Published in final edited form as:

J Am Soc Mass Spectrom. 2016 May ; 27(5): 795–799. doi:10.1007/s13361-016-1367-3.

Ion Mobility Separation of Peptide Isotopomers

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Abstract

Differential or field asymmetric waveform ion mobility spectrometry (FAIMS) operating at high electric fields fully resolves isotopic isomers for a peptide with labeled residues. The naturally present isotopes, alone and together with targeted labels, also cause spectral shifts that approximately add for multiple heavy atoms. Separation qualitatively depends on the gas composition. These findings may enable novel strategies in proteomic and metabolomic analyses using stable isotope labeling.

Graphical Abstract

Introduction

Since its origins a century ago, mass spectrometry (MS) has been intertwined with isotopic analyses [1]. The existence of isotopes was the first major discovery made by MS [2], and their preparative separation [3] in 1940-s was the original industrial application. Today, numerous quantification and pathway tracking strategies in proteomics, metabolomics, and

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Supporting Information

Mass spectra, further FAIMS spectra with graphs of separation properties, and statistics of peak positions and widths for dialanine ions.

Dr. A. A. Shvartsburg has interest in Heartland Mobility, LLC that produces high-definition FAIMS systems and ion funnel interfaces such as those utilized in this work.

environmental applications of MS (for instance, SILAC, ICAT, and ITRAQ), rely on stable-isotope labeling [4–10] - typically by D, ^{13}C , ^{15}N , or ^{18}O . The explosion of isotope-labeling methods in MS has been enabled by modern (time-of-flight and Fourier-transform) instruments providing a resolving power (R) of $\sim 10^5 - 10^6$, which allows resolution not only of isotopologues with unequal nominal masses, but commonly of those with same nominal mass but different stoichiometry: e.g., ^{15}N and ^{13}C differ by 6 mDa and can be distinguished for peptides [11].

Identifying and quantifying isotopomers (isomers with variant position of one or more labels) is also important, but much more challenging. They can be disentangled to a limited extent by proton NMR and better by ^{13}C NMR spectroscopy [7, 12] which requires purified compounds in bulk. Tandem MS can characterize some isotopomers, but is hampered by uninformative fragments and spectral congestion. As with all MS analyses of complex samples, prior fractionation of mixtures is desired. The traditional tools of gas or liquid chromatography and electrophoresis broadly separate structural isomers, but never isotopomers (though LC can resolve non-isobaric isotopologues for various compounds, including labeled peptides) [13–14].

Those methods are now replaced or augmented by rapid ion mobility separations (IMS) in gases that have unique selectivity [15]. Linear IMS based on absolute mobility (K) at moderate electric field strength (E) can separate structural isomers and elucidate their geometries. Unlike with MS, peaks display no isotopic envelopes [16] even at the highest attained $R \sim 200$. That comports with theory [17], where the isotopic shift in IMS follows from the reduced-mass term in the Mason-Schamp equation (relating K to the ion-molecule collision cross section, Ω) and scales as the inverse square of ion mass, m . As the result, separation of isotopologues differing by 1 Da at $m > 100$ Da requires $R > 10^3$ that is beyond known technology [17, 18]. Within that model, IMS will not distinguish isotopomers at any R .

The drift of all ions in any gas becomes nonlinear at elevated E , with the mobility dependent on E . Differential IMS (FAIMS) extracts the increment of K between two E levels using a periodic field established in a space between two electrodes [19–22]. This differential mobility is correlated to the ion mass much weaker than Ω , hence FAIMS is far more orthogonal to MS than linear IMS is [19], and generally separates isomers better at equal resolving power (as has been proven for lipids [23], peptides [24 – 27] and proteins [28, 29]). The performance is notably sensitive to the buffer gas identity. In particular, adding light gases (e.g., He) to heavier gases (such as N_2) tremendously improves the resolution of peptide, protein, and lipid isomers [23 – 29] because of higher ion mobility in lighter gases and prominent non-Blanc effect in mixtures of molecules with disparate masses [30]. In FAIMS chips, where microscopic gaps allow extreme fields by the Paschen law, He/ N_2 buffers with $\sim 80\%$ He are optimum [31]. However, the electrical breakdown in macro devices employed here constrains the He fraction to $\sim 50 - 75\%$ [23 – 28].

