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# Isotopic Effect on Ion Mobility and Separation of Isotopomers by High-Field Ion Mobility Spectrometry

**Alexandre A. Shvartsburg,*,† David E. Clemmer,‡ and Richard D. Smith†**

*Biological Sciences Division, Pacific Northwest National Laboratory, P.O. Box 999, Richland, Washington 99352, and Department of Chemistry, Indiana University, Bloomington, Indiana 47405*

**Distinguishing and separating isotopic molecular variants is important across many scientific fields. However, discerning such variants, especially those producing no net mass difference, has been challenging. For example, single-stage mass spectrometry is broadly employed to analyze isotopes but is blind to isotopic isomers (isotopomers) and, except at very high resolution, species of the same nominal mass (isobars). Here, we report separation of isotopic ions, including isotopomers and isobars, using ion mobility spectrometry (IMS), specifically, the field asymmetric waveform IMS (FAIMS). The effect is not based on the different reduced masses of ion−gas molecule pairs previously theorized to cause isotopic separations in conventional IMS, but appears related to the details of energetic ion−molecule collisions in strong electric fields. The observed separation qualitatively depends on the gas composition and may be improved using gas mixtures. Isotopic shifts depend on the position of the labeled site, which allows its localization and contains information about the ion geometry, potentially enabling a new approach to molecular structure characterization.**

Since early 1900s, when vacuum techniques and ion detectors first enabled investigations of gas-phase ions, two approaches to their separation and characterization have emerged, mass spectrometry (MS) and ion mobility spectrometry (IMS).[1,2] Though both exploit the motion of charged species in an electric field, MS is performed in vacuum and is thus based only on the ion mass/charge ($m/q$) ratio while IMS involves buffer gases and relies on ion transport properties. The first major discovery enabled by MS was the existence of isotopes by Thomson and Aston,[3] and isotopic analyses have since been integral to MS. In particular, the preparative separation of U isotopes using Lawrence's Calutron was the first industrial MS application,[4] and isotopic labeling is intrinsic to MS quantification methods. The issue of isotopes was largely ignored in IMS as the resolving power ($R$) was generally too low for their separation.

A theory for isotopic separations in conventional IMS, where absolute ion mobility ($K$) is measured at relatively low electric field intensities ($E$), has been presented by Valentine and Clemmer.[5] Postulating that isotopic variations do not affect the ion−molecule collision cross sections ($\Omega$) significantly, they suggest that isotopic shifts in mobility would primarily reflect the difference in reduced mass in the Mason−Schamp equation:[5]

$$K = \frac{3}{16}\sqrt{\frac{2\pi mM}{(m+M)kT}}\frac{q}{N\Omega} \quad (1)$$

where $T$ and $N$ are the gas temperature and number density, and $M$ is the gas molecule mass. Thus, lighter isotopes have higher mobilities, and the relative mobility shift between an ion L with the mass $m$ and its heavier isotope H with the mass $(m + \Delta m)$ is[5]

$$\frac{K_L}{K_H} = \sqrt{\frac{m^{-1} + M^{-1}}{(m+\Delta m)^{-1} + M^{-1}}} \quad (2)$$

One typically has $\Delta m \ll m$, and eq 2 may be condensed to

$$K_L/K_H \cong 1 + \Delta mM/[2m(m+M)] \quad (3)$$

Usually ions are (much) heavier than the buffer gas molecules and the shift by eq 3 is (much) smaller than the relative mass difference. For example, the mobilities of isotopes weighing 100 and 101 Da would differ by 0.1% in nitrogen and 0.02% in helium commonly used in IMS, and their resolution would require $R$ of ~1000 and ~5000, respectively (based on the peak width at half-maximum).[5] For heavy ions where $m \gg M$, eq 3 further reduces to

$$K_L/K_H \cong 1 + \Delta mM/(2m^2) \quad (4)$$

Hence, the IMS resolving power needed scales with the ion mass squared. As conventional IMS is currently limited[6−8] to $R \sim 200$,

* To whom correspondence should be addressed.
† Pacific Northwest National Laboratory.
‡ Indiana University.

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the absence of reports or interest in isotope separation by IMS is not surprising. The recently developed cyclotron IMS[9] potentially allows a dramatic increase of $R$ to $\sim 10^3$ and higher, but the real separation power of such devices remains to be shown.

