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Separation of a Set of Peptide Sequence Isomers Using Differential Ion Mobility Spectrometry

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Abstract

Protein identification in bottom-up proteomics requires disentangling isomers of proteolytic peptides, a major class of which are sequence inversions. Their separation using ion mobility spectrometry (IMS) has been limited to isomeric pairs. Here we demonstrate baseline separation of all seven 8-mer tryptic peptide isomers using differential IMS. Evaluation of peak capacity of the method implies that even larger libraries should be resolved for heavier peptides with higher charge states.

Introduction

The focus of proteomics is shifting to the identification and quantification of protein and peptide isoforms, especially isomers that are often not differentiated in analyses. Of topical interest are regioisomers created by variant localization of post-translational modifications (PTM).[1–5] Other isomer types are enantiomers, diastereomers (e.g., due to enzymatic racemization *in vivo*), isopeptides with isomeric residues (such as leucine vs. isoleucine or aspartic acid vs. isoaspartic acid), and sequence inversions where the residues are permuted.[5] While sequence isomers are frequently easier to tell apart than the other types based on fragment ions in MS/MS, collision-induced dissociation cleaves the bonds adjacent to the N-terminus and other basic sites poorly and distinguishing inversions of the first two or neighboring basic residues is a challenge.[5] Electron capture or transfer dissociation (EC/TD) severs the backbone bonds on unmodified peptides more uniformly, but works only for multiply-charged precursors and yields no structurally informative backbone fragments in the presence of "electron predator" PTMs with high electron affinity such as nitration.[6–8] Sequence isomer mixtures are generally separated prior to MS analyses, commonly by liquid chromatography (LC). However, that takes a long time (typically, ~1 hr.)[5] and has insufficient peak capacity to characterize large peptide libraries that consist of thousands of species.[9,10] Conventional IMS, based on the absolute ion mobility (K) in gases, can resolve sequence inversions[11] in <0.1 s. The speed of IMS permits nesting it between the LC and MS steps for greater total peak capacity and number of identifications in complex samples.[9,10]

Differential or field asymmetric waveform IMS (FAIMS) relies on the difference of mobility between high and low electric field intensity, measured directly using a periodic asymmetric electric field and superimposed fixed "compensation field" (E_C) established in the gap between two electrodes.[12,13] Since integration into electrospray ionization (ESI)

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MS, FAIMS was used to separate proteolytic digests, including isobaric ions,[14,15] and peptide conformers.[16] The capability to resolve isomers has been demonstrated for localization variants,[4,17,18] but not sequence inversions beyond dipeptides with the charge state (z) of 1 and two possible isomers (GA vs. AG and GS vs. SG).[19] Likewise, only two sequences (GRGDS and SDGRG) were resolved by conventional IMS.[11] Proteolytic peptides processed in bottom-up proteomics normally have ~5 – 20 residues, allowing many more than two sequences. ESI sources tend to produce multiply protonated ions for those peptides; for tryptic digests, 2+ ions typically dominate. Here we show that FAIMS can broadly separate sequence isomers of tryptic peptides in the above size range, including the modified peptides that could not be sequenced by EC/TD,[7,8] by fully resolving the seven species derived by exhaustive permutation of residues (except for the tryptic C-terminus) in a model octapeptide nYAAAAAAK (781.4 Da), where nY is nitrotyrosine.

Experimental methods

The sequence isomers (**1 – 7**), where the number gives the nY position, were synthesized by AltaBiosciences (Birmingham, UK). The peptides or their mixtures were dissolved to ~10 μM in 50/49/1 water/methanol/acetic acid and infused to the ESI source at ~0.5 μL/min. Analyses used a high-resolution FAIMS unit with a 1.88-mm gap coupled to a modified LTQ ion trap mass spectrometer (Thermo Fisher).[20,21] The He/N_2 gas was flown at the "standard" rate of Q = 2 L/min (leading to the ion residence time in the gap of t = 0.2 s)[21,22] or reduced Q = 1.2 – 0.6 L/min for longer t (~0.33 – 0.67 s) and hence better FAIMS resolution.[23] The asymmetric waveform had the peak voltage (dispersion voltage, DV) of 4.0 or 5.4 kV, with the maximum He fractions to avoid electrical breakdown being ~70% and ~50%, respectively.[21–23]

