Published in final edited form as:
Anal Chem. 2008 September 15; 80(18): 6888. doi:10.1021/ac8004988.

Assessing the dynamic range and peak capacity of nanoflow LC-FAIMS-MS on an ion trap mass spectrometer for proteomics

Jesse D. Canterbury, **Xianhua Yi**, **Michael R. Hoopmann**, and **Michael J. MacCoss**
Department of Genome Sciences, University of Washington, Box 355065, Seattle, WA 98195-5065

Abstract

Proteomics experiments on complex mixtures have benefited greatly from the advent of fast-scanning ion trap mass spectrometers. However, the complexity and dynamic range of mixtures analyzed using shotgun proteomics is still beyond what can be sampled by data-dependent acquisition. Furthermore, the total liquid chromatography-mass spectrometry (LC-MS) peak capacity is not sufficient to resolve the precursors within these mixtures, let alone acquire tandem mass spectra on all of them. Here we describe the application of a high-field asymmetric waveform ion mobility spectrometry (FAIMS) device as an interface to an ion trap mass spectrometer. The dynamic range and peak capacity of the nanoflow LC-FAIMS-MS analysis was assessed using a complex tryptic digest of *S. cerevisiae* proteins. By adding this relatively simple device to the front of the mass spectrometer, we obtain an increase in peak capacity >8 fold and an increase in dynamic range of >5 fold, without increasing the length of the LC-MS analysis. Thus, the addition of FAIMS to the front of a table top mass spectrometer can obtain the peak capacity of multidimensional protein identification technology (MudPIT) while increasing the throughput by a factor of 12.

Introduction

In shotgun proteomics, a complex mixture of proteins is digested to create a mixture of peptides. This mixture is then typically separated by microcapillary liquid chromatography followed by the detection and characterization of the peptides using tandem mass spectrometry. Improvements in the sensitivity, dynamic range, and scan speed of ion trap mass spectrometers have greatly improved the automated acquisition of tandem mass spectra (MS/MS) of peptides using data-dependent acquisition from a complex mixture[1,2]. The implementation of automatic gain control on ion trap mass spectrometers further improves the detection of low abundance molecular species because the instrument accumulates low abundance ions to fill the trap. However, the dynamic range in the context of a complex mixture is often limited because the ion trap can fill with coeluting high abundance species as opposed to low abundance ions.

To enhance the mass spectrometer's ability to handle extremely complex mixtures of peptides, several groups either biochemically simplify the intact protein mixture prior to digestion or add an extra dimension of liquid chromatographic separation on the peptide level prior to measurement by mass spectrometry (i.e. multidimensional protein identification technology; also known as MudPIT)[3–6]. By improving the separation of peptides and thus reducing the complexity of the mixture entering the mass spectrometer at any one point in time, ion trap mass analyzers can now accumulate low abundance ions with reduced interference from much higher abundance species. These multidimensional separation methods interfaced with fast

Address Correspondence to: Michael J. MacCoss, Ph.D., Department of Genome Sciences, University of Washington, Foege Building, Box 355065, Seattle, WA 98195-5065, maccoss@u.washington.edu, Voice: (206) 616-7451, Fax: (206) 685-7301.

scanning mass spectrometers are very powerful and have improved our ability to identify large numbers of proteins from complex mixtures.

While effective, this approach is inherently slow. A single analysis on a single sample can often take 12–30 hours[4;5;7;8], making the general approach not particularly amenable to large-scale comparative analyses. Furthermore, replicate analyses to produce data with appropriate measures of variance for differential comparisons are rarely obtained because they are impractical given the lengthy nature of these experiments. Therefore, to meet the demand for proteome-scale analyses that acquire sufficient replicate measurements to obtain statistically meaningful results, the overall throughput for these experiments must be increased.