Molecular species can have isotopomers with same labeled atom(s) in different locations, e.g., $NH_2CH_2{}^{13}CO_2H$ vs $NH_2{}^{13}CH_2CO_2H$ glycines. Such ions have equal masses and, by eq 2, mobilities. There also are "pseudoisotopomers" with identical nominal mass but differing isotopic content and slight mass difference due to unequal atomic mass defects, e.g., $NH_2CH_2{}^{13}CO_2H$ (76.035 Da) and $^{15}NH_2CH_2CO_2H$ (76.029 Da). Such ions can be distinguished[10] by high-resolution Fourier-transform MS, but separation by conventional IMS would, according to eq 3, require an unrealistic $R$.

Another IMS approach, the differential or field asymmetric waveform IMS (FAIMS), is based on mobility differences in weak and strong electric fields.[11] In practice, ions are filtered in a gap between two electrodes carrying an asymmetric high-voltage waveform (with the peak amplitude called "dispersion voltage", DV) plus weak fixed "compensation voltage" (CV). Only some species pass the gap at a given CV, and scanning CV produces the spectrum of species present. This method has separated mixtures, including isomers and isobars, and often resolves species undistinguishable by conventional IMS.[11−16] A single report of isotopic separation had appeared[17] for atomic ions: $^{35}Cl^-$ and $^{37}Cl^-$ that differ in mass by 6%. That work involved a FAIMS unit of cylindrical shape and hence inherently modest[14] $R$ of $\sim$10.

Here, we demonstrate that the recently developed high-resolution FAIMS[15,16] can separate isotopes with a much smaller mass difference of $\sim$1%, and, most importantly, isobars with different isotopic content and isotopomers. This capability may enable a new method for isotope separation in a small-scale format at ambient pressure and aid localization of labeled sites in molecules. As the observed isotopic shifts depend on the label position, they potentially provide detailed structural information not obtainable for gas-phase ions using conventional IMS or MS.

We employed a planar FAIMS device with $R \sim 100$, coupled to an ion trap MS. We used the peak waveform amplitude of 4.0 or 5.4 kV and the $N_2$, He/$N_2$, or He/$CO_2$ mixtures for the gas with the He fraction limited to $\sim$70% at 4 kV and $\sim$40% at 5.4 kV (to avoid electrical breakdown in the gap).[15,16] To maintain a level ion flux past FAIMS, at higher He content the separation time was reduced from $\sim$200 to $\sim$50 ms by increasing the flow rate from 2 to $\sim$8 L/min. We studied $\sim$1:1 pairwise mixtures of normal glycines or alanines and their isotopically pure analogues: $NH_2CH_2{}^{13}CO_2H$ (C1 labeled), $NH_2{}^{13}CH_2CO_2H$ (C2 labeled), $NH_2{}^{13}CH_2{}^{13}CO_2H$, $^{15}NH_2CH_2CO_2H$, and $NH_2CD_2CO_2H$ glycines, and $NH_2CH_2CH_2{}^{13}CO_2H$ (C1 labeled) and $NH_2{}^{13}CH_2CH_2CO_2H$ (C3 labeled) alanines (Sigma Aldrich, the label numbers counted from the carboxylic end). Ions were generated by electrospray ionization (ESI), where amino acids yield protonated 1+ ions that produce a single feature in the FAIMS spectrum. Samples were dissolved in 50:49:1 water/methanol/acetic acid and infused to the ESI source at $\sim$0.5 $\mu$L/min.