All species (**1 – 7**) produced 1+ and 2+ ions. The E_C scale was again calibrated using the Syntide 2 (St) peptide.[4,18,21–23] That has proven beneficial but less accurate here, likely because of disparate sizes and E_C values for present analytes and St ions. Thus, once a certain isomer was found to not overlap with some others in the spectra under given conditions, that isomer was adopted as an internal reference for final E_C adjustment. All data were validated by analyses of various isomer mixtures.

Results

The resolving power of planar FAIMS devices for peptides overall improves for higher ion charge states and He content in He/N_2 mixtures, as the peaks narrow while E_C values increase.[21–23] The resolution of differing localization variants scales in proportion, generally maximizing for z = 3 or 4 (rather than 2) at the highest feasible He fraction.[4,18] The same trends are observed here for sequence isomers.

We have employed DV = 5.4 kV and Q = 2 L/min first (Fig. 1). For z = 1, all isomers exhibit single peaks of comparable intensity. In N_2, the features for **1, 3, 5, 6,** and **7** nearly coincide while **2** and **4** are partly separated from them at lower and higher E_C, respectively. Adding He does not change the order of peaks, but, as usual, narrows them and raises the resolving power, R. In agreement with published benchmarks,[22–24] between 0% and 40% He the mean peak width (fwhm) decreases by ~25% (from w = 2.4 to 1.9 V/cm) and the average R improves by 2.3 times (from 15 to 34). At 40% He, **2** and **4** are resolved baseline (at ~10% height) from each other and from **1, 3,** or **6,** but the cluster {**1, 3, 5, 6, 7**} is still merged. As usual for planar-geometry FAIMS, the ion signal generally diminishes upon He addition because of greater losses in the gap (due to both faster diffusion and larger amplitude of ion oscillations in the waveform cycle).

The resolving power and resolution are much superior for $z = 2$ (Fig. 1). In N_2, the features for **1, 4, 6,** and **7** are baseline-resolved from each other and merged {**2, 3, 5**}, and **1, 4,** and **7** are several times less intense than others. The first and second highest E_C belong to **4** and **7**. Adding He now has effects beyond improved resolution. First, multiple minor features for **1** (**a1** and **b1**), **4,** and **7** (**a7, b7, c7,** and **d7**) surface and grow at lower E_C, while the total signal drops and vanishes into the noise by 30% He for **4** and 40% He for **1** and **7**. Second, the feature **3** progressively moves to lower E_C with respect to the major peaks for other isomers that retain their relative positions. The minimum peak widths for $z = 2$ at 40% He are $w = 1.6 – 1.8$ V/cm and R is ~70 – 90, similar to the metrics for 2+ ions of St or bradykinin (Bk) under same conditions.[22] Shifting of **3** enables its complete separation from other isomers at ~10% He, but **2** and **5** remain unresolved at any He percentage. Whereas several isomers exhibit minor peaks as stated above, overall conformations appear less diverse than those[16,21–23] for Bk^{2+}. This might reflect smaller size of present peptides, although most 2+ tryptic peptides (including the larger ones) and St^{2+} show a single major peak in FAIMS spectra.[21–23]

