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Split-Field Drift Tube/Mass Spectrometry and Isotopic Labeling Techniques for Determination of Single Amino Acid Polymorphisms

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A combination of split-field drift tube/mass spectrometry and isotopic labeling techniques is evaluated as a means of identifying single amino acid polymorphisms (SAAPs) in proteins. The method is demonstrated using cytochrome*c* (equine and bovine) and hemoglobin (bovine and sheep). For these studies, proteins from different species are digested with trypsin, and the peptides are labeled at primary amine groups [using either a light (H_3)- or heavy (D_3)-isotopic reagent]. SAAP analysis is carried out by mixing the light-labeled peptides of one species with the heavy-labeled peptides of the other and electrospraying the resulting mixture into a split-field drift tube/mass spectrometer. Peptides having the same sequence in both species appear as doublets in the mass spectrum [shifted in mass-to-charge (*m*/*z*) according to the number of incorporated labels]; additionally, these species have identical mobility distributions. Peptides having sequences that differ by one amino acid appear as peaks in the mass spectrum that are shifted in *m*/*z* according to the mass difference associated with the SAAP and the number of incorporated labels. The ion mobility distributions for these peptides (differing by only a single amino acid) can often be rationalized by their expected similarities or differences providing additional evidence that they are related. In all, 12 and 26 peptide variants (between species) corresponding to 5 and 11 amino acid polymorphisms have been identified for the cytochrome *c* and hemoglobin protein samples, respectively.

Keywords: ion mobility spectrometry • single amino acid polymorphism (SAAP) • isotopic labeling • time-of-flight mass spectrometry • single nucleotide polymorphism (SNP)

Introduction

Missense gene mutations [i.e., nucleotide substitutions that result in a single amino acid polymorphism (SAAP)] are one of the most common forms of genetic alteration.[1] Such events account for ~53% and ~46% of mutations catalogued in the Human Gene Mutation Database[2] and the Online Mendelian Inheritance in Man[3] databases, respectively.[1,4] The substitution of one amino acid for another can alter protein structure and function, as well as its solubility and stability,[1] and may lead to disease; recently, it has been estimated that roughly 60% of SAAPs in the Swiss-Prot protein database are linked to human diseases.[5] For example, mutations have been found that disrupt protein activation; protein−protein interactions; as well as DNA-binding, ATP-binding, and catalytic protein domains.[1] One of the more widely known examples of a SAAP-associated disease is sickle cell anemia which results from the single amino acid substitution of a glutamic acid residue for a nonpolar valine residue in the β-chain of hemoglobin.[6] Many other SAAP-related pathologies exist, ranging from psychiatric disorders to cardiovascular disease.[7−13] A relatively recent study determined the role of SAAPs in the development of graft versus host disease indicating the relevance of SAAP determination for tissue typing.[14]

SAAPs not only play a significant role in the development of certain diseases but may also affect treatment strategies.[7,15] Polymorphisms in drug-metabolizing enzymes are of interest because they have been implicated as the primary source of variability in interindividual drug response.[16] For example, genetic data have been used to group individuals into drug-metabolizing categories ranging from "poor" to "ultrarapid" metabolizers.[16] Polymorphisms within drug metabolic enzymes may result in individuals being categorized as poor or intermediate responders; utrarapid metabolism, however, primarily arises from gene duplication.[16] Because drug toxicity is associated with metabolism, the analysis of SAAPs within metabolic enzymes is of considerable interest.[7,15,16]

Generally, the presence of a genetic mutation associated with a disease is determined by single nucleotide polymorphism (SNP) analysis.[17,18] Multiple analytical platforms exist for SNP analysis, and several offer advantages in sensitivity (e.g., those that amplify the molecules of interest via the polymerase chain reaction) and throughput (e.g., multiplexed gene chip approaches).[7,19−21] However, SNP analyses suffer from several drawbacks, especially, as they relate to SAAPs. First, the

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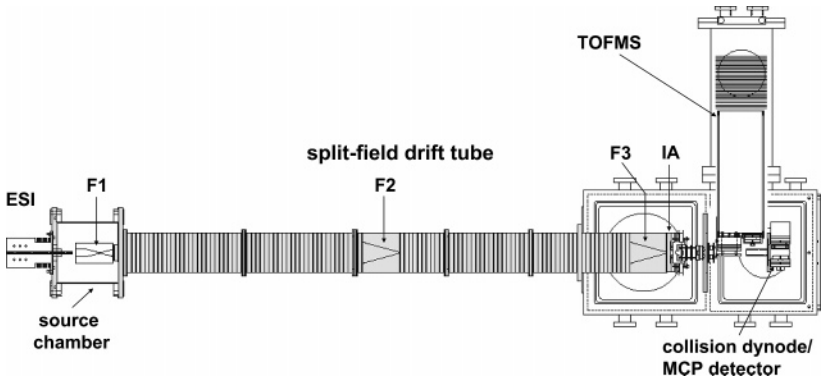


Figure 1. Schematic diagram of the IMS-MS instrument used in the current studies.

presence of a SNP provides no information about the expression level of a particular gene product.[15] Thus, such an analysis may not provide important information about the onset of disease. Second, only 1% of SNPs are actually manifested as SAAPs; therefore, many observed SNPs are not relevant at the protein level.[7,22] Third, as with any other screening techniques, SNP analyses can, at times, be inaccurate.[19,20] Because of such limitations, analytical methods for directly determining SAAPs offer several advantages and serve as an important cross check of SNP assignments. Overall, SAAP analysis will be highly complementary with results from SNP analyses.

At present, the determination of SAAPs within proteins can be cumbersome for mass spectrometry (MS) techniques.[7,15,23] Typically, a purified protein is enzymatically digested, and the masses of the resulting peptides are measured and compared with expected masses obtained from a sequence database. Peptides with molecular weights that do not match theoretical values are candidate SAAP peptides (mismatches may also result from post-translational modification). Problems can then arise due to the completeness or fidelity of the database used in the comparison.[7] Additionally, finding all SAAPs within a protein requires the observance of peptides across the entire protein sequence (i.e., complete sequence coverage).[15] As obtaining high sequence coverage of known peptides may be difficult with enzymatic digestion due to digestion efficiency as well as the fact that some SAAPs are expected to occur at the cleavage sites of specific enzymes, the use of multiple enzymes may at times be required to reveal protein polymorphisms.[23]

Having said this, the identification of amino acid variations in proteins from MS data has been demonstrated using different approaches. Lim et al. determined SAAPs in transthyretin using multiple enzyme digests and accurate mass information, as well as de novo sequencing of tandem mass spectrometry (MS/MS) data.[23] Another method has been demonstrated by Yates and co-workers where genes from a database have been manipulated in silico to produce all possible SNPs for the α- and β-chains of hemoglobin.[15] The MS/MS data are then searched against all SNP permutations that have been translated into peptide sequences.