Perhaps the most impressive example is the resolution of isobaric isotopologues and isotopomers [18], achieved for protonated glycine (G) and alanine (A) ions labeled by D, ^{13}C , ^{15}N in specific positions. The lack of correlation to ion mass came through in further

facts: (1) non-isobaric species (e.g., ^{15}N -G at $m/z = 77$ and $^{13}\text{C}_2$ -G at $m/z = 78$) were often separated much less than isobars (e.g., $^{13}\text{C}_2$ -G and D_2 -G at $m/z = 78$); (2) separations strongly depended on the buffer gas, with the peak order inverting between N_2 and CO_2 ; (3) adding He to either N_2 or CO_2 expanded the separation space, while the impact of reduced mass was proportional to the gas molecule mass and therefore diminished at higher He fractions. Isotopic substitutions slightly alter all ion geometries, more so for the H/D exchange. However, the D labeling was not always associated with the largest peak shift, revealing that other phenomena are in play [18].

Of note was the additivity of shifts: those for $^{13}\text{C}_2$ -G equaled the sum of those for G with ^{13}C on the 1st and 2nd carbons (in all buffers). While FAIMS agglomerates multiple physical causes, isotopomeric shifts appear mainly due to the center of mass of the ion transposed within its frame, which changes the partition of collision energy into the rotational and translational degrees of freedom. Then, in contrast to the reduced-mass factor [17], the isotopic shifts in FAIMS may increase for larger ions that permit farther moves of the labels and thus the center of mass. A better resolution of species with ^{13}C labels separated by two C-C bonds in A vs. one bond in G support that argument [18]. Whether this trend persists for larger biomolecules such as peptides and lipids is the key question that would decide the practical utility of FAIMS for isotopomer separations.

Here, we show full resolution of a dipeptide with the 1st or 2nd labeled residues - the best isotopomer separation found so far. This extension of the capability to >100 Da range opens the door to real bioanalytical applications.

Experimental Methods

The planar FAIMS unit coupled to the Thermo LTQ XL ion trap via a slit aperture/funnel interface is an upgrade on the previous system [27]. The gap width (g) is now adjustable by varying the thickness of ceramic washers and was set to 1.25 or 1.88 mm. The bisinusoidal waveform is produced by a high-definition generator (Heartland Mobility, Wichita) protected from electrical shorts that lets trying new combinations of the field and gas composition without risking equipment damage. The amplitude (dispersion voltage, DV) was 4.00 kV with $g = 1.88$ mm and 3.20 or 3.84 kV with $g = 1.25$ mm. These values correspond to the dispersion field (E_D) of 21.3 kV/cm ("moderate"), 25.6 kV/cm ("high"), and 30.7 kV/cm ("maximum").

The buffer gas was formulated by digital flow meters (MKS Instruments) controlled from a PC and delivered at a rate of 1.1 – 3.5 L/min to balance the resolution and sensitivity. Electrical breakdown limited the He fractions in He/ N_2 and He/ CO_2 to ~70% for $g = 1.88$ mm and ~35% for $g = 1.25$ mm and DV = 3.20 kV. At DV = 3.84 kV, no He could be used.

The regular dialanine (AA, 160.1 Da) and analogs labeled by $\{^{13}\text{C}_3; ^{15}\text{N}\}$ (mass increment = 4 Da) on the 1st residue (A^*A) and 2nd residue (AA^*) were synthesized by Thermo Scientific. The ~0.2 mM solutions of standards or their mixtures in 50:49:1 methanol/water/acetic acid were infused at 0.4 $\mu\text{L}/\text{min}$ to the electrospray emitter biased at ~2.5 kV above the FAIMS inlet.