For 2+ ions, an inordinate E_C for peptide **4** suggests a more compact geometry compared to other isomers, and the evolution of features for **1, 3, 4,** and **7** indicates isomerization (unfolding) as higher He content augments the field heating in the FAIMS gap.[18,21–23] To rationalize these observations, we note that all isomers must be protonated on the N-terminus and lysine (K) side chain, with the nitro group of nY carrying positive charge on the N atom and negative charge shared by the two oxygens. Positive and negative sites on peptides often form salt bridges that define the global ion shape, subject to steric constraints.[25,26] The ECD and infrared multiphoton dissociation studies have revealed that the N-terminus and nY interact (presumably via a salt-bridge) only in **4, 5, 6,** and **7** (and **3** to a much lesser extent), which thus apparently requires a separation of three or more alanines to overcome the steric hindrance.[7,8] Assuming the same for the salt bridge between K and nY, only **4** would have two bridges and thus be the most compact isomer by far. The next most compact ones would perhaps be **1** and **7** with minimum backbone tails outside of the cycles linked by bridges. The geometries with positive N-terminus and K not spaced apart by other residues (**1, 4,** and **7**) are likely to be less stable because of Coulomb repulsion, with **4** further destabilized by greater strain in smaller rings. This picture is consistent with higher E_C for **4** and **7** and the loss of **4**, and then **1** and **7** at higher He content by the "self-cleaning" mechanism.[23,27] However, a middle-of-the-range E_C for **1** and shifting of the peak for **3** remain to be explained.

Considering that the isomerization of 2+ ions attributed to field heating proceeded already at the lowest He content of 0 – 20%, we have repeated the analyses for **1 – 5** at a lower DV = 4 kV where ions are colder[22] (Fig. 2). For unmodified and phosphorylated peptides,[18,22] reducing DV from 5.4 to 4 kV is near-equivalent to subtracting ~20 – 30% from the He fraction (for example, the spectra at DV = 5.4 kV and 40% He are closest to those at DV = 4 kV and 60 – 70% He). This holds here too, with (i) the spectra for **4** at 0% and 20% He in Fig. 1 most resembling those at 20% and 40 – 50% He in Fig. 2, respectively, (ii) the signal for **4** disappearing at 30% He in Fig. 1 and 60% He in Fig. 2, and (iii) the distinct three-feature pattern for **1** emerging at 20 – 30% He in Fig. 1 but 50 – 60% He in Fig. 2. Continuing the trend in Fig. 1, the peak **3** moves to yet higher relative E_C at lower He content and now lies to the right of 5 at 0 – 20% He. Again, all species could be resolved at some He fraction, except **2** and **5** moving in lockstep. As one would expect from the independence of spectral patterns for $z = 1$ of the He fraction at DV = 5.4 kV (Fig. 1), the results at DV = 4 kV essentially reproduce those in Fig. 1.

The present separations for 1+ and 2+ ions are nearly orthogonal at any He fraction, with slight correlation being only due to the peak for **4** that has by far the highest E_C in both

charge states (Fig. 3a, b). This matches the findings for localization variants of phosphopeptides[18] and presumably reflects the sensitivity of separation properties to peptide conformations that depend on the residue charging scheme. The E_C values in either charge state are also uncorrelated with the nY position (Fig. 3c, d) and often differ for the isomers with single-residue nY shift (such as **3** and **4** or **4** and **5** for either *z*) much more than for those with larger shifts (such as **1** and **7** for $z = 1$ or **2** and **5** for $z = 2$). With phosphopeptides, the E_C difference is similarly uncorrelated with the magnitude of PTM shift along the backbone.[18]

While resolution of any two species is more probable in higher *R* separations, a substantially orthogonal method of lower *R* might still outperform. Here, **2** and **5** are separated better as 1+ than 2+ ions (Fig. 1), despite higher resolving power for the latter as described above. In planar FAIMS devices, *w* fundamentally scales as $t^{-1/2}$ or $Q^{1/2}$, thus slowing the gas flow through the gap enhances separation[23,28] without changing E_C. Then, reducing *Q* by 40% (to 1.2 L/min) and 70% (to 0.6 L/min) should narrow the peaks and increase *R* by 1.29 and 1.82 times, respectively. The actual gains are slightly greater at 1.32 and 2.16 times on average (Fig. 4), which has been noted previously and explained as a consequence of finite separation onset time that effectively reduces *t* by a constant amount.[23] As the result, the {**2; 5**} pair (and also the {**3; 5**} and {**3; 7**} pairs) are baseline resolved at $Q = 0.6$ L/min.