An alternative to multidimensional chromatography is the use of a mass spectrometer with sufficient resolution and peak capacity to handle the complexity of the mixture with only a single dimension of chromatographic separation. Recent advances in commercially available hybrid Fourier transform mass spectrometers have facilitated the routine acquisition of mass spectra at >50,000 resolution, with accurate mass, and on a chromatographic timescale[9;10]. While the data-dependent acquisition of MS/MS spectra cannot be acquired on everything that can be separated and detected with these mass spectrometers in a short 1D-chromatographic separation, these nanoLC-MS datasets can still be used to find the most important differences between samples[11–15]. These differences can then be used to direct the acquisition of MS/MS spectra in subsequent experiments or can be used to query a database of peptides using just the accurate mass and normalized chromatographic retention time[16–18]. Using this approach is analytically appealing because MS/MS data is acquired on molecular species that are changing between samples and not just the most abundant ions that are normally fragmented using data-dependent acquisition. A downside to this approach is that the cost, complexity, and even footprint of these instruments limits the number of labs that have the resources available to purchase and operate these instruments.

In this work we describe an intermediate solution in which the dynamic range and peak capacity of a bench-top ion trap mass spectrometer can be increased by the addition of a high-field asymmetric waveform ion mobility spectrometry (FAIMS) device to the front of the instrument. FAIMS works by subjecting ions traveling between two electrodes at atmosphere to a high-voltage asymmetric RF waveform[19]. These ions take on a high-field mobility during the high-voltage portion of the waveform and a low-field mobility during the low-voltage portion. The ratio between the two mobilities is termed the differential mobility and the net motion of an ion under these conditions will drive the ion into one of the electrodes. Application of a small DC voltage, termed a compensation voltage (CV), steers the ion back into a region where it can successfully traverse the electrodes and enter the mass spectrometer. FAIMS can thus be implemented as a filter, sequentially passing ions of a single differential mobility – making it ideal to couple with ion trap mass spectrometers like the LTQ. The FAIMS interface minimizes isobaric interferences and facilitates the detection of low abundance peptides by reducing the complexity of the mixture stored in the ion trap at any one point in time.

FAIMS was first applied to the measurement of peptides by Guevremont and coworkers[20–22]. While instrumental, its potential for proteomics was unclear because this initial application was targeted at simple peptide mixtures and was not interfaced with nanoflow liquid chromatography. Recently, the compatibility of FAIMS with nanoflow chromatography and a quadrupole time-of-flight mass spectrometer with application to the identification of proteins in a biologically relevant sample has been examined[23]. FAIMS adds an additional dimension of separation in the gas phase and has been shown to reduce chemical noise and improve the overall detection limit of low abundance species in complex mixtures[22]. Here we perform experiments with the specific goal of evaluating the capability of FAIMS as a tool for increasing the peak capacity and dynamic range of proteomics analyses on an ion trap mass spectrometer.

In this work, we evaluate the relative benefits and weaknesses of FAIMS with an LTQ ion trap mass spectrometer in the context of profiling experiments on a complex peptide mixture.

Methods

Materials

All reagents, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, Mo.).

Sample preparation

A complex yeast lysate (*S. cerevisiae*) was prepared by growing strain S288C in 500 mL of yeast extract/peptone/dextrose medium, harvested at OD 1.2, and lysed in a BeadBeater (BioSpec Products, Inc. Bartelsville, OK) in 50 mM ammonium bicarbonate at pH 7.8. Unbroken cells and cell debris were removed by centrifugation at 5000*g* for 10 min. Final protein concentration was 1.5 μg/μL as derived from a Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). A 45-μL aliquot of the supernatant was mixed with 5 μL of 1% PPS (Protein Discovery, Knoxville, TN), treated serially with dithiothreitol and iodoacetic acid for 30 min each as described previously[24]. The reduced and alkylated protein mixture was digested to peptides with the addition of trypsin at a 1:50 enzyme/substrate ratio. The mixture was incubated at 37 °C for 4 h and quenched by acidification with HCl per the manufacturer's directions. The digest was centrifuged at 14,000 rpm at 4 °C in a microcentrifuge, and the supernatant stored at −80 °C until analyzed by mass spectrometry.

Liquid chromatography

The nanoflow liquid chromatography (Figure 1) setup consisted of an Agilent 1100 binary pump operating at 200 μL/min flow rate, connected to an open split that was adjusted to obtain a flow rate of ~350 nL/min at the open tip of a fused silica column (length 40 cm, inner diameter 75 μm). This column was packed with Jupiter Proteo C12, 4 μm particle size chromatography material (Phenomonex, Torrance, CA). The microcapillary column was inserted into a New Objective nanospray probe that was used in place of the standard ThermoFisher pneumatically assisted electrospray probe in the FAIMS apparatus and Ion Max source.