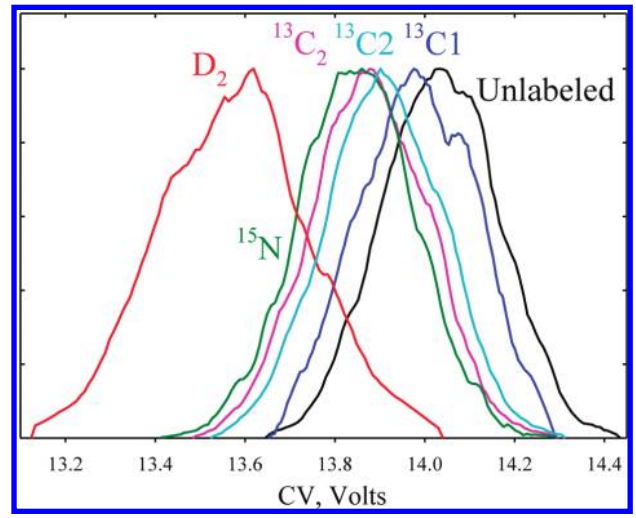


**Figure 1.** Composite FAIMS spectra for labeled glycines at DV = 4 kV in $N_2$, relative to the normal glycine.

First, we set DV to 4 kV. With $N_2$ gas (Figure 1), the peak for glycine ($m/z = 76$) has CV = 14 V, the width of 0.3 V, and thus $R \sim 45$, which is far too low to separate isotopes based on the reduced mass effect by eq 2. In fact, all labeled glycines ($m/z =$ 77 and 78) have notably lower CVs (Figure 1) and shifts not controlled by the mass difference: the $^{13}C_2$ peak ($m/z = 78$) lies close to and between those for $^{15}N$ and $^{13}C2$ ($m/z = 77$), while the $D_2$ peak ($m/z = 78$) has a much lower CV. The physics of this separation clearly differs from that considered in ref 5 and isobars, e.g., $^{13}C_2$- and $D_2$-glycines, can be distinguished.

The shifts in Figure 1 allow only limited separation. The FAIMS resolution can be enhanced using gas mixtures where non-Blanc phenomena (deviations from the Blanc's law for mobility and diffusion in gas mixtures that occur at high $E$) expand the separation space,[18] especially He/$N_2$ with 50−75% He.[15,16] Upon He addition, the CVs of small ions first increase and then decrease and cross to negative values.[15,18] This applies to isotopic glycines, with CVs maximizing at 22−23 V at $\sim$55% He and decreasing to 20−21 V at 66% He (Figure 2a). The covered CV range expands roughly in proportion, from 0.5 V in $N_2$ to 0.9 V at 60% He (Figure 2b). For $^{15}N$- and $D_2$-glycines, the CV shifts vs the normal one nearly double at $\sim$60% He, varying little in relative terms. The peak widths change little because the flow is adjusted for constant sensitivity (as stated above), and the resolution scales approximately with the CV shifts between relevant features. The order of peaks is retained, except all $^{13}C$ labeled and the


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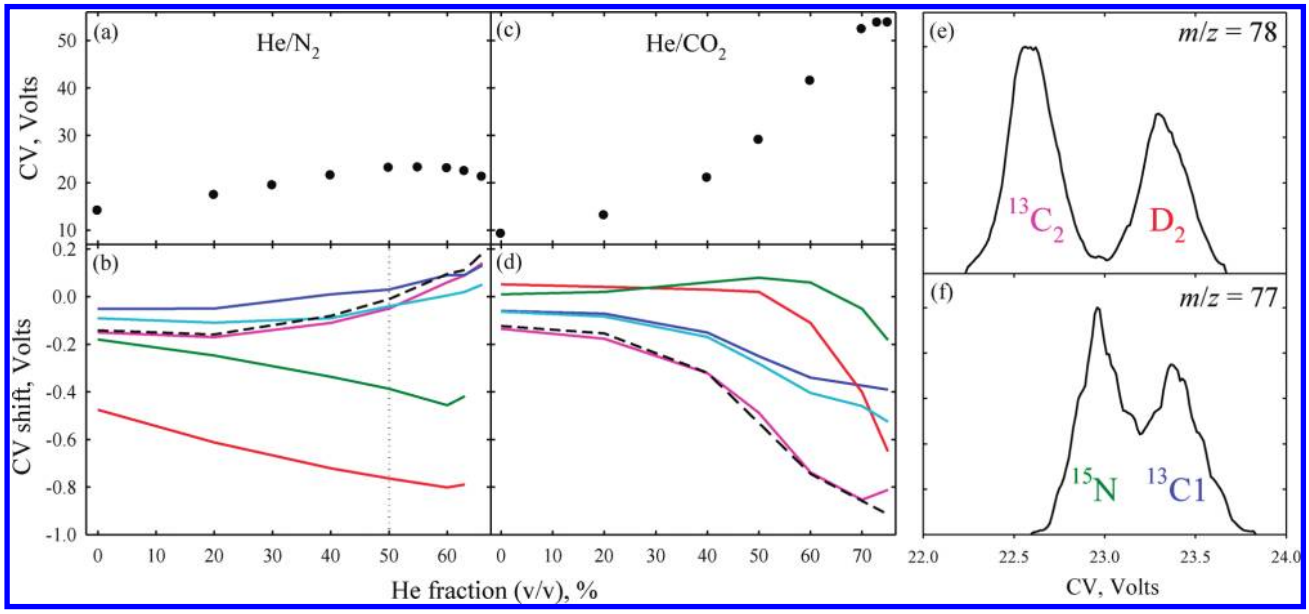