Separations in both charge states at all gas compositions were confirmed by analyses of binary mixtures and, finally, a mixture of all seven isomers (Fig. 5). Each isomer may be baseline-resolved from any other, although not simultaneously. For example, one may filter **2** and **4** as 1+ ions at 40% He (Fig. 4), **1** and **6** as 2+ ions at 0% He, and distinguish **3**, **5**, and **7** as 2+ ions at 20% He. Many other options exist, but at least three regimes appear needed to baseline-resolve all 21 isomer pairs.

Conclusions

In the first ion mobility separation of a significant set of peptide sequence inversions, all seven isomers of a tryptic octapeptide have been resolved using FAIMS. This outcome is enabled by impressive independence of the FAIMS and MS dimensions, evidenced by twofold difference between the E_C values for isomeric 2+ ions (Fig. 1). In contrast, in conventional IMS tryptic peptides of a given charge state lie within ~10% (on average, 3%) of the mean mobility determined by mass,[29–32] and isomers (with equal intrinsic size parameters by definition)[30–32] deviate even less. For example, GRGDS and SDGRG differed in mobility by 5% as 2+ ions and none as 1+ ions.[11] Here, even a moderate resolving power of $R \sim 70 – 90$ for $z = 2$ creates a substantial peak capacity (pc) of ~30 (based on fwhm). Species merged in one charge state may lie apart in others: we resolve as 1+ ions the two sequences not fully separated as 2+ ions. Separations of same isomers with different *z* have proven nearly orthogonal, and, with pc of ~10 for 1+ ions, the overall pc for both charge states is ~40. The resolving power of FAIMS for 3+ or 4+ peptides reaches ~300, or ~2 – 3 times that for 2+ ions under same conditions, because of higher E_C and narrower peaks.[22,23] That has translated into similar or greater gains for the resolution and peak capacity for localization variants,[4,18] e.g., the pc for variant phosphorylated τ-protein sequences[18] was 13 for $z = 2$ but 63 for $z = 3$. The effect ought to be similar for sequence isomers, allowing separations of libraries comprising dozens of species for larger peptides that normally acquire 3+ and 4+ charge states in ESI. This capability may be particularly useful for nitrated peptides and possibly others with basic residues and/or “electron predator” PTMs that resist sequencing by EC/TD methods.

