D(t_f)$ distribution. In the former data, 23 peaks are resolved; in the latter, there is evidence for 35 resolved $[M + H]^+$ peaks. Here, a feature in the data is considered a peak when an ion signal is observed in three or more adjacent drift time windows (across a single flight time). In a number of cases, it appears that it is possible to separate isomers that were not resolved in the HPLC separation. For example, an ion mobility distribution at m/z = 510.5 shows evidence for two different isomers having drift times of 17.2 and 18.2, respectively. A very low intensity spike at t_D = 17.7 may also correspond to a peptide. It is generally possible to differentiate low-intensity peaks in the mobility distributions from noise on the basis of experimental peak widths. Peaks due to library peptides should have widths consistent with the typical resolving power of the high-pressure drift tube used in these studies ($t/\Delta t$ = 100−

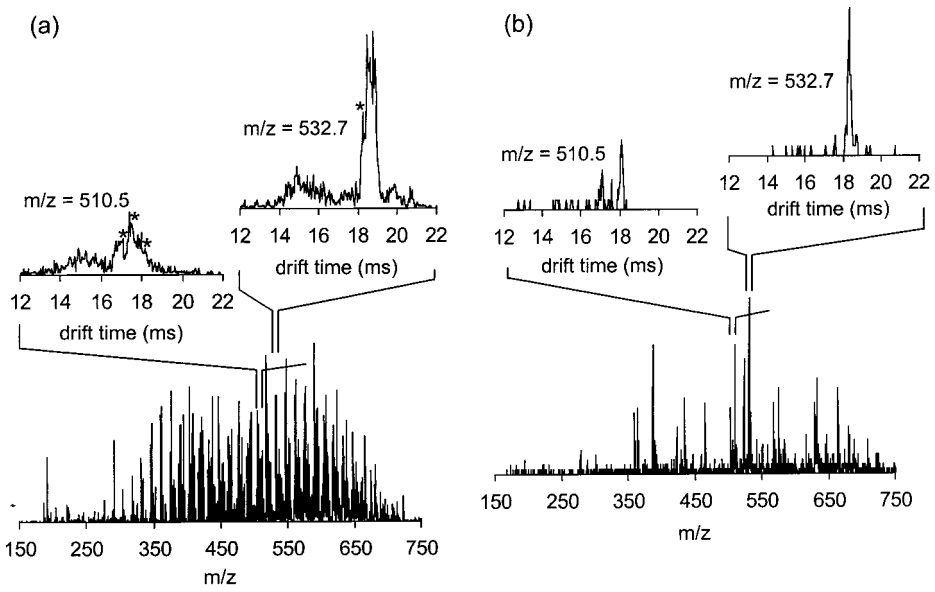

Figure 8. Part a shows the TOF mass spectrum obtained by integration of the direct ESI $t_D(t_f)$ data ($[M + H]^+$ ions only) for the 4000-component mixture (bottom). Ion mobility distributions for the direct ESI analysis of the library mixture are also shown for $m/z = 510.5$ and 532.7 (top). These mobility slices show broad, unresolved features that are consistent with the presence of numerous isomeric peptides having very similar mobilities. Part b shows the integrated $[M + H]^+$ mass spectrum obtained from the $t_r[t_D(t_f)]$ data for the $t_r = 43.7-44.7$-min window. Also shown are the corresponding mobility distributions at $m/z = 510.5$ and 532.7 for this t_r window (top). The features indicated with asterisks in the mobility distributions in part a correspond to the sharp features observed in the distributions for the $t_r = 43.7-44.7$-min window shown in part b.

150). Analysis of this single $t_D(t_f)$ window provides evidence for multiple $[M + H]^+$ peptides at m/z values of 510.5, 524.6, 532.6, and 631.8. Additionally, multiple peptides appear to be resolved for several $[M + 2H]^{2+}$ peaks. For example, at $m/z = 333.0$, three distinct peaks are resolved. A more detailed comparison of the number of different components that are resolved within the $t_r[t_D(t_f)]$ distribution is given below.

Finally, we have considered how the ion mobility distributions are influenced by the LC separation. Figure 8 shows a comparison of the $t_D(t_f)$ data obtained for direct ESI of the library mixture and the data from the $t_r[t_D(t_f)]$ dataset taken at $t_r = 43.7-44.7$ min. The integrated mass spectrum for the $[M + H]^+$ ions from the direct ESI-IMS-TOF data is significantly more complex than the mass spectrum for the single LC window. Additionally, ion mobility slices across the datasets show that peaks are much sharper in the $t_r[t_D(t_f)]$ dataset. This comparison corroborates the idea presented above that in the direct $t_D(t_f)$ analysis, the broad features in the ion mobility data were due to many unresolved peptides.