**Figure 2.** Separation properties for the glycines in Figure 1, depending on the $He/N_2$ (a, b) and $He/CO_2$ (c, d) mixture composition; (a, c) CV for normal glycine; (b, d) CV shifts for labeled glycines from the values for the normal analogue; (e, f) separation of the $^{13}C_2/D_2$ and $^{15}N/^{13}C1$ glycine ion pairs in 1:1 $He/N_2$ (dotted line in part b). The dashed line in parts b and d is the sum of shifts for the $^{13}C1$ and $^{13}C2$ glycines.

normal glycine switch at ~40−60% He and $^{13}C2$ and $^{13}C_2$ switch at ~50% He. These results confirm that the separation is not due to the reduced mass difference, which decreases at higher He fractions and never changes sign.

The FAIMS separations in $CO_2$ and $He/CO_2$ mixtures[19] differ from those in $N_2$ or $He/N_2$. As He and $CO_2$ diverge in terms of mass, size, or polarizability more than He and $N_2$, the non-Blanc behavior would be stronger[18] in $He/CO_2$ than in $He/N_2$. Indeed, the maximum CV of 54 V (at ~75% He) is ~2.3 times that in $He/N_2$ mixtures and 6 times that at 0% He (Figure 2c). The CV range for isotopic glycines also expands drastically with He addition but from a lower base than in $N_2$ and never exceeds 0.9 V reached in $He/N_2$ (Figure 2d). The peak order strikingly differs from that with $He/N_2$: the $^{15}N$- and $D_2$-glycines now have the highest CVs up to ~50% He. Their relative CVs rapidly drop above ~50% He, but up to the maximum 75% He, the CV for $^{15}N$-glycine exceeds that for all $^{13}C$ labeled ones and that for $D_2$- exceeds that for $^{13}C_2$-glycine. At low He %, the $^{15}N$ and $D_2$ peaks are inverted compared to the $He/N_2$ case. The drop of CVs for $^{15}N$- and $D_2$-glycines relative to the normal one at high He fractions is expected as the data in $He/N_2$ and $He/CO_2$ must converge in the high-He limit, yet the shifts for the $^{13}C$ labeled species exhibit no clear upturn up to 75% He. Tailoring the gas composition may help resolving specific targets, even in a narrower separation space. For example, the $^{13}C_2$- and $^{13}C1$-glycines separate by >0.5 V in 7:3 $He/CO_2$ vs <0.15 V in $He/N_2$ mixtures. Therefore, again, the separation is not derived from the reduced mass difference, the sign of which is independent of the gas.

In any $He/N_2$ or $He/CO_2$ composition, the sum of shifts for $^{13}C1$- and $^{13}C2$-glycines (relative to the normal one) approximates that for the $^{13}C_2$-glycine (Figure 2b,d). Whether the isotopic CV shifts are additive in general, allowing the prediction upon multiple labeling from the values for individual labels, remains to be determined.

By Figure 2, all pseudoisotopomer pairs ($^{15}N/^{13}C1$, $^{15}N/^{13}C2$, and $^{13}C_2/D_2$) are best separated in ~3:2 $He/N_2$, with CV shifts of ~0.5 V or more resulting in clear peak partitioning. The peak width variation maximizes the resolution at ~50% He, with the separation at baseline for $^{13}C_2/D_2$ and half-height for $^{15}N/^{13}C1$ (Figure 2e,f). For the $^{13}C1$ and $^{13}C2$ isotopomers, the greatest shift is 0.1 V or $^1/_3$ of the peak width, precluding observable peak separation.

Isotopomers may be easier to separate when the labeled atoms are not adjacent as in $^{13}C1$ and $^{13}C2$ glycines but spread apart such as carbons 1 and 3 in alanine. Indeed, the CV shifts for $^{13}C1$- and $^{13}C3$-alanines relative to the normal analogue and each other (Figure 3a) have the same sign and dependence on the $He/N_2$ ratio as those for $^{13}C1$- and $^{13}C2$-glycines (Figure 2b). Although the maximum CV drops to 15 V, the greatest CV difference between the isotopomers increases to 0.24 V (here at ~60% He). This is close to the peak width, which clearly broadens the feature for the mixture (Figure 3b).