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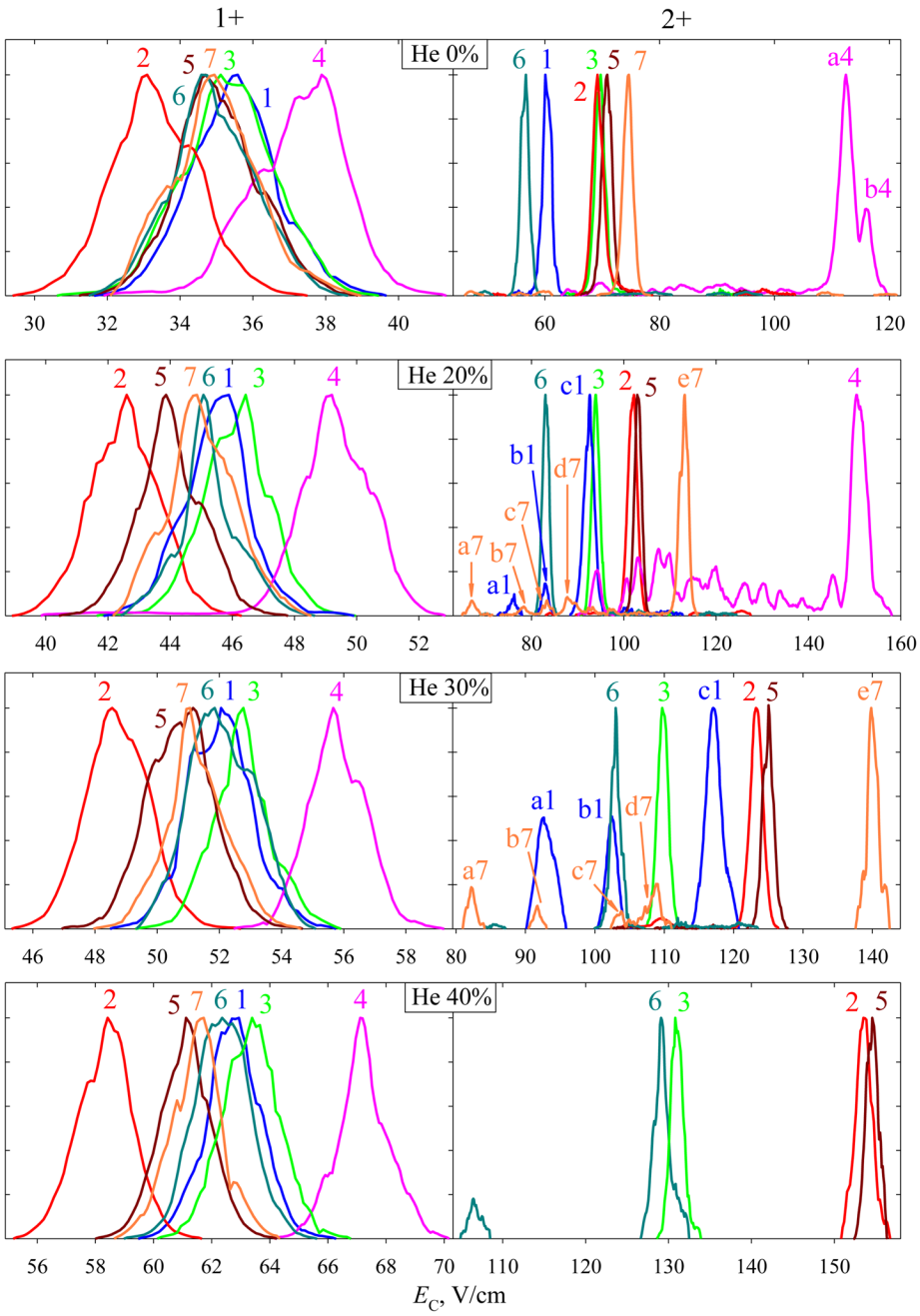

Fig. 1.

Normalized mass-selected FAIMS spectra for **1 – 7** with z = 1 (left column) and 2 (right column) at DV = 5.4 kV, Q = 2 L/min, and 0 – 40% He (as labeled). The features mentioned in the text are marked.

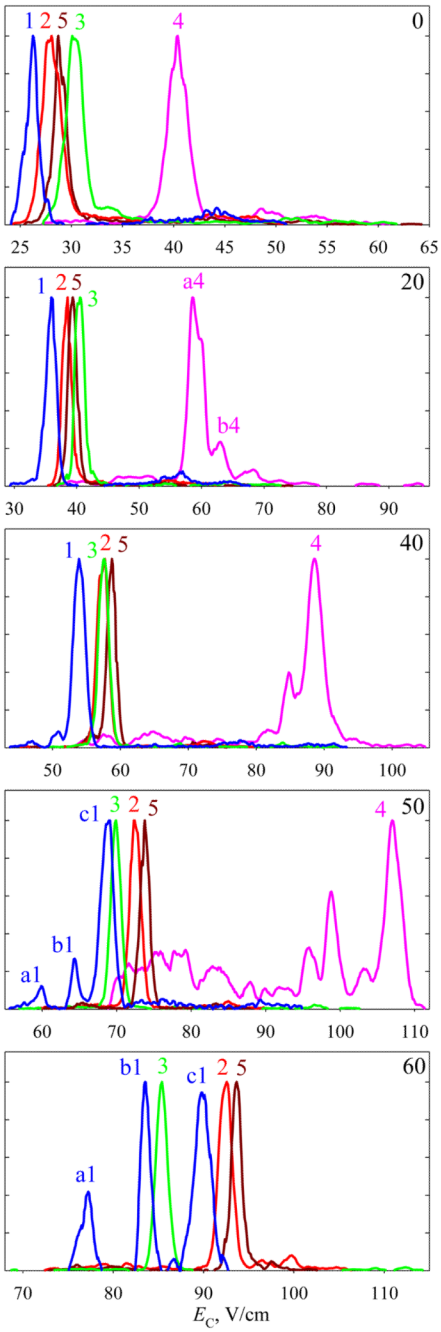

Fig. 2.
Same as Fig. 1 for **1 – 5** with $z = 2$ at DV = 4 kV and 0 – 60% He, as labeled.