The work presented below is closely related to a recent report by Liu and Regnier that utilizes stable isotope coding for SAAP determination.[7] They compared albumin proteins from four different canine breeds. All peptides of the same sequence appeared as mass spectral doublets, characterized by a specific mass difference associated with the isotopic coding. SAAP peptides found in different breeds were identified from peaks that appeared as singlets and were shifted by the m/z difference associated with the polymorphism.

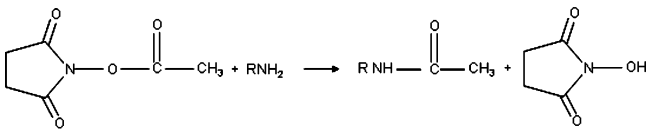
In the work reported here, we investigate the use of a gas-phase separation technique, ion mobility spectrometry (IMS) combined with mass spectrometry (MS) for SAAP analysis. Tryptic digests of two proteins, cytochrome *c* (bovine and equine) and hemoglobin (bovine and sheep), have been used to create identical peptides, as well as those differing by a single (or multiple) amino acid(s). Although the single amino acid variant peptides come from different animal species, we hereafter refer to them as SAAP peptides. The global internal standard technology (GIST)[24,25] has been used to isotopically label the primary amines of all tryptic peptides with the light (H_3)- or heavy (D_3)-labels (for the respective proteins in each pair). Digest mixtures are then analyzed with IMS-MS instrumentation. Here, we present evidence showing that the mobility distributions between complementary SAAP peptides are often similar (though they may be shifted in overall drift time) and can thus be used as a signature to link related SAAP peptides. Differences in mobilities may also be useful for interpreting spectra. Overall, the IMS-MS approach reveals many identical peptides and peptide variants resulting in relatively high sequence coverage for both protein pairs.

Experimental Section

General. Ion mobility spectrometry has been used previously with time-of-flight (TOF) mass spectrometry to characterize tryptic digests of purified proteins, as well as tryptic peptide mixtures produced from proteomics samples.[26−33] Detailed reviews of the history, instrumentation, and theory of IMS have been given elsewhere.[34−41] Here, only a brief description of the split-field IMS instrumentation[42] used for the SAAP analysis is presented. Figure 1 shows a schematic diagram of the instrument used in these studies. Peptide ions are produced by electrospray ionization (ESI) of an infused protein digest solution. Ions are stored in an ion funnel trap similar to that described by Smith and co-workers.[43−45] Periodically, a pulse (100 μs wide) of ions from the funnel is introduced into a split-field drift tube. In the first drift region (200 cm), ions are separated based on differences in their mobilities through a buffer gas (~2.8 and 0.2 Torr He and N_2, respectively) under the influence of a weak electric field (~10 to 11 V·cm^{-1}). The field in the second region of the drift tube (1.3 cm) can be set to transmit precursor ions or to induce fragmentation. Ions exiting the drift tube are extracted into the source region of an orthogonal-extraction TOF mass spectrometer. Here, high-

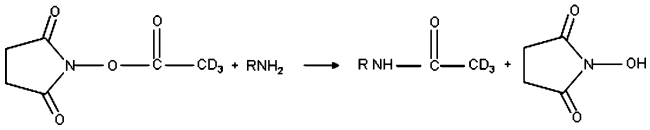
Scheme 1

bovine (cytochrome *c* and hemoglobin)



N-acetoxysuccinimide

equine (cytochrome *c*) and sheep (hemoglobin)



frequency, high-voltage pulses synchronous with the mobility measurement pulse are used to initiate flight time (t_F) measurements.

The time required for an ion to traverse the drift tube (drift time, t_D) is dependent on the mobility (K) of the ion as given by the expression $K = (V_D \cdot E^{-1})$.[35] Here, V_D is the drift velocity, and E is the drift field. The transit time of an ion (and thus V_D) is dependent on the overall charge state of the ion, as well as its collision cross section. That is, more highly charged ions will have shorter t_D values than those of lower charge ions, and more compact ions will have shorter t_D values than those of more elongated species. Additionally, because the time scale of the drift measurement is significantly longer than that of the TOF measurement [milliseconds (ms) versus microseconds (μs)], it is possible to record flight time distributions within individual drift time windows. This has been termed a "nested" drift(flight) time [$t_D(t_F)$] measurement.[46] Alternatively, t_F values can be converted to mass-to-charge ratios (m/z) to produce $t_D(m/z)$ datasets.

Tryptic Digestion of Proteins. Hemoglobin (bovine and sheep, no purity given) and cytochrome *c* (horse ≥95% and bovine ≥95%) have been purchased from Sigma-Aldrich and used without further purification. Each protein is dissolved in 0.1 M ammonium bicarbonate buffer solution (pH 7.5) with 1 M urea to a final protein concentration of 20 mg·mL^{-1}. TPCK-treated trypsin (Sigma-Aldrich) was added at a ratio of 1:50 (w/w) to each protein solution and incubated for 24 h at 37 °C. The digest solution was then filtered using a 3 kDa molecular weight cutoff microconcentrator (Amicon Bioseparations). The filtered solution is then subjected to solid-phase extraction (Oasis HLB) to clean the digest peptides. The remaining peptides are dried using a CentriVap Concentrator (Labconco).

Isotope Labeling of Tryptic Digests. Isotopic labeling strategies for MS have a rich history and have been reviewed extensively;[47−50] here, we present only a brief description of the labeling procedure used in the current studies. *N*-Hydroxysuccinimide, acetic anhydride, and acetic anhydride-D_6 (99% purity, Sigma-Aldrich) have been used for synthesizing *N*-acetoxysuccinimide and its D_3-analogue according to a procedure described previously.[24] The reactions to produce the light (H_3)- and heavy (D_3)-labeled peptides are shown in Scheme 1, where R represents a tryptic peptide. A 100-fold excess of *N*-acetoxysuccinimide and *N*-acetoxy-[D_3]succinimide is added individually to two equal aliquots of 1 mg/mL digest solution (0.1 M sodium phosphate buffer at a pH of 7.5). The final light (H_3)- and heavy (D_3)-labeled protein samples are stirred for 5 h at 300 K. Following this, 0.5 mL of *N*-hydroxylamine is added per 1 mL aliquot of the labeling reaction solution, and a 5 M NaOH solution is added to a pH of ∼11. After 10 min, the pH of each light (H_3)- and heavy (D_3)-labeled peptide solution is adjusted to ∼7−7.5 using 0.1 M HCl. The labeled peptides of each protein are extracted and purified by solid-phase extraction (Oasis HLB), and subsequently, they are dried using the CentriVap Concentrator (Labconco). Complete labeling results in the incorporation of an isotopic label at the amino terminus of each tryptic peptide, as well as at each lysine residue.[24,25]