The FAIMS resolving power improves at higher DV, but the electrical breakdown constraint reduces the maximum He content with an opposite effect.[11,16] At DV = 5.4 kV (the limit of existing waveform generators), the highest He fraction was ~50% and the resolution for amino acids and peptides at best slightly exceeded that at DV = 4 kV.[16] Raising the DV from 4 to 5.4 kV in same gas about doubles the CVs for normal glycine to 31 V in $N_2$ and 40 V at 35% He (Figure 4a). The isotopic shifts also nearly double, reaching 1.5 V with the CV range expanding to 1.6 V (Figure 4b). However, the relative shifts and thus the peak order are retained (Figure 4c), and the shifts for $^{13}C1$- and $^{13}C2$-glycines again add to that for the $^{13}C_2$-one (Figure 4b). The same peak order was likewise observed for amino acids or peptides at DV of 4 and 5.4 kV with the maximum He content for each.[16] The peak widths in

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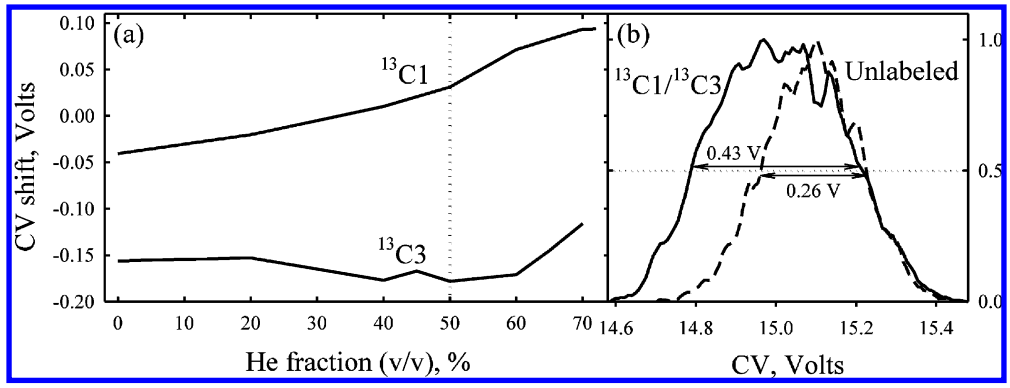


**Figure 3.** Separation of isotopomers: (a) CV shifts for $^{13}$C1 and $^{13}$C3 alanine from the values for the normal analogue, depending on the He/$N_2$ composition at DV = 4 kV and (b) broadening of the peak ($m/z$ = 91) relative to that for the normal analogue ($m/z$ = 90) in the $^{13}$C1/$^{13}$C3/normal alanine mixture analyzed at 50% He.

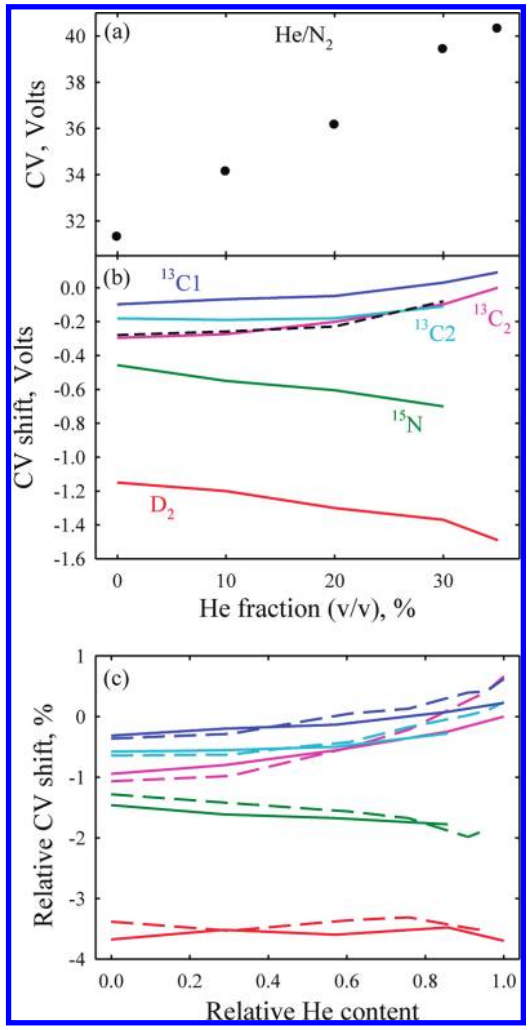


**Figure 4.** Panels a and b replicate Figure 2a,b at DV = 5.4 kV, and panel c shows the CV shifts as a percentage of CV for normal glycine at DV = 5.4 kV (solid lines) and 4 kV (dashed lines). The horizontal axis in panel c is in terms of the fraction of maximum He content at each DV (assumed 66% for 4 kV and 35% for 5.4 kV).

planar FAIMS units scale[11] approximately with $K^{-1/2}$. Hence, with other conditions equal, peaks are broader at the DV of 5.4 kV than 4 kV where ions are more mobile because of higher He content, and the resolving powers at 4 and 5.4 kV are close despite very different CVs.[16] Here the separation time is adjusted for level ion signal and the peak widths at 5.4 kV are the same at ~0.3 V. This results in a much better resolution at the greater DV.