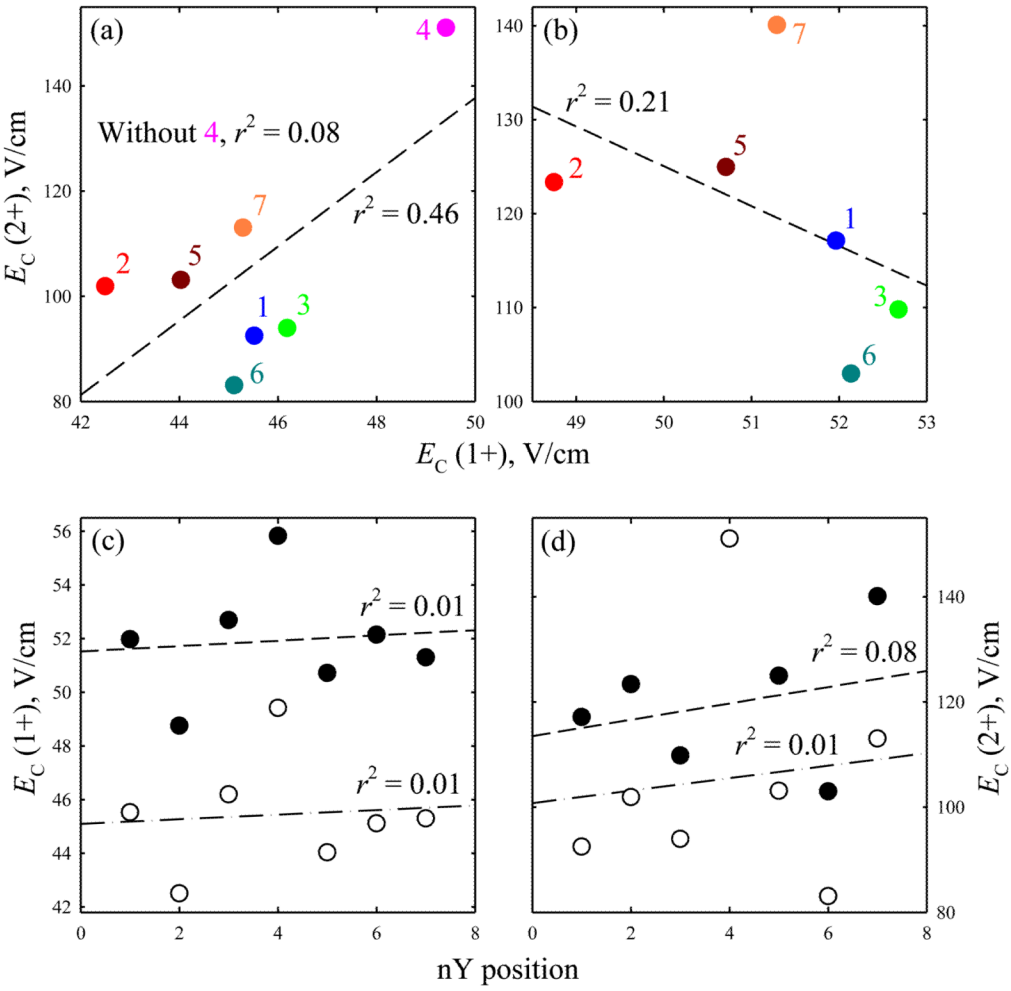

Fig. 3.