Table 1. Complete List of Amino Acid Variations between Species for These Proteins

protein	residue[a]	mass difference[b]
cytochrome *c*	$S_{47} \rightarrow T_{47}$	14.02
cytochrome *c*	$G_{60} \rightarrow K_{60}$	71.07
cytochrome *c*	$G_{89} \rightarrow T_{89}$	44.03
hemoglobin α chain	$G_8 \rightarrow S_8$	30.01
hemoglobin α chain	$H_{20} \rightarrow N_{20}$	23.02
hemoglobin α chain	$A_{22} \rightarrow G_{22}$	14.02
hemoglobin α chain	$E_{23} \rightarrow A_{23}$	58.00
hemoglobin α chain	$A_{60} \rightarrow E_{60}$	58.00
hemoglobin α chain	$E_{71} \rightarrow G_{71}$	72.02
hemoglobin α chain	$A_{79} \rightarrow T_{79}$	30.00
hemoglobin α chain	$E_{82} \rightarrow D_{82}$	14.02
hemoglobin α chain	$S_{111} \rightarrow C_{111}$	15.98
hemoglobin α chain	$S_{115} \rightarrow N_{115}$	27.01
hemoglobin α chain	$A_{12} \rightarrow G_{12}$	14.02
hemoglobin α chain	$G_{24} \rightarrow A_{24}$	14.02
hemoglobin α chain	$S_{43} \rightarrow H_{43}$	50.02
hemoglobin α chain	$T_{49} \rightarrow N_{49}$	13.00
hemoglobin α chain	$A_{86} \rightarrow Q_{86}$	57.02
hemoglobin α chain	$K_{103} \rightarrow R_{103}$	28.01
hemoglobin α chain	$N_{116} \rightarrow H_{116}$	23.02
hemoglobin α chain	$F_{117} \rightarrow H_{117}$	10.01
hemoglobin α chain	$F_{119} \rightarrow N_{119}$	33.03
hemoglobin α chain	$R_{143} \rightarrow K_{143}$	28.01

[a] Variant amino acid residues and positions. The residues on the left and right correspond with those found in bovine and equine cytochrome *c* and bovine and sheep hemoglobin, respectively. [b] Mass differences are obtained from the monoisotopic values for each residue.

Electrospray Conditions. Light (H_3)- and heavy (D_3)-labeled digest peptide solutions are reconstituted by dissolving ∼1 mg of each in 1 mL of water. Then two aliquots (100 μL each of the light (H_3)- and heavy (D_3)-labeled peptide solutions) are mixed together and diluted to a final peptide concentration of ∼5 μg·mL^{-1} in a water−acetonitrile−formic acid (49:49:2, v/v/v) solution. This solution is used directly for ESI−IMS-MS analysis. Positively charged (protonated) ions are formed by electrospraying the peptide mixture solutions. The ESI needle is biased at +2000 V relative to the entrance of the desolvation region, and the solution flow rate is held at 0.25 μL·min^{-1}. For the present studies, datasets have been collected for 60 s (∼1−2 ng of sample consumed); however, shorter analysis times (requiring less sample) can be utilized.

Results and Discussion

Protein Sample Selection and Amino Acid Variations. To demonstrate the utility of IMS-MS analyses for characterization of SAAPs, we have selected two simple, model protein systems, cytochrome *c* and hemoglobin. Cytochrome *c* consists of a single polypeptide chain that is 104 amino acid residues long. Hemoglobin contains α- and β-polypeptide chains such that the total number of amino acid residues is ∼2.8 times larger. The choice of species is aimed at generating different levels of amino acid variance and thus numbers of SAAPs. Table 1 shows all amino acid variations and their position within the protein