The MS spectra were dominated by protonated dialanine ions (Fig. S1). Their FAIMS spectra were acquired by scanning the compensation voltage (CV) at the speed of 0.3 – 1 V/min. Measuring A⁺A and AA⁺ individually with AA as the internal standard allowed determining the isotopic CV shifts with the precision of ~0.01 V (per replicate statistics). Separation of A⁺A and AA⁺ has been confirmed by the spectra for their mixtures in various ratios.

Results and Discussion

For consistency across systems, we express CV as the compensation field E_C , set as positive when $K/dE < 0$. As expected for this m/z range and N₂ or CO₂ gases, dialanines are type A ions where E_C is negative and becomes more negative at higher E_D (Fig. 1). Low polarizability of He compared to N₂ or CO₂ makes for a shallower interaction potential with any ion and thus harder scattering, and adding He eventually converts [32,33] ions to type C, where $E_C > 0$ and increases at higher E_D . At moderate E_D , however, E_C turns slightly more negative (toward higher absolute values) between 0 and 20% He because of the non-Blanc effect [30]. The effect escalates with more dissimilar components and so is greater for He/CO₂ than He/N₂ compositions (Fig. 1).

The H⁺A⁺A and H⁺AA⁺ ions ($m/z = 165.1$) track these trends with reproducible isotopic shifts (E_{iso}) that vary as a function of He concentration. We define:

$$\Delta E_{iso} = E_C(\text{labeled}) - E_C(\text{unlabeled}) \quad (1)$$

In He/N₂ mixtures (Fig. 2), the shift for A⁺A ($m/z = 165.1$) is positive (meaning a less negative E_C) and substantial (1.4 – 2.1 V/cm) at all He fractions, with a maximum at ~55% He. The shift for AA⁺ is small, nudging from 0.5 V/cm in N₂ to 0 at ~60% He and –0.3 V/cm at 65% He. Accordingly, the isotopomeric shift

$$S_{iso} = \Delta E_{iso}(\text{AA}^+) - \Delta E_{iso}(\text{A}^+\text{A}) \quad (2)$$

smoothly increases in magnitude from 0.9 V/cm in N₂ to 2 V/cm at 65% He. These and other key data in the paper have been verified by multiple replicates (e.g., Table S1). This pattern qualitatively copies that for single amino acids labeled by ¹³C on the N- and C-terminal carbons (the respective analogs of A⁺A and AA⁺) [18].

Indeed, at same E_D , the isotopic shifts for the first (¹³C1) isotopomers of G and A were positive with a slight maximum (~1 V/cm) near 50% He, whereas those for the second (¹³C2 for G; ¹³C3 for A) isotopomers were near-zero, gradually decreasing from 0.3 V/cm in N₂ to –0.4 V/cm at 65% He (all values for G) [18]. Thus S_{iso} values were similarly negative, with the magnitude increasing from 0.6 V/cm in N₂ to 1.3 V/cm at 60% He. The E_{iso} value for N-terminally labeled species is about doubled here, which may reflect a greater transposition of the ion center of mass because of higher total mass of the label (4 vs. 1 Da) and perhaps its more distant move in an ion of larger dimensions. This translates into a greater S_{iso} that improves the isotopomer resolution. As is common [24], the optimum (though still

incomplete) separation is achieved somewhat below the maximum He fraction because the signal declines with rising He content.

Our standards were not isotopically depleted at unlabeled atoms and thus include isotopologues conforming to the stoichiometry ($C_6H_{12}N_2O_3$) and natural isotopic abundances for unlabeled atoms of constituent elements: 0.011% (D), 1.07% (^{13}C), 0.37% (^{15}N), 0.04% (^{17}O), and 0.20% (^{18}O). Hence nearly all species at 1 Da increment (96% for the AA and 92% for the A^{*}A and AA^{*} samples) are due to the doping by ^{13}C or ^{15}N that compose the labels in A^{*}A and AA^{*} (Table S2). One of these isotopes replacing one ^{12}C or ^{14}N atom in A ($NH_2CH_2CH_2CO_2H$) creates eight isotopomers for AA at the nominal 161 Da mass and four for A^{*}A or AA^{*} at 165 Da.