Reverse-phase gradients were run for 90 minutes for all experiments. A complete liquid chromatography run consisted of a 15-minute loading phase, a 90-minute elution phase, and a 15-minute re-equilibration phase, for a total of 120 minutes of chromatography run time. Peptides were loaded directly on to the microcapillary column (no trapping column was used). During the elution phase, solvent composition began at 95% buffer A (95% H_2O, 4.9% acetonitrile, 0.1% formic acid) and 5% buffer B (80% acetonitrile, 19.9 % H_2O, 0.1% formic acid). Over the course of the 90-minute gradient, solvent composition changed linearly from 5% buffer B to 35% buffer B. In the final 5 minutes (at minute 85 in the elution phase), the composition was changed to 95% buffer B to elute all remaining peptides. The flow rate at the column tip during elution was about 350 nL/min. During the re-equilibration phase, solvent composition returned to 95% buffer A and 5% buffer B. The same column and same chromatography conditions were used for all experiments.

FAIMS-MS apparatus

The FAIMS apparatus (Figure 1) used in these experiments was a prototype commercial device interfaced with an LTQ ion trap mass spectrometer (ThermoFisher, San Jose, CA). The FAIMS interface consists of two stainless steel electrodes mounted coaxially between two PEEK endcaps and insulated from each other by Delrin rings. The inner and outer cylinders have radii of 6.5mm and 9.0mm, respectively, with the analytical region between the cylinders forming a gap of 2.5 mm. This assembly is mounted on a housing which has a spring-loaded interface

plate that provides soft contact to the source cone on the LTQ mass spectrometer. An entrance plate is mounted on the outside of the electrode assembly, and fixed at a potential of 1000 V. The entire interface, electrode assembly plus entrance plate, is secured to a source mount similar to the IonMax source that ships with LTQ mass spectrometers. A connection housing extending from the left side of the source mount provides all gas and electrical connections (RF waveform, electrode biases, and the entrance plate voltage). The RF waveform consists of a sine wave at 750 kHz added to its phase-shifted first harmonic (at approximately 1500 kHz), resulting in the asymmetric waveform necessary for FAIMS[19]. The electronics consists of a doubly-resonant inductor-capacitor network tuned for maximum amplification at 750 and 1500 kHz, coupled to an autotuning feedback circuit[25;26].

In all experiments described in this paper, the FAIMS dispersion voltage (DV) – the magnitude of the voltage from zero to the peak amplitude at the high portion of the asymmetric waveform – was set at −5 kV. Additionally, ambient temperature, although not directly measured, was likely somewhat higher than room temperature due to the close proximity of the LTQ's heated capillary to the FAIMS apparatus. Ambient pressure was very near that at sea level, about 775 torr.

Instrument control of both the FAIMS and the LTQ was provided by a modified version of ThermoFisher's Xcalibur software. This software permitted the synchronization of the FAIMS compensation voltage with the mass spectrometer's scan events. Other FAIMS parameters, such as outer electrode bias and electrode temperature, are also adjustable. The temperature control module was not used for these experiments due to its heavy gas consumption.

Experimental Designs

Three different experiments were designed to evaluate FAIMS as an interface to an LTQ mass spectrometer. Because this device is being used to provide a gas-phase separation of ions for use in proteomics studies, these experiments were designed to evaluate the key metrics in handling complex mixtures: dynamic range and peak capacity.

Experiment 1: NanoLC-FAIMS-MS—To examine the peak capacity and dynamic range of FAIMS, we performed several experiments spanning the range of useful values of the compensation voltage (−3 to −19 V, for operation with nitrogen as the carrier gas; see Figure 1). In these experiments, one full scan from m/z=400 to m/z=1400 was performed at each odd value of CV starting at −3V and stepping to −19V. These scans were acquired in full profile mode (no centroiding) to facilitate post-process data analysis.

Additionally, we acquired nanoLC-MS data without the use of the FAIMS device for the purpose of comparison. All nanoLC-MS data acquired without FAIMS used the same sample and same chromatography conditions (same column) as those data acquired with FAIMS. Instrument tuning parameters, however, changed between FAIMS and non-FAIMS operation to optimize the signal in each mode of operation.