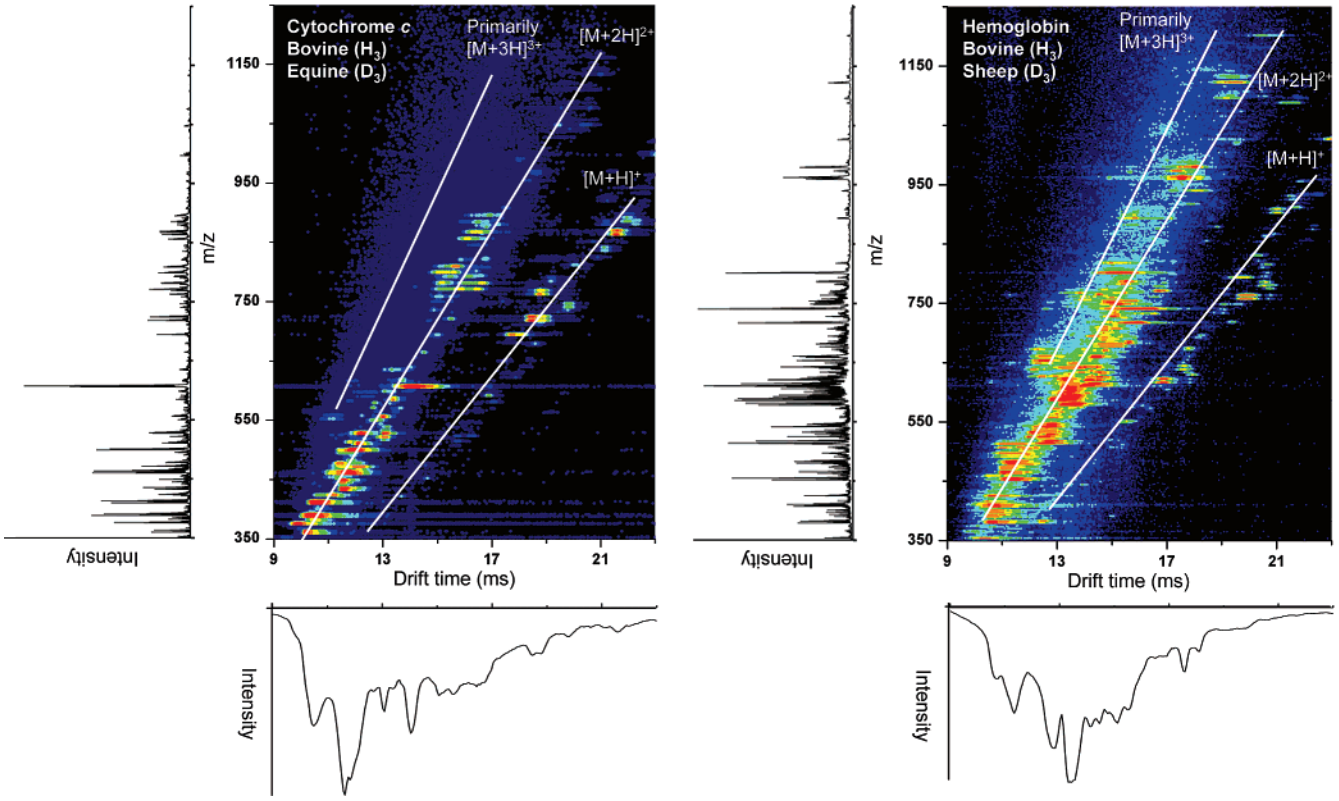


Figure 2. Two-dimensional (2D) $t_D(m/z)$ dot plots for the cytochrome *c* (left) and hemoglobin (right) digest mixtures. On the left of each 2D plot are total-ion mass spectra obtained by integrating all ion counts across the drift time range at each *m*/*z* value. On the bottom of each 2D plot are total ion drift time distributions obtained by integrating all ion counts across the entire mass spectrum at each drift time bin. Charge-state families in both 2D plots are delineated with solid lines, and peak intensities are indicated as a color map. An intensity threshold of 4 counts was used for each 2D plot, and the maximum color intensity represents a count of 300. In these experiments, peptides from bovine proteins are labeled with the light (H_3) label, while peptides from equine (cytochrome *c*) and sheep (hemoglobin) are labeled with the heavy (D_3) label.

sequence, as well as the sample source and mass difference. The information in Table 1 can be used to describe how SAAPs are distinguished with MS techniques including those employed here. Consider, for example, the substitution of the amino acid serine by threonine (S → T) at residue position 47 in bovine and equine cytochrome *c*, respectively. A pair of peptides containing this single variation would be distinguished by a shift in *m*/*z* corresponding with the sum of the mass difference from the SAAP (~14 Da for the S → T variation) and the mass difference associated with the incorporated isotopic label(s) divided by the peptide charge state.

Recognizing Peptide Sequence Variants from a Two-Dimensional $t_D(m/z)$ Analysis of Digest Mixtures. Figure 2 shows nested $t_D(m/z)$ distributions for isotopically labeled mixtures of bovine/equine cytochrome *c* and bovine/sheep hemoglobin digest mixtures. Typically, features in $t_D(m/z)$ datasets fall into mobility families based on peptide charge state. Such families consist of a low-mobility, singly charged ion family, as well as higher mobility doubly and triply charged ion families. Under the spray conditions employed, the doubly and triply charged families are favored in both protein datasets. Visual inspection of the two datasets demonstrates the increased complexity in the hemoglobin mixture; many more high-abundance features are evident. Figure 2 also demonstrates a major advantage of the IMS-MS analysis. That is, the mobility dispersion of all species makes it possible to observe many low-abundance species (e.g., features in the singly charged, $[M + H]^+$, family) because they are removed from regions of interfering higher abundance signals. This advantage is illustrated and discussed in more detail below.

Figure 3 shows an expanded region of the two-dimensional plot of the cytochrome *c* data shown in Figure 2 for several typical peptides. In examining the plot in detail, we find that peptides (A and B) from different species having the same sequence exhibit essentially identical mobility distributions. The 6.03 *m*/*z* difference between the experimentally determined values of 762.50 and 768.53 for this $[M + H]^+$ pair indicates that two labels have been incorporated into the peptides and that the sequences are identical. Additionally, two labels suggest that the C-terminal residue is lysine. The combined information is used to identify these peaks; we assign this pair to the light (H_3)- and heavy (D_3)-labeled peptide ion $[YIPGTK+H]^+$ from bovine and equine cytochrome *c* [*m*/*z* (calculated) of 762.38 and 768.38, respectively].

Peptides with amino acid sequence variations are observed as peaks that differ in *m*/*z* depending upon the amino acid variation. Figure 3 shows two peptide sequence variants (C and D) assigned as $[TGQAPGFSYTDANK+2H]^{2+}$ ($m/z = 770.84$) and $[TGQAPGFTYTDANK+2H]^{2+}$ ($m/z = 780.84$) from bovine and equine cytochrome *c*, respectively. The experimentally determined *m*/*z* difference, 10.00, corresponds to the $S_{47} \rightarrow T_{47}$ substitution (bovine → equine) plus the isotopic shift associated with incorporation of two labels for each peptide. A complete list of all SAAP peptides, as well as unique peptides (i.e., those found in only one of the protein samples), is given in Tables 2 and 3 for cytochrome *c* and hemoglobin, respectively. In total,