The intensities of MS peaks at $m/z = 162.1$ (vs. 161.1) and 166.1 (vs. 165.1) match the calculated 7% and 4% (Fig. S1). The absolute E_C shifts for the features at $m/z = 166.1$ vs. those from the same sample at $m/z = 165.1$ (Fig. 3a) are less than those in Fig. 2, which is reasonable with the 4-fold smaller mass increment. The shifts for A^{*}A and AA^{*} differ as well: the 1st decreases from 0.2 V/cm in N_2 to 0 at 65% He, whereas the 2nd increases from 0.2 V/cm in N_2 to 0.4 V/cm at 65% He. As a control, the E_{iso} values for AA feature at $m/z = 162.1$ in the scans for AA/A^{*}A and AA/AA^{*} mixtures are equal, amounting to 0.2 V/cm at all He fractions (Fig. 3b). This is the first report of shifts in FAIMS resulting from a natural isotopic distribution.

The features at $m/z = 166.1$ are not split or much broadened: at 50% He, the mean peak width exceeds those at $m/z = 161.1$ or 165.1 by ~15% (Table S1). Hence all fairly abundant isotopomers have close E_C values. Then, if the isotopic shifts are additive, E_{iso} from ^{13}C or ^{15}N in either A would equal ¼ of E_{iso} from the $\{^{13}C_3; ^{15}N\}$ block in *same* residue. The curves thus obtained from experimental E_{iso} for A^{*}A and AA^{*} at $m/z = 165.1$ clearly track the observations for, respectively, AA^{*} and A^{*}A at $m/z = 166.1$ (Fig. 3a). The small discrepancies are within the combined uncertainty of two E_{iso} measurements and the error margin of our approximation of equal shifts from all ^{13}C and ^{15}N labels within a residue, not even considering the currently unquantifiable contribution of up to 8% of isotopomers at $m/z = 166.1$ comprising ^{17}O or D (Table S2). The ensemble of AA isotopomers at $m/z = 162.1$ is a 1:1 mixture of the pools doped on the 1st and 2nd residues. Then the mean E_{iso} for AA should equal the average of E_C shifts for A^{*}A and AA^{*} in Fig. 3a, in line with the data (Fig. 3b). These findings uphold a wide additivity of isotopic shifts.

The He/ N_2 and He/ CO_2 mixtures elicited the opposite peak order for many G and A isotopologues (including isobaric ones) [18], but not isotopomers labeled by $^{13}C1$ and $^{13}C3$. Here, the isotopic shifts are almost switched: E_{iso} for A^{*}A is near-zero, increasing from -0.2 V/cm in N_2 to 0.7 V/cm at 65% He, and E_{iso} for AA^{*} is positive and substantial, with a maximum of 1.7 V/cm at ~55% He (Fig. 4). The isotopomeric shift thus amounts to 0.7 – 1.5 V/cm, maximizing near 40% He. The peaks are wider in He/ CO_2 than He/ N_2 with equal He percentage (Fig. S2). This is because the peak widths in planar FAIMS devices scale as $\sim K^{-1/2}$, and all ions are less mobile in CO_2 than N_2 with smaller and lighter molecules. A lower S_{iso} and broader peaks vs. those with He/ N_2 buffers mean worse separation, and we

did not pursue the analyses in He/CO₂ mixtures in detail. Nonetheless, these data demonstrate the likely utility of varying the gas composition for isotopomer separations.