Top: correlations between separations of 1+ and 2+ ions (the major peaks of each isomer) from Fig. 1 at 20% (a) and 30% He (b). Lines are the 1st-order regressions through the data. Bottom: correlations between the nY position and separation parameters for 1+ (c) and 2+ (d) ions at 20% He (blank circles, dash-dot lines) and 30% He (solid circles, dashed lines).

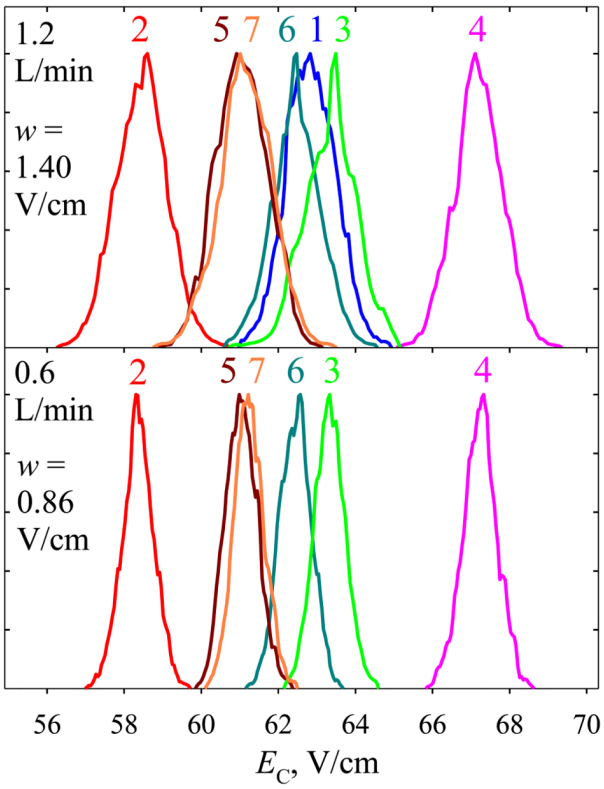

Fig. 4.

Normalized mass-selected FAIMS spectra for **1 – 7** with $z = 1$ using 40% He and reduced gas flow rates, as labeled (no data for **1** were acquired at $Q = 0.6$ L/min). The mean peak widths are given in both panels.

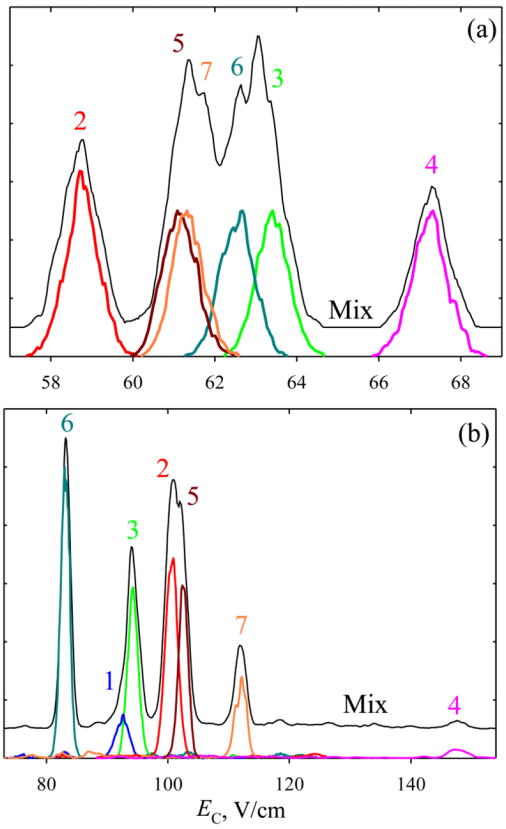

Fig. 5.

Mass-selected spectra for an equimolar mixture of **1 – 7** and overlays for individual isomers (with intensities scaled to match the peak heights in the total) obtained for $z = 1$ using 40% He and $Q = 0.6$ L/min (a) and $z = 2$ using 20% He and $Q = 2$ L/min (b). The spectra for mixtures are vertically offset for clarity. The results with other He fractions and flow rates similarly verify the separations in Figs. 1 and 4.