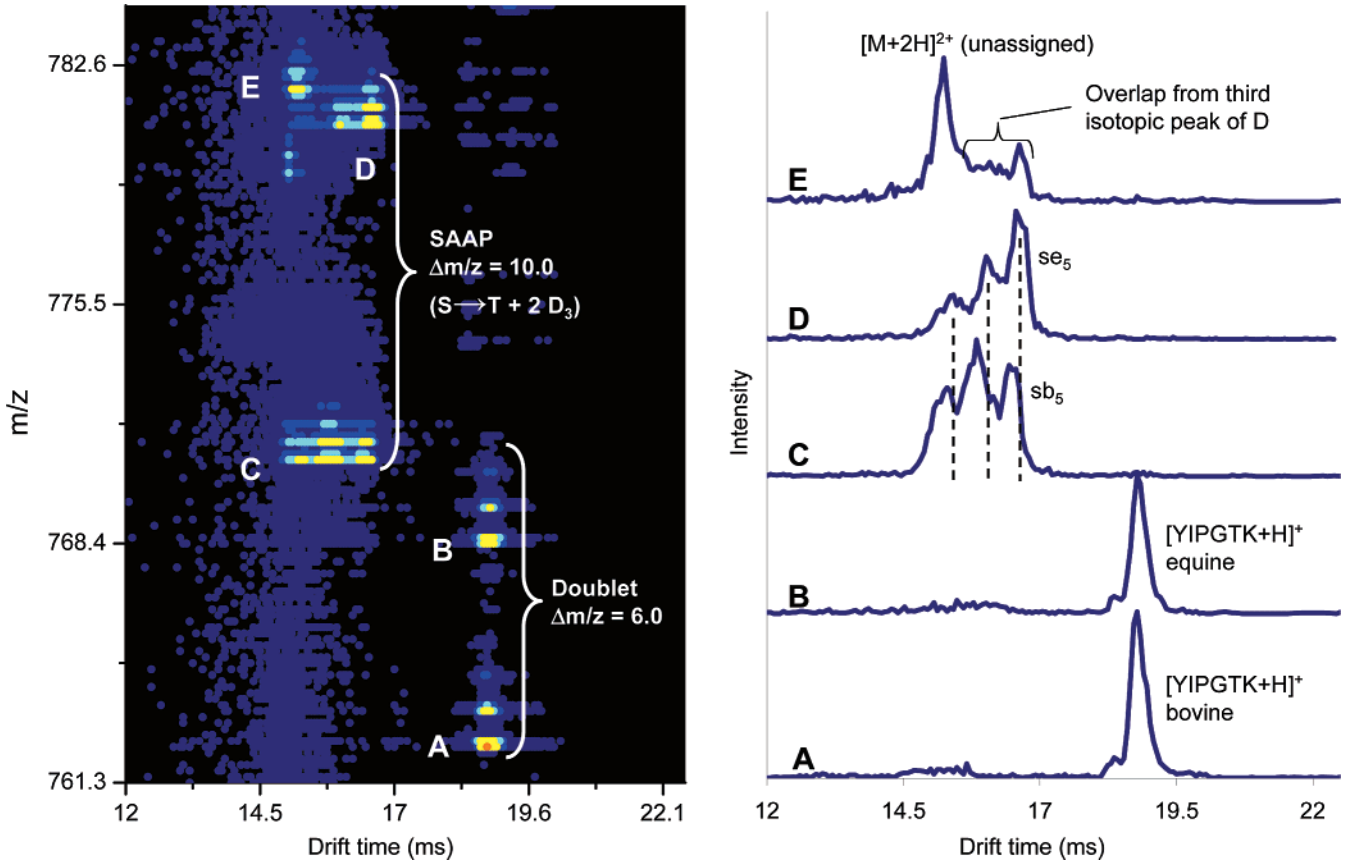


Figure 3. Two-dimensional $t_D(m/z)$ dot plot showing an expanded region of Figure 2 for the cytochrome *c* digest mixture. The mobility distributions for features labeled A−E in the 2D plot are shown on the right. The mobility distributions are obtained by integrating all drift bins across the 2D plot range at the *m/z* value corresponding with the monoisotopic peak for each feature. The identity of the assigned doublet feature is given as the peptide sequence. The SAAP peptide sequences are given in Table 2.

Table 2. Assignments for Peptide Ions from a Cytochrome *c* Digest Mixture

peptide sequence						
bovine	equine	charge[a]	no. labels[b]	experimental *m/z*[c]	calculated *m/z*[d]	peak[e]
	GITWK	1	1, 2	649.39, 694.39	649.34, 694.34	ue_1, ue_2
	NKGITWK	1, 2	1	891.57, 446.32	891.48, 446.24	ue_3, ue_4
	TYTDANK	2	2	451.76	451.69	se_1
	TYTDANKNK	2	1	550.30	550.26	se_2
KTGQAPGFSY		2	2	570.32	570.26	sb_3
KGEREDLIAY	KTEREDLIAY	2	2	639.31, 664.33	639.31, 664.32	sb_4, se_4
TGQAPGFSYTDANK	TGQAPGFTYTDANK	2	2	770.84, 780.84	770.84, 780.84	sb_5, se_5
	EETLMEYLENPK	2	2	793.42	793.35	ue_5

[a] Charge (*z*) of the assigned peptide ion. Two numbers indicate the peptide was observed as a singly and doubly charged ion. [b] The total number of isotopic labels incorporated into the peptide. Two numbers correspond with the observance of the same peptide as two different dataset features with the incorporation of one or two isotopic labels. [c] The experimentally determined *m/z* value for the monoisotopic peptide ion. [d] Calculated *m/z* values are obtained for digest peptides from the Peptide Mass program (http://ca.expasy.org/tools/peptide-mass.html). To these values, the masses of the incorporated labels are added, as well as the total number of attached protons. The summed value is then divided by the charge state of the ion. Two values correspond either with the SAAP peptides from different sources, the same peptide of different charge state, or the same peptide with differeing numbers of attached labels. [e] Peaks observed in the cytochrome *c* dataset. Here ue, ub, se, and sb are unique equine, unique bovine, SAAP equine, and SAAP bovine peptides, respectively.

we observe 12 and 26 peptide variants corresponding to 5 and 11 amino acid polymorphisms for cytochrome *c* and hemoglobin, respectively.

An interesting issue that arises in the IMS distributions for the $[TGQAPGFSYTDANK+2H]^{2+}$ and $[TGQAPGFTYTDANK+2H]^{2+}$ ions is the degree of similarity. As discussed in some detail, many factors contribute to the establishment of gas-phase peptide ion conformation, including peptide amino acid composition and primary sequence, the overall length of the peptide, as well as the cation (for positively charged ions) used in the electrospray ionization process.[51−60] Because the mobility separation distinguishes peptide ions based on differences in overall collision cross section (as well as charge), the nature of an amino acid substitution must be considered when assessing the utility of the mobility dimension as a differentiator of SAAP peptides. Figure 3 also shows the drift time distributions for the two cytochrome *c* SAAP peptides (C and D). Notably, both distributions contain three peaks at similar drift times. That three peaks are resolved requires that at least three conformation types are stable at 300 K over the time scale of the experiment. Additionally, the width (~2.5 ms) at the base of the triplet peaks is nearly the same for both

Table 3. Assigned Peptides for a Hemoglobin Digest Mixture

peptide sequence							
bovine	sheep	chain[a]	charge[b]	no. labels[c]	experimental m/z[d]	calculated m/z[e]	peak[f]
GNVK		α	1	1	459.25	459.24	sb_1
NFGK		*β*	1	1,2	507.27, 549.23	507.24, 549.24	ub_1
GHGAK	GHGEK	α	1	2	553.29, 617.30	553.25, 617.25	sb_2, ss_2
AAVTAFWGK	AAVTGFWGK	*β*	1	2	1034.59, 1026.57	1034.50, 1026.49	sb_3, ss_3
GNVKAAWGK	SNVKAAWGK	α	2	2	507.70, 525.76	507.76, 525.76	sb_4, ss_4
AAVTAFWGK	AAVTGFWGK	*β*	2	2	517.71, 513.78	517.75, 513.75	sb_5, ss_5
VDEVGGEALGR	VDEVGAEALGR	*β*	2	1	572.29, 578.29	572.28, 578.28	sb_6, ss_6
	VVAGVANALAHK	*β*	2	2	620.33	620.34	ss_7
AAVTAFWGKVK		*β*	2	2	631.39	631.34	sb_8
	GHGEKVAAALTK	α	2	2	636.23	636.33	ss_9
VKVDEVGGEALGR	VKVDEVGAEALGR	*β*	2	1	706.79, 694.40	706.86, 694.37	sb_{10}, ss_{10}
GTFAALSELHCDK	GTFAQLSELHCDK	*β*	2	1,2	716.87, 769.81	717.33, 769.84	sb_{11}, ss_{11}
	VGGNAGAYGAELER	α	2	1	740.40	740.35	us_1
EFTPVLQADFQK		*β*	2	2	753.89	753.86	ub_2
VVAGVANALAHRYH	VVAGVANALAHKYH	*β*	2	2	781.77, 770.35	781.40, 770.40	sb_{12}, ss_{12}
VGGHAAEYGAEALER		α	2	1	786.44	786.37	ub_3
	HHGNEFTPVLQADFQK	*β*	2	2	979.47	979.45	us_2
	FFEHFGDLSNADAVMNNPK	*β*	2	2	1122.15	1121.99	us_3