Experiment 2: NanoLC-FAIMS-MS/MS—To evaluate the ability of the FAIMS apparatus to separate charge states of peptides in a complex mixture, we performed tandem mass spectrometry experiments. An initial survey mass scan was followed by two MS/MS scans on the most abundant peaks found in the survey scan. This procedure was performed at each of five different values of compensation voltage, −7, −9, −11, −13, and −15 V. Between each CV, there was a delay of 100 ms between scan events to allow for ions at the new CV value to traverse the electrodes and enter the mass spectrometer – this delay is referred to as the FAIMS dwell time. Assuming that the time for an ion to traverse the FAIMS electrodes and enter the mass spectrometer is roughly the same as the time the vacuum system would take to evacuate the volume encompassed by the FAIMS electrodes, a simple conductance calculation gives

about 70 ms for the FAIMS dwell time. Setting the dwell time at 100 ms on the instrument is thus conservative. CV values in addition to the five cited above were not used because the combined dwell time and total data acquisition time (each set of scans at each CV) was close to the typical width of our chromatographic peaks.

Experiment 3: Direct infusion—To investigate the potential of FAIMS to handle the analysis of complex mixtures without chromatographic separation, we desalted our peptide digest as follows. We loaded our sample offline on to a column packed with reverse phase material (Jupiter C12, as described above). We then desalted the sample by washing the column with buffer A, and eluted the sample from the column using buffer B. We lyophilized the eluted sample and resuspended the dried sample in buffer A (see above for definitions of buffers A and B). All these desalting procedures were done offline using a pressure bomb.

Direct infusion of the desalted yeast digest was performed through an empty 100 μm fused silica capillary (tip pulled to approximately 5 μm diameter using a Sutter Instruments CO_2 laser puller) at a flow rate of 3 μL/min using a Harvard apparatus syringe pump.

Data Analysis

NanoLC-MS Data Analysis—The mass spectrometer's data system output files (ThermoFisher Scientific's RAW format) contained all spectra acquired sequentially, labeled by both CV and retention time. Data was extracted from the RAW files and stored in the MS1 format[27] using an in house program called MakeMS2 (available at http://proteome.gs.washington.edu). A separate MS1 file was stored for each CV resulting in nine separate files for each RAW LC-MS file. Each of these files contained all mass spectra acquired at a single CV.

A program called MScout was used to evaluate our data to quantify: 1) number of peptide-like signals detected; 2) the intensity range over which those signals were present; 3) the chromatographic peak capacity; and 4) the peak capacity of the FAIMS separation. First, individual mass spectra from extracted CV-chromatograms were read sequentially into memory and peaks detected based on signal-to-noise ratio. The signal-to-noise ratio (S/N) was computed using a modified form of the approach reported by Horn *et al*[28]. Briefly, each spectrum was divided into windows, each of which contained 10% of the total ion current in that spectrum. Noise level was then estimated by assuming that the most frequently measured abundance was equivalent to the baseline noise. Ions of S/N greater than 3 (user-selectable) were then eliminated from the window and the noise level was then recomputed for the remaining peaks (using the same method). This procedure attempted to account for conditions where an intense ion in a single window might account for nearly all the ion current in that window. S/N was then recomputed using the new noise level for all detected peaks in the original spectrum. Peaks of S/N greater than 3 (the same user-selectable value used in the first part of the calculation) were retained and the rest discarded.

The detected ions were then analyzed for their persistence in the time domain. Using an approach described previously[29], ions of the same m/z that were detected in consecutive mass spectra were retained and grouped. Typical settings required the signal to persist for >0.2 minutes but <3 minutes. Each persistent signal was stored as an m/z value and the time range over which the signal persisted. Additionally, the intensity and signal-to-noise ratio for the beginning, maximum, and end of each persistent signal were stored. Output from MScout consisted of a short summary of values describing peak capacity, overlap of signals in adjacent FAIMS CVs, dynamic range, and the number of signals (along with the reported maximum and minimum values of intensity and S/N, with the m/z and retention time of each). Additionally, MScout output a tab-delimited file containing the list of detected signals. MScout was written in C++ and compiled with GNU g++ version 3.4 on a desktop computer running

Linux. The software is freely available for non-commercial use at http://proteome.gs.washington.edu.