Estimate of the Number of Components in the Library. One approach for summarizing the experimental data and analysis presented above is to simply count the number of expected and resolved peaks at each m/z ratio across all of the LC retention times and IMS drift times. For the integrated LC/MS form of the data, we have combined an automated and manual analysis to determine the number of peaks that are present. In total, we observed 2069 different $[M + H]^+$ peptides (i.e., those with intensities that are above a background cutoff level). A direct count of the total number of peaks resolved in the $t_r[t_D(t_f)]$ dataset is more difficult, primarily because an automated approach for identifying the positions of peaks in the two-dimensional data does not exist yet. It is useful to examine the results of a manual peak count over some limited ranges. For example, we calculate that the synthesis should produce 24 different $[M + H]^+$ sequences having $m/z = 423.4$. An analysis of the MS data for all retention time windows shows that at this m/z ratio, ions are observed in 10 different t_r windows. Upon examining the $t_D(t_f)$ data for each of these windows, we find evidence for 19 peaks. This analysis shows that for the sequences at $m/z = 423.4$, incorporation of the mobility separation with the LC and TOF approaches permits a large fraction (~79%) of the expected library components to be resolved.

For other m/z values, the ability to resolve different isomers with a combined LC/IMS separation varies. Figure 9 provides a summary of LC/MS and LC/IMS-MS peak counts for 11 different groups of isomers (chosen randomly).[27] In all of these cases, the LC/MS experiment can resolve at least 50% of the different isobars; the average fraction of components resolved by LC/MS is 68 ± 28% of the total expected number. When the two-dimensional $t_D(t_f)$ distributions are examined, we find that 82 ± 25% of the expected number of peaks are observed. From this very limited analysis of 11 out of 601 expected m/z ions, we estimate (very roughly) that ~3280 of the anticipated 4000 components have been resolved and detected.

Another test of the rough assessment of the data can be obtained by carrying out detailed peak counts for a limited number of retention time windows and then scaling these results over the entire distribution. This type of analysis allows us to locate the positions of individual peaks within the complete array of

(27) The number of peaks that are observed for ions at $m/z = 593.7$, 623.8, and 637.8 is greater than the number of expected library peptides. Several explanations may help to explain this result. First, some isomers could exhibit multiple stable conformations such that an individual component is observed as two resolved peaks in the drift-time distribution. We note that most small peptides (<6 residues) do not display such behavior (for experiments conducted at 300K). It is also possible that some synthetic impurities are present, as discussed previously in ref 6.

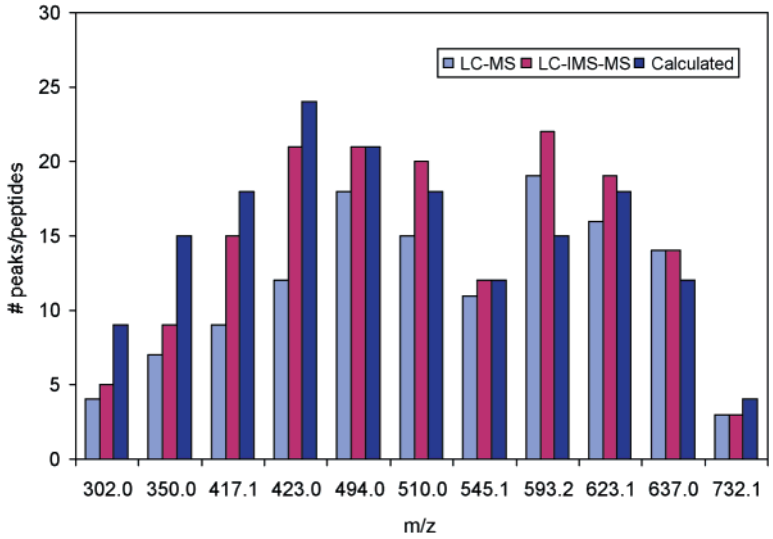

Figure 9. Comparison of the number of peaks obtained from an LC/MS and LC/IMS-TOF analysis of the $t_r[t_D(t_f)]$ data with the number of expected peptides for 11 different *m*/*z* ratios (*m*/*z* = 302.4, 349.3, 417.4, 423.4, 494.5, 510.5, 545.6, 593.7, 623.8, 637.8, and 731.9). The LC/MS data presented in the figure were obtained by determining the number of different t_r windows in which ions at these 11 *m*/*z* ratios are observed. Data for the LC/IMS-TOF analysis was obtained by determining the number of mobility-resolved peaks at these 11 *m*/*z* values for the retention time windows identified in the LC/MS analysis.