[a] Hemoglobin chain in which the peptide is found. [b] Charge (z) of the assigned peptide ion. [c] The total number of isotopic labels incorporated into the peptide. Two numbers correspond with the observance of the same peptide as two different dataset features from the incorporation of one or two isotopic labels. [d] The experimentally determined m/z value for the monoisotopic peptide ion. [e] Calculated m/z values are obtained for digest peptides from the Peptide Mass program (http://ca.expasy.org/tools/peptide-mass.html). To these values, the masses of the incorporated labels are added, as well as the total number of attached protons. The summed value is then divided by the charge state of the ion. Two values correspond either with the SAAP peptides from different sources or the same peptide with differing numbers of attached labels. [f] Peaks observed in the cytochrome *c* dataset. Here ub, us, sb, and ss are unique bovine, unique sheep, SAAP bovine, and SAAP sheep peptides, respectively.

peptides. The similaritities in these distributions, associated as a signature for the two SAAP peptides, can be contrasted by the disparity of the mobility distribution for the unassigned, doubly charged feature E shown in Figure 3. This distribution is largely comprised of a single peak at ~15 ms corresponding to ions with relatively high mobilities. Thus, one simple approach is to examine IMS peak shapes and positions as a means of confirming SAAPs that are found from m/z analysis.

As also illustrated in Figure 3, the two mobility distributions for the SAAP peptides are dissimilar in some respects. For example, the relative abundances of the three features are not identical. The middle peak dominates the bovine distribution, while the lowest-mobility ions dominate the equine distribution. In addition to the difference in relative abundances, the entire distributions occur over different t_D ranges; that is, all three features from the higher molecular weight peptide are observed at slightly longer times (see Figure 3). Such a shift is expected because of the correlation of size and mass.[57] In this case, the observed shift in t_D results from the increasing peptide size originating with the S → T substitution (associated with a difference of a single methyl group).

While the data presented in Figure 3 demonstrate the possibility of using mobility information to both relate SAAP peptides (through similarities in mobility distributions) as well as distinguish these species (through distribution differences), the utility of mobility information alone may be limited for some peptides. For example, the S → T amino acid substitution described above might be expected to produce the similar mobility distributions (triplet peaks), as both amino acids are polar and of relatively similar size. Previous studies, however, have shown that substitutions of other types of amino acid residues (nonpolar or charged) or substitutions occurring at key positions within the peptide sequence can result in significant changes in mobility distributions (including large shifts in t_D as well as changes in the number of stable structures).[53,57,60] Thus, for some cases, the mobility information should be combined with sophisticated molecular modeling[51,53−55,61−63] and cross section calculations[57,64−66] to confirm the presence of SAAPs. This type of analysis has already proven useful in identifying peptide sequences having identical m/z values (but different cross sections).[61] Although such an approach is computationally demanding, it may prove to be important for some types of assignments.

Detecting Low-Abundance SAAPs. One of the problems of MS analyses of complex mixtures such as the hemoglobin sample (Figure 2) is that the signal from low-abundance species (or those with low-ionization efficiencies) can be obscured by isobaric ions.[67] As described previously, IMS separation can reduce chemical noise.[67] Figure 4 shows an expanded region of bovine and sheep hemoglobin data shown in Figure 2. The selected region highlights the low-m/z, low-mobility (singly charged) peptide ions. Many features are evident that would otherwise not be observed with MS analysis alone; they would be buried in the higher-mobility chemical noise. This is illustrated by the mass spectral insets associated with specific regions of the data. Integrating all drift bins at each m/z value (i.e., the mass spectrum that would be obtained in the absence of mobility separation) shows a relatively complicated spectrum. The baseline of this spectrum over the region shown is ~500 ion counts. A narrow slice across the $[M + H]^+$ family shows two distinct features corresponding with the SAAP peptide ion $[GHGAK+H]^+$ and the unique peptide ion $[NFGK+H]^+$ (see Table 3). The observation of these ions illustrates the ability to observe low-intensity features with the combined IMS-MS approach that are not observed with MS alone.

The ability to identify low-abundance (or low-response) species leads to relatively high sequence coverage for the proteins, even with the short analysis times employed here (≤60 s). The percent sequence coverage from IMS-MS analysis is ~76% and ~94% for bovine and equine cytochrome *c*, respectively, compared with only ~52% and ~68% for MS analysis alone. In addition to providing high sequence coverage, we note that almost all of the amino acid variations (~83%) between