Alanine ions follow the same trend: raising DV to 5.4 kV does not affect the peak order or qualitative dependence of the CV shifts on He content but increases the maximum CV to 27 V and the greatest CV difference between $^{13}$C1 and $^{13}$C3 isotopomers to 0.37 V (at ~30−40% He), Figure 5a. This permits a conclusive though still partial separation of these species (Figure 5b). Further resolution gain by ~1.5−2 times, which should be achievable using more effective rectangular waveforms,[11,20] would allow full separation of these isotopomers.

The physics behind the present isotopic effects and resulting separations remains unclear but is not based on the variation of reduced mass of the ion−gas molecule pair theorized to cause separation of nonisobaric isotopic ions in low-field IMS.[5] Isotopic substitutions also affect the ion geometry (particularly bond lengths) and thus the mobility. The greatest mass ratio for any isotopes (two) is for D and H, and deuteration has the maximum isotopic effect on molecular geometries.[21,22] The observation of largest isotopic shift in He/$N_2$ mixtures for $D_2$-glycine (Figure 2b) is consistent with this mechanism, but an unremarkable shift for this species in He/$CO_2$ combinations (Figure 2d) tells that the geometry change is not decisive in at least some cases. Another factor is the shift of the ion center of mass, which may have a nonintuitively large impact on high-energy collision dynamics[23] that underlies the high-field ion mobility in FAIMS. Experiments for a more extensive and diverse set of small molecules should clarify the trends of shifts as a function of the gas composition and the nature and position of isotopic substitution, from which a physical explanation for the effect would hopefully emerge.

The relative mass difference between the glycines examined here (76 vs 77 Da or 77 vs 78 Da) is the same 1.3% as that between $^{235}$U and $^{238}$U. The capability to separate such isotopes in a simple compact device at atmospheric pressure may be significant. The presence of isotopes in samples (natural or due to labeling) would notably broaden or split the FAIMS peaks, which would otherwise be ascribed to structural

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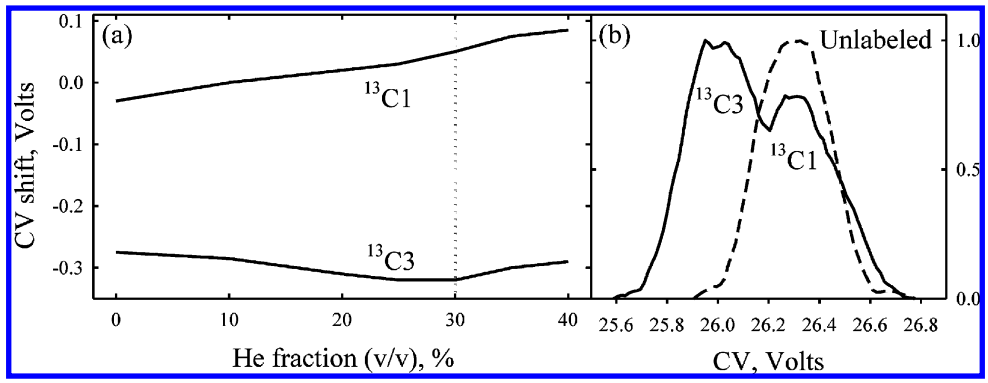


**Figure 5.** Same as Figure 3 at DV = 5.4 kV, and part b is at 30% He.

isomers. The ability to distinguish isotopomers may aid localization and deconvolution of labeled sites (e.g., in the H/D exchange context), simplify analyses involving isotopic labels and particularly multiple labels (such as used for metabolities in pharmaceutical studies), and enable quantification schemes for FAIMS using stable isotope labeling, in parallel to the MS methods. As the isotopic shifts are sensitive to the labeled atom position, they contain structural information and may provide insights into subtle features that are presently the domain of very different tools such as NMR or molecular spectroscopy, which require large amounts of pure sample and are not applicable to ions. In contrast, the ion mobility methods readily work for chemical traces in complex samples.

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