Analysis of MS/MS Spectra—MS/MS spectra were extracted from RAW files to the MS2 format using MakeMS2 as described above. These spectra were then searched against the yeast open reading frames using the database searching algorithm SEQUEST[30]. SEQUEST's output was passed to the Percolator algorithm[31], which output a file in a modified SQT format[27]. This modified SQT file was then filtered using DTASelect[32] to retain only those peptide-spectrum matches of q-value ≤ 0.01 and assemble the matches into protein identifications. The charge states of the spectra matching a peptide at these thresholds were used to assign the correct charge state of the low resolution tandem mass spectrum.

Results

Application of a Serially Stepped Compensation Voltage on an Ion Trap Mass Spectrometer

FAIMS is normally used as a filter to pass ions of a single differential mobility at any point in time; however, the fast scan speed of the LTQ makes the acquisition of mass spectra across the entire useful CV range possible on a chromatographic timescale. Mass spectra were acquired using 9 different scan events, with each scan event synchronized with a different compensation voltage. The acquisition of mass spectra in parallel with a stepped CV adds an extra dimension to the analysis without increasing the analysis time. Figure 2 shows the base-peak chromatograms at each CV acquired during the 120 minute (90 minute gradient) nanoLC-FAIMS-MS analysis. The chromatograms at each CV are visibly different with only minimal mass spectral similarity between adjacent CVs.

Figure 2 also shows mass spectra at selected CVs for a single retention time (73.1 min; dashed vertical line). As expected from the differences in the chromatograms, these data illustrate a difference in the mass spectra from the same chromatographic retention time but at different CVs. At this retention time there is a large signal at m/z = 688 that reaches its maximum intensity at CV = −11 V (not shown); however, this peptide is undetectable in CVs other than −11, −13, and −15 V. Because the use of FAIMS eliminates this abundant ion from other CVs, the dynamic range is improved at other CVs and ions of low signal abundance can be accumulated in the ion trap to improve their detection. Over the course of the whole experiment, this effect leads to an increase in the number of detected independent features. From the mass spectra obtained using the 9 different CVs over the length of the whole chromatographic gradient, we can detect a total of 14,481 molecular species with S/N > 3, compared with <6000 for a similar experiment without FAIMS. Furthermore, from the data acquired from all 9 CVs, there are signals with S/N > 3 that span >20,000 fold difference in intensity – a dynamic range that is normally associated with a Fourier transform mass spectrometer.

An alternative way to illustrate these data is to plot the extracted mass spectra at retention time 73.1 minutes in three dimensions, as shown in Figure 3. Each mass spectrum is plotted against CV, and several different peptide peaks are visible, each at different values of m/z and different values of CV. Extracting the CV spectra from a single point in the chromatographic retention time (i.e., plotting intensity versus CV at fixed values of retention time and m/z) can be used to assess the peak capacity of the FAIMS separation. In Figure 3, three different m/z peaks are shown with Gaussian curves, obtained from a least-squares fit to the data (Microcal Origin), drawn in the CV dimension. These are extracted on the right-hand side of Figure 3 to show the CV-traces only, along with the best fit Gaussians. The full-widths at half-maximum (FWHM) of these selected CV peaks range from 2.0 to 2.6V, and have an average value of 2.3V. Peak capacity n_c is usually calculated as

$$n_c = \frac{R}{w} \quad (1)$$

where R is the range over which measurements are made and w is the average peak width. The peak width can be taken as full-width at half-maximum or baseline-to-baseline, depending on the particular method of separation used. Because the data are distributed over a 16-volt range, we can therefore estimate the FAIMS peak capacity using these selected signals as 16/2.3 = 6.96.[33]

We used MScout to estimate the FAIMS peak capacity for the entire LC-FAIMS-MS data set depicted in Figure 2. MScout measured peak width in the FAIMS CV dimension as the difference between the last and the first CV in which a particular m/z value appeared and computed the peak capacity as described above. Most of the detected peaks appeared at only one CV. Ions detected at only one CV were assigned a peak width of 2 V, because our step size in the CV dimension was 2 V. The value of peak capacity in the FAIMS dimension for this global assessment was 8.9, averaged over all detected ions in the entire experiment. This value is a rough estimate but it is similar to the value given by the Gaussian fits above. Because of our relatively low sampling frequency in the CV dimension, values for peak widths likely lie somewhere between our first-and-last detected CV and the actual baseline-to-baseline values. However, because our simple sampling approach results in a peak capacity measure similar to that obtained by fitting a Gaussian to representative data we believe this is a reasonable estimate. Furthermore, because most signals were detected at only a single CV, our estimate is likely conservative. Figure 4 shows a linked Venn diagram showing which signals overlap between CVs, and illustrating that most signals appear at just a single CV.