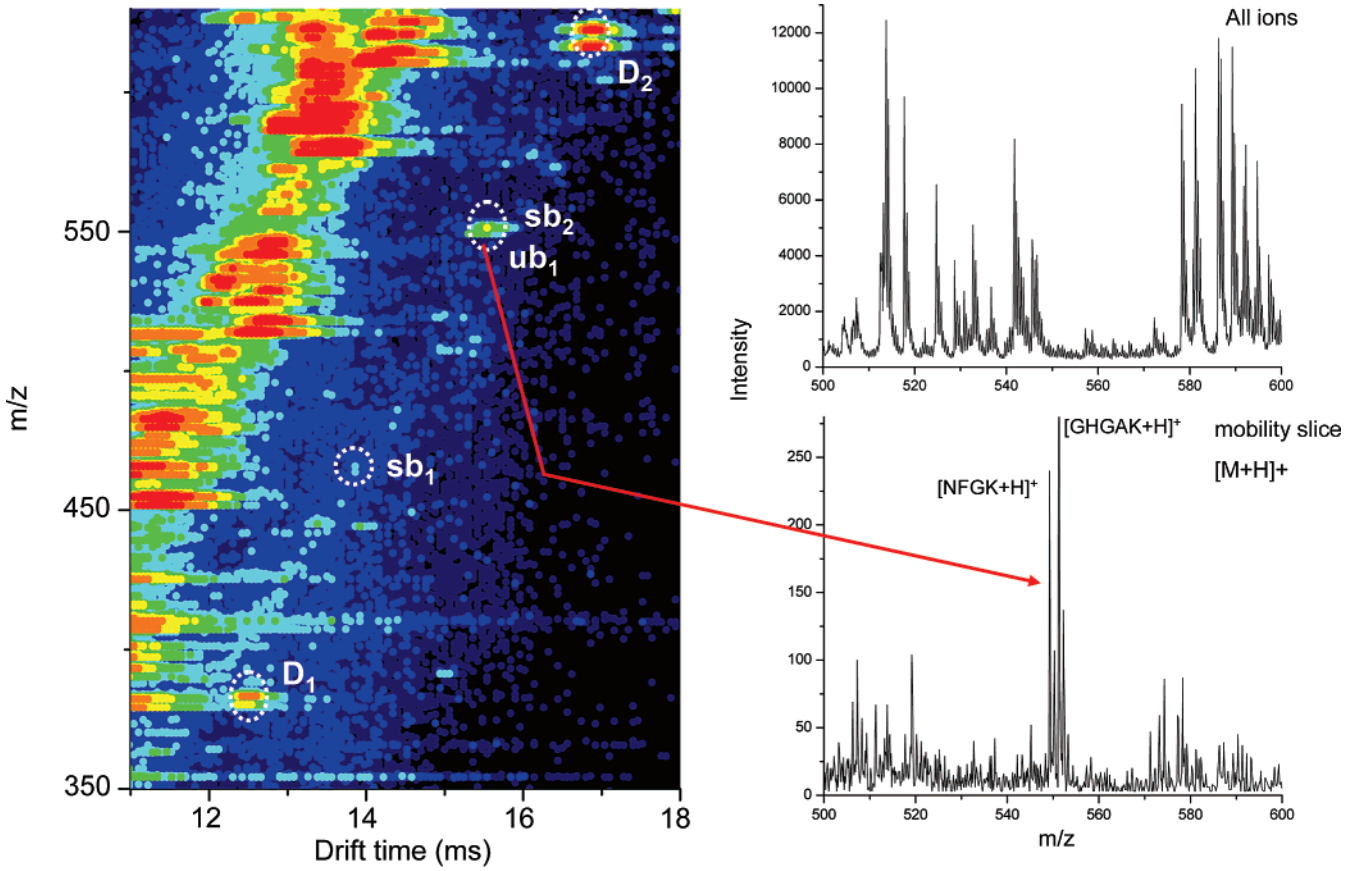


Figure 4. An expanded region of the 2D dot plot of the hemoglobin digest mixture shown in Figure 2. Here, the lower-mobility, singly charged peptide ions are highlighted, and assigned features are labeled. The sequences for the SAAP and unique peptides are given in Tables 2 and 3. Two doublets representing identical peptides are indicated as D_1 and D_2. On the right are mass spectra obtained by integrating all ion counts across the entire drift time distribution (top trace) and by integrating only ions within a narrow drift bin tolerance (±3 bins) of the $[M + H]^+$ mobility family line shown in Figure 2 (bottom trace). A SAAP peptide and a unique peptide (see Table 3) not observed in the total-ion mass spectrum are labeled in the mobility-selected mass spectrum.

the two cytochrome *c* molecules are accounted for with at least one peptide. The observed variant peptides constitute a significant portion of these proteins, ~24% and ~25% of the total sequence of bovine and equine cytochrome *c*, respectively. A relatively large fraction of each hemoglobin protein has also been observed (~64% and ~73% for IMS-MS analysis compared with ~52% and ~59% for MS analysis alone for bovine and sheep hemoglobin, respectively). Variant peptides again constitute a significant fraction of the protein sequence, comprising ~29% and ~42% of the bovine and sheep hemoglobin sequences, respectively. Only ~50% of the sequence variations is accounted for in the hemoglobin analysis.

The sequence coverage for cytochrome *c* and hemoglobin obtained from all features observed in these studies is shown in Schemes 2 and 3, respectively. It is instructive to consider the tryptic peptides containing amino acid variations that are not observed in the present experiments. Such peptides include GITW**G**EETLMEYLENPK from bovine cytochrome *c*, as well as AV**E**HLDDLPG**A**L**S**ELSDLHAHK, LLSHSLLVTLA**S**HLP**S**DFTPAVHASLDK, and FFE**S**FGDLS**T**ADAVMNNPK from bovine hemoglobin, and AV**G**HLDDLPG**T**LS**D**LSDLHAHK and LLSHSLLVTLA**C**HLP**N**DFTPAVHASLDK from sheep hemoglobin. The calculated masses of these peptides are 2008.95, 2366.19, 2968.60, 2088.95, 2310.16, and 3011.59, respectively; all of these values fall within ~33% of the value for the molecular weight cutoff filter employed in the sample cleanup steps (see above).

Scheme 2. Complete Sequences of Bovine and Equine Cytochrome *c*[a]

CYTOCHROME *C* BOVINE

*GDVEKGKKIF VQK*CAQCHTV EKGGKHK*TGP NLHGLFGRKT GQAPGFSYTD* **ANK**NKGITW**G** EETLMEYLEN PK*KYIPGTKM IFAGIKKKGE* **REDLIAY***LKK ATNE*

CYTOCHROME *C* EQUINE

*GDVEKGKKIF VQK*CAQCHTV EKGGKHK*TGP NLHGLFGRKT GQAPGFTYTD* **ANKNKGITWK** EETLMEYLEN PK*KYIPGTKM IFAGIKKKTE* **REDLIAY***LKK ATNE*

[a] Peptides observed with the IMS-MS are shown in bold (SAAP peptides), italic (identical peptides observed as MS doublets), and underline (unique peptides or an identical peptide observed from only one protein sample). Amino acid variations are shown in red. For a list of observed SAAP peptides, as well as peptides that are, in fact, unique to each protein, see Table 2.

Thus, such species may not comprise an appreciable amount of the sample peptides. This argument does not preclude other factors (such as trypsinization or ionization efficiency), which may also contribute to the nonobservance of these and other variant peptides.