For labeled G and A, raising E_D to 28.7 kV/cm in He/N₂ buffers has retained the CV spectra overall while expanding the isotopic shifts (despite a lower He content limited by the breakdown) [18]. Same happens here at $E_D = 25.6$ kV/cm: the maximum E_{iso} goes up to 3.3 V/cm for A^{*}A and 0.8 V/cm for AA^{*} ($m/z = 165.1$), with the absolute S_{iso} growing to $\sim 2 - 3$ V/cm (Fig. 5). The extra shifts for A^{*}A and AA^{*} at $m/z = 166.1$ similarly expand up to 0.7 V/cm (for AA^{*}) and roughly match the values derived from measured E_{iso} at $m/z = 165.1$ (Fig. S3). The average of those shifts again agrees with E_{iso} for AA at $m/z = 162$. Thus the additivity of isotopic shifts holds across dispersion fields.

At the maximum $E_D = 30.7$ kV/cm, the isotopic shift for A^{*}A reaches 4.2 V/cm while that for AA^{*} remains at 0.7 V/cm: thus abs. S_{iso} expands to 3.5 V/cm. The E_C shifts at $m/z = 166.1$ also increase to 0.4 V/cm (A^{*}A) and 0.8 V/cm (AA^{*}), while E_{iso} for AA at $m/z = 162.1$ increases to 0.5 V/cm. This regime has provided the best (better than half-maximum) resolution of A^{*}A and AA^{*}, enabling perfect filtering of either at its peak apex (Fig. 6). This is the first isotopomer separation of that quality.

The relative isotopic shift for AA^{*} and A^{*}A at $m/z = 166.1$ vs. 165.1 (Fig. 3a) is of opposite sign compared to S_{iso} at $m/z = 165.1$ (Fig. 2). This shrinks the isotopomeric shifts at $m/z = 166.1$ at any E_D , hence resolving A^{*}A and AA^{*} at that mass was not sought. This makes sense given that random doping by natural isotopes blunts the distinction between deliberately labeled species.

Conclusions and Outlook

High-definition FAIMS can resolve isotopomers for species over 100 Da, such as peptides with labeled amino acids. The optimum condition is near the breakdown threshold in N₂, which differs from the He/N₂ mixtures with 50 – 75% He that worked well thus far [23 – 28]. This indicates testing the new regime in proteomic and metabolomic analyses. The separation in He/CO₂ buffers is all but opposite to that in He/N₂, showcasing the crucial role of gas properties and suggesting the exploration of other compositions, especially H₂/N₂ that is excellent for protein conformer isolation [29]. The isotopomer separation turns better for dipeptides than for labeled single amino acids, suggesting a substantial and perhaps greater effect for larger biomolecules more relevant to topical applications. We also observed the previously unknown shifts for species with natural and hybrid natural/labeled isotopic atoms that expand the analytical scope of approach. These shifts apparently exhibit the pairwise additivity noted for labeled amino acids [18], showing its broad validity that should help rationalize the isotopic effects in FAIMS. These directions are under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Gordon Anderson (GAACE) and Dr. Keqi Tang (PNNL) for aiding us to develop and integrate the FAIMS/MS system, and Matt Baird and Dr. Rinat Abzalimov for collaboration. This research was funded by NIH K-INBRE (P20 GM103418) and NSF EPSCoR (EPS-0903806).

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Figure 1. Separation parameters for regular dialanine in He/N₂ and He/CO₂ buffers at three E_D levels

Figure 2. Isotopic and isotopomeric shifts for the labeled dialanines with He/N₂ at the “moderate” field. Lines are the quadratic regressions through each curve.

Figure 3.

Shifts of E_C for A^*A and AA^* at $m/z = 166.1$ vs 165.1 (a) and isotopic shifts for AA at $m/z = 162.1$ (b): measured (points) and modeled based on the experimental shifts for A^*A and AA^* at $m/z = 165.1$ vs 161.1 as described in the text (lines).

Figure 4.
Same as Fig. 2 for He/CO₂ buffers

Figure 5.
Same as Fig. 2 at the “high” field.

Figure 6.

FAIMS spectral window obtained for A^*A , AA^* , and their equimolar mixture at the maximum E_D . The spectra for three lower A^*A/AA^* ratios are in Fig. S4. To compare, the result at moderate field is shown in Fig. S5.