FAIMS separates ions by differential ion mobility in the presence of a carrier gas and not mass-to-charge in a vacuum as with a mass spectrometer. While FAIMS separates ions using a different mechanism than mass spectrometry, the separation is still not entirely orthogonal. Thus, while there is some correlation between differential mobility and m/z, we can still estimate the maximum peak capacity of the entire experiment by multiplying the peak capacity of FAIMS by the peak capacity of our mass spectrometer. For these experiments, our full scan mass spectra ranged in m/z from 400 to 1400. Typical peak widths for this instrument (linear ion trap mass spectrometer) are 0.75–1 Da. Therefore a conservative estimate for the peak capacity of the mass spectrometer is 1000, and thus the maximum peak capacity for just the FAIMS-MS dimensions is 8.9 × 1,000 = 8,900. Additionally, our average chromatography peak width in these experiments was 0.47 minutes; therefore, acquiring data while running a 90-minute gradient leads to a peak capacity for our liquid chromatography of 90 / 0.47 = 191.5. Thus, an estimate of peak capacity for our entire experiment is 191.5 × 8.9 × 1,000 = 1.7 × 10^6. This peak capacity is comparable to that reported previously for MudPIT[5]. The advantage with FAIMS is that these data were all acquired on a single 90-minute reversed-phase gradient and not over 24 hours of mass spectrometer time as with MudPIT. Thus, nanoLC-FAIMS-MS offers an approximately 12 fold increase in throughput over MudPIT for handling a mixture of similar complexity.

Signal intensity and dynamic range

Because ions in the current configuration are spread throughout the analytical region between the electrodes of the device with no confinement in the lateral dimension, absolute maximum signal intensity decreases. We find that the absolute signal intensity of the most abundant peptides decreases by at least an order of magnitude over that obtainable without the FAIMS device on the instrument. However, compensating for this decrease in the absolute signal intensity is a reduction in the noise level of FAIMS measurements by roughly three orders of

magnitude over that possible without the FAIMS device on the instrument. This reduction in background leads to an increase in dynamic range of FAIMS measurements over non-FAIMS measurements by >5-fold.

The effect of FAIMS on dynamic range was evaluated by performing several experiments without the FAIMS device connected to the instrument, and then performing identical experiments but with the FAIMS device connected to the instrument, as described in the Methods section. Using the software described in the methods, we evaluated the dynamic range of our measurements by looking only at peaks above a signal-to-noise threshold of 3 and persisting in time for >0.2 minutes and <3 minutes, i.e. peaks with characteristics typical of actual peptide peaks. The dynamic range was computed by dividing the intensity of the maximum detected peak by the intensity of the minimum detected peak. Using these methods, measurements were performed with and without the FAIMS interface using the same mass spectrometer, the same sample, and the same chromatography conditions. The non-FAIMS analyses had a mean dynamic range of 2,372 over five replicate analyses. Analyses with FAIMS had a mean dynamic range of 12,907 over five replicate analyses. Thus, analyses with FAIMS increased the dynamic range by >5 fold. This improvement in dynamic range is shown in Figure 5 and is a result of the drop in background noise level in the measurements using FAIMS. In the non-FAIMS measurements (Figure 5A), the most frequently measured intensities lie in the 10^4-10^5 counts range, roughly halfway between the minimum at 10^3 counts and the maximum at ~10^6 counts. However, in the FAIMS experiments the absolute intensity of the measured intensities is less than that measured without the FAIMS, but the range over which the signals is detected is greater (Figure 5B). Some of the reduction in absolute intensity is not unexpected and will be unavoidable. By adding an extra dimension of separation between the chromatography column and the mass spectrometer, peptide signals will be spread over a range of CV values. This dispersion of the signal in the CV dimension will reduce the absolute signal measured at any single CV compared to the same measurement performed without this extra dimension of separation. Interestingly, when signal-to-noise, instead of absolute counts, is plotted versus frequency, the two profiles look much more similar (Figures 5C and 5D).

Separation of charge states

The FAIMS interface is extremely powerful in selecting ions based on charge-state. FAIMS was used to separate ions that passed through the device at selected CVs and these ions were selected for tandem mass spectrometry using data-dependent acquisition. The resulting spectra were searched at multiple charge-states using SEQUEST. The fraction of peptide spectrum matches at each charge state were plotted as a function of the CV in which they were observed (Figure 6). The greatest number of 1+ spectra were sampled at a CV of −7 V and decreased at more negative CVs. Likewise, <10% of the 2+ spectra were detected at -7 V while the maximum number of 2+ ions were transmitted into the mass spectrometer between −9 and −11 V. There was a reduction in 2+ ions of ~75% between CVs of −11 and −13 V. Additionally, 3+ peptide ions were most frequently observed at −13 V. Because 2+ and 3+ peptide ions tend to produce the highest quality MS/MS spectra using collision induced dissociation (CID), FAIMS could be used to selectively direct the ion accumulation and MS/MS spectral acquisition on peptides transmitted between −9 and −13 V with minimal loss of peptide identifications. Likewise, FAIMS could be used to direct exclusively high charge state ions into the mass spectrometer using an activation technique like electron transfer dissociation[34;35] which is more efficient for 3+ and higher charge states[36;37].

Direct infusion

In a separate experiment, a yeast peptide digest was infused directly into the FAIMS-MS instrument without chromatographic separation. In these experiments, we infused the digest continuously while increasing the CV in steps of 0.5 V, acquiring 10 microscans at each CV

before moving on to the next. We scanned over a range of 0 to −30 V. The data, shown in Figure 7, took less than 2 minutes to acquire and show that FAIMS provides a modest degree of separation without any liquid chromatography. Using an estimated peak width of 2 V, we computed a peak capacity of ~15, using Equation (1) above. Several groups have investigated similar gas-phase high-throughput experiments, but usually on homebuilt instruments incorporating multiple dimensions of liquid chromatography and ion mobility separation[38–42] and not with commercially available instrumentation. This experiment was performed on a benchtop ion-trap instrument, with the only separation being provided by the FAIMS electrodes added onto the front of the instrument. While this sample mixture is undoubtedly too complex to analyze comprehensively in this way, this does point the way towards highly sensitive, high-throughput analyses of less complex but still biologically relevant samples such as protein complexes.

Discussion

Proteomics methodology is faced with the measurement of a very large number of molecular species with abundances expressed over a very large dynamic range. To avoid sampling and detecting only the most abundant components within a mixture, separation techniques are routinely used on both the protein and the peptide level to simplify the mixture and to increase the dynamic range over which different molecular species can be detected. This fractionation facilitates the accumulation and detection of low abundance ions in ion trap mass spectrometers without interference from high abundance species. However, most of these strategies extend the peak capacity and dynamic range of LC-MS measurements at the expense of overall analysis time. This increase in per sample analysis time often results in minimal, if any, biological replicates. Thus, to handle the throughput required for biological replicates to compute appropriate measures of variance, we need to extend this dynamic range and peak capacity without extending the overall analysis time.

A particularly elegant approach to improve the peak capacity is to incorporate a very rapid gas phase separation between the microcapillary liquid chromatography separation and the mass spectrometer. Ion mobility spectrometry (IMS) has been used extensively for this purpose [38; 39;42–47]. In IMS, ions are separated by their mobility in the presence of a carrier gas at a low electric field. This technique has been successfully coupled to time-of-flight mass spectrometers. Because the flight times of ions in a time-of-flight mass spectrometer are significantly shorter than the residence time of ions in the drift tube of the IMS, mass spectra can be acquired for all drift times without loss of information. However, because the scan speed of ion trapping mass spectrometers is slower than the IMS separation, only a portion of the drift region will be sampled by the mass spectrometer[48]. Additionally, because the IMS drift tube separates ions in packets and the ions are not continuously emitted, an ion trap mass spectrometer cannot accumulate ions to fill the trap.

In contrast to IMS, FAIMS acts as a filter allowing only ions of a single differential mobility to pass through the filter under any set of conditions. Because FAIMS filters ions according to differential mobility in a continuous fashion, ions can be accumulated in the ion trap using automatic gain control to maximize the signal-to-noise ratio of low abundance ions in the absence of most chemical interferences. Additionally, the FAIMS CV can be scanned, and as long as the scan rate is slower than the mass spectrometer scan rate, the complete spectrum of differential ion mobility and m/z can be measured in a single analysis. In cases where the FAIMS CV cannot be scanned on a chromatographic timescale, the CV can be stepped rapidly as described here, allowing multiple selected differential ion mobilities to be measured during the chromatographic separation in real-time.

Reduction of Chemical Background Increases the Detection of Low Abundance Signals

Using FAIMS as a separation technology for proteomics experiments is promising because the filtering action of FAIMS separates ions based on their differential mobilities in a high and low electric field prior to the separation by m/z within an ion trap mass spectrometer. This separation facilitates the discrimination of singly charged solvent ions from multiply charged peptides while also aiding in the accumulation of low intensity molecular species in an ion trap by minimizing the interference from high intensity ions and chemical noise. It is important to note that the signal intensity on an ion trap mass spectrometer is a function of both the number of ions and the ion accumulation time (known simply as the ion time). Thus, a low intensity signal does not necessarily mean that there are a small number of ions in the trap. In fact we have been able to detect peptides from as little as 10 counts and in some cases acquire high quality product ion spectra at these levels. The only downside of minimizing the chemical background and filling the trap with lower abundance species is that the ion time increases significantly – noticeably slowing the overall scan speed.

A Modified Paradigm for Shotgun Proteomics

Traditionally in shotgun proteomics, methods have been developed that focus on acquiring MS/MS spectra on as many molecular species as possible without any care for whether those peptides are of interest or not. This semi-random sampling of peptides by data-dependent acquisition focuses on the most abundant peptides first and then samples lower intensity peptides in subsequent scan events. While improvements in mass spectrometer scan speeds have improved the depth to which peptides can be sampled, this approach will still spend a majority of the instrument time sampling the most abundant molecular species in the sample. Furthermore, because the sampling of peptides is a semi-random process, the ability to detect the same peptides between analyses is complicated and unreliable. However, useful biological information is more likely to originate from differential protein abundance between two samples that differ by some perturbation or condition. In this case, samples are first analyzed for peak abundances that change in a statistically meaningful way and then analyzed again to assign identities to the changing peaks. This modified experiment is similar to decades-old proteomics experiments performed using 2-dimensional gel electrophoresis. Gels were run first to identify the changing spots and then efforts were taken to determine the identities of those changing proteins. In this modified experiment, speed is less important than reliable identification of changing quantities, especially the low-abundance peptides that might have might never have been sampled in traditional data-dependent experiments.

An inherent disadvantage of FAIMS is that more time is needed to accumulate ions to fill the trap. This time reduces the number of peptides that can be sampled on a chromatographic timescale and makes LC-FAIMS-MS an unlikely tool for profiling the contents of a single protein sample by data-dependent acquisition. However, FAIMS coupled with an ion trap mass spectrometer is an ideal tool for a modified experimental paradigm in which proteomics experiments are optimized for throughput, dynamic range, and peak capacity to identify low-abundance molecular species with a differential abundance between samples using technical and biological replicates.

Nevertheless, there is still room for improvement. In the current electrode configuration the dynamic range of the combined FAIMS-MS measurement is *increased* even though the absolute signal intensity decreases. Thus, the dynamic range can be increased even further by improving the transmission of ions from the FAIMS into the mass spectrometer. A simple explanation for the loss of ions through the FAIMS is the lack of lateral confinement of ions in the current electrode configuration. The current electrode configuration will create a ribbon-like ion beam similar to what has been reported using parallel plate electrodes[49]. This ribbon shaped beam is problematic because the entrance into the mass spectrometer is a small circular

orifice provided by the LTQ's heated ion transfer tube. Losses can be minimized using this electrode configuration using a slit-shaped entrance into an intermediate vacuum region and an ion funnel to refocus the beam from a ribbon shape to a circle[49]. Additionally, this problem can also be minimized using a more traditional "finger" electrode design[50–52] that has a hemispherical end on the inner and outer electrodes to focus ions into the mass spectrometer inlet.

Increase in Peak Capacity of Peptide Separation without Loss in Analysis Time

Data dependent acquisition, even if implemented on a mass spectrometer twice as fast as current state of the art, will have difficulty sampling all the proteins in an unfractionated mixture. There are simply too many components in a tryptic digest