Identifying SAAPs with IMS-MS Methods. Distinguishing sequence variants is the first step in SAAP peptide analysis; however, an ultimate goal is to determine the nature of the polymorphism itself (i.e., the position and identities of the

Scheme 3. Complete Sequences of Bovine and Sheep Hemoglobin[a]

HEMOGLOBIN α-CHAIN_BOVINE

VLSAADKGNV K*AAWGK*VGGH AAEYGAEALE R*MFLSFPTTK TYFPHFDLSH GSAQVK*GHGA K*VAAALTK*AV EHLDDLPGAL SELSDLHAHK *LRVDPVNFK*L LSHSLLVTLA SHLPSDFTPA VHASLD*KFLA NVSTVLTSKY R*

HEMOGLOBIN β-CHAIN_BOVINE

MLTAEEK**AAV TAFWGKVKVD EVGGEALGR***L LVVYPWTQRF* FESFGDLSTA DAVMNNPK*VK AHGKK**VLDSF SNGMKHLDDL** *K***GTFAALSEL HCDK**LHVDPE NF**K**LLGNVLV VVLAR**NFGKE** FTPVLQADFQ K*VVAGVANAL* **AHRYH**

HEMOGLOBIN α-CHAIN_SHEEP

VLSAADK**SNV K***AAWGK*VGGN AGAYGAEALE R*MFLSFPTTK TYFPHFDLSH GSAQVK**GHGE K***VAAALTK*AV GHLDDLPGTL SDLSDLHAHK *LRVDPVNFK*L LSHSLLVTLA CHLPNDFTPA VHASLDK*FLA NVSTVLTSKY R*

HEMOGLOBIN β-CHAIN_SHEEP

MLTAEEK**AAV TGFWGK**VKVD **EVGAEALGR***L LVVYPWTQRF* FEHFGDLSNA DAVMNNPKVK AHGKK*VLDSF SNGMKHLDDL* *K***GTFAQLSEL HCDK**LHVDPE NF**R**LLGNVLV VVLAR**HHGNE** FTPVLQADFQ K**VVAGVANAL AHKYH**

[a] Both chains are shown for each protein. Peptides are indicated in a similar fashion as those in Scheme 2. For a list of observed SAAP peptides, as well as peptides that are, in fact, unique to each protein, see Table 3.

amino acids). For these protein systems where the sequence differences are known, it is fairly straightforward to use the parent mass, as well as the number of added isotopic labels, to determine the identity of peptide variants. However, for systems where the peptide variants are not known or where the mixture contains multiple proteins, such an approach is intractable. In these cases, it is useful to examine collision-induced dissociation (CID) data. In the present IMS-MS system, CID is implemented using a split-field approach described previously.[42,68] Briefly, the field in the second, short mobility region at the back of the drift tube can be modulated to transmit precursor ions or create fragments. Thus, precursor MS and CID-MS information can be obtained.

Figure 5 shows two CID-MS spectra for the peptide $[TGPNLHGLFGR+2H]^{2+}$ from cytochrome *c* (bovine and equine), as well as the SAAP peptide $[VVAGVANALAHK+2H]^{2+}$ from sheep hemoglobin. Both spectra contain a homologous y-type ion series, and both series are quite extensive. For example, the y_2-y_{11} and the y_2-y_{10} fragment ions are observed for the former and latter peptides, respectively. In this type of defined system, the fragment ion assignments can be interpreted manually. The approach can also be combined with database search methods as outlined previously.[29−33]

Summary

This paper illustrates the combination of split-field drift tube/mass spectrometry and isotopic labeling techniques for the analysis of SAAPs. The methods are demonstrated using bovine/equine cytochrome *c* and bovine/sheep hemoglobin digest mixtures. The approach has potential as a high throughput strategy and offers advantages in selectivity and identification. This said, a limitation of this type of analysis is that it may miss SAAPs because of incomplete protein sequence coverage, and some remain difficult to observe (e.g., nonretained species or those containing a Leu/Ile substitution). Additionally, C-terminal arginine peptides containing a moiety blocking N-terminal labeling (e.g., post-translational modifications) will only appear as mass spectral singlets. We note that these are also limitations in commercially available mass spectrometry methods, and in the long run, innovative new approaches should have value. Perhaps the most serious limitation associated with broad use of the current techniques is that only a few prototype IMS-MS instruments have been constructed, and much remains to be done with data analysis. We are currently working on solutions to these issues. Additionally, because the combination of LC with IMS-MS techniques has been shown to provide more than an order of magnitude improvement in the resolution of components in complex biological mixtures,[26−33] efforts are underway to use combined condensed- and gas-phase separations with isotopic labeling for SAAP characterization of more complex systems.

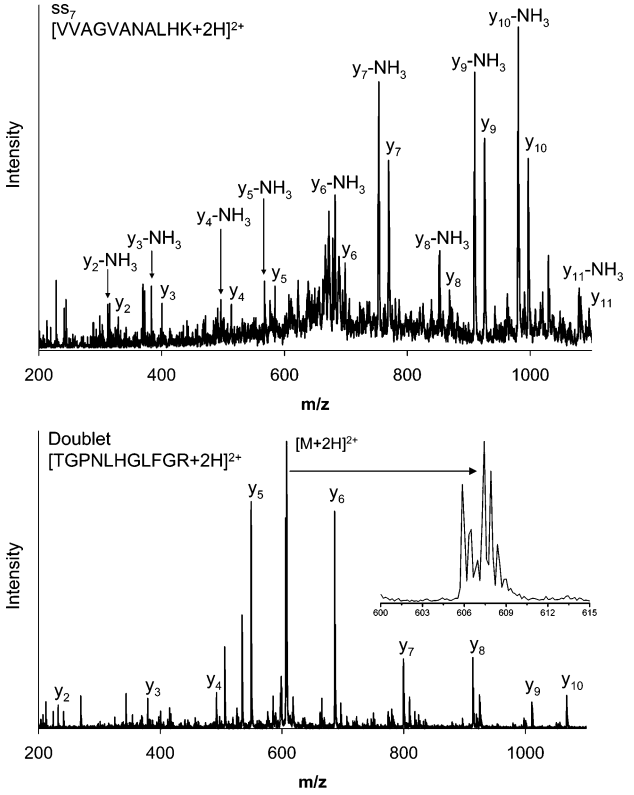


Figure 5. Mobility-selected MS/MS spectra obtained by integrating a narrow range of drift bins (centered about the precursor ion drift time) for each *m/z* value. Precursor ions, as well as fragment ions from the homologous y-series, are labeled. Here, the top mass spectrum is obtained upon collisionally activating the indicated SAAP peptide (sheep hemoglobin), while the bottom mass spectrum has been obtained upon collision-induced dissociation of a peptide doublet with the given sequence from the cytochrome *c* digest mixture. The precursor mass spectral doublet, indicating the incorporation of a single label, is shown as an inset in the bottom mass spectrum.

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