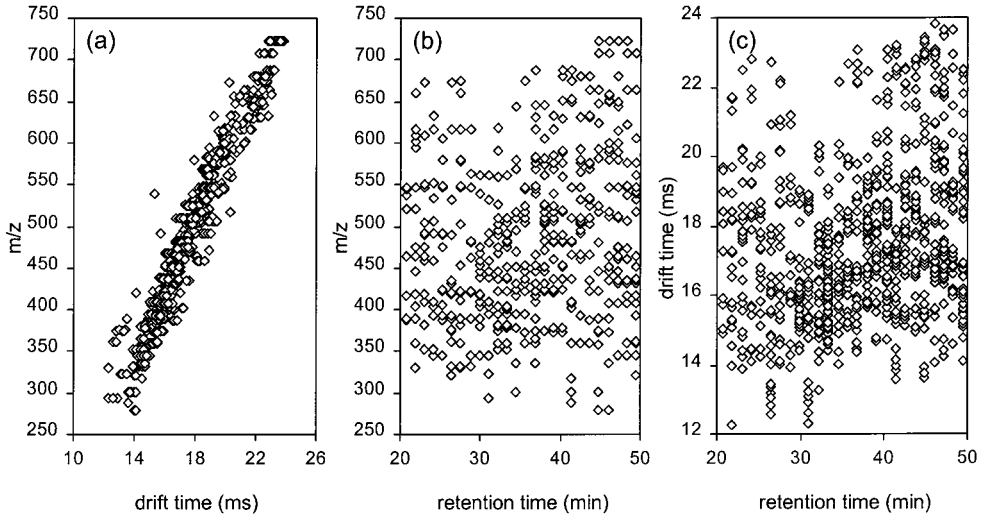

Figure 10. A summary of the data obtained from a complete analysis of the $t_r[t_D(t_f)]$ data for the 4000-component library over a limited range of retention times (t_r = 20−50 min) illustrates the relationship between the individual LC, IMS, and MS dimensions. Data for the plots were generated by determining the experimental drift times and *m*/*z* ratios for all of the peaks observed in the $t_r[t_D(t_f)]$ data for the t_r = 20−50 min range. Plots of experimental drift time versus *m*/*z* ratio (part a), retention time versus *m*/*z* ratio (part b), and retention time versus drift time (part c) are given in the figure.

$t_r[t_D(t_f)]$ volumes (~400 × 10^6 possible t_r, t_D, and t_f elements, 147 × 512 × 5120 different bins). Figure 10 shows a summary of peak positions determined from the $t_D(t_f)$ peaks found in 26 different retention time windows (t_r = 20−50 min). The plots in Figure 10 provide insight regarding the degree to which the different separation techniques are correlated. Clearly, the drift times of ions increase with increasing *m*/*z* ratios. This can be understood by considering that the size of a peptide increases with its mass. There are not obvious correlations of retention time with either *m*/*z* ratios or drift times. Across these 26 different windows, we determine that there are 848 unique peaks. In our LC/MS analysis, we found that peaks are observed in only 111 of the total 147 different retention time windows. Assuming that a similar number of isomers is resolved across each of the remaining $t_D(t_f)$ windows where MS peaks were found (111 windows), we estimate that ~3620 peptides are resolved. Although this value is only an estimate, it corroborates the discussion of Figure 9. That is, the LC/IMS-TOF approach provides detailed information about complex isomer mixtures by allowing a large number of individual isomer components to be resolved.

SUMMARY AND CONCLUSIONS

Condensed-phase LC separations have been combined with ion mobility/time-of-flight analysis for the characterization of a peptide library expected to contain 4000 different peptides having the general form: NH_2-Xxx-Xxx-Xxx-CO_2H, NH_2-Ala-Xxx-Xxx-Xxx-CO_2H, NH_2-Ser-Ala-Xxx-Xxx-Xxx-CO_2H, and NH_2-Leu-Ser-Ala-Xxx-Xxx-Xxx-CO_2H (where Xxx represents Ala, Arg, Asp, Glu, Gly,

Leu, Lys, Phe, Ser, and Val). The incorporation of the mobility separation provides several advantages over standard LC/MS experiments. One important feature of the technique involves the separation of mixture components into distinct charge-state families. This separation makes it possible to obtain integrated mass spectra for specific families of ions and permits identification of ion charge states for unambiguous mass assignment. In addition, many isomeric library peptides that have identical LC retention times can be separated on the basis of differences in their gas-phase mobilities in the $t_r[t_D(t_f)]$ data. For the 4000-component library studied here, the presence of numerous sequence and structural isomers results in only 601 unique peptide masses. In many cases, there are as many as 20−30 isomers expected at a given m/z ratio.

In the analysis presented here, on average, fewer than ~68% of the library peptides would be distinguished on the basis of differences in retention time and m/z ratio. From the analysis of representative regions of the $t_r[t_D(t_f)]$ data, we estimate that it is possible to resolve ~82% of the 4000 library components using the combined LC/IMS-TOF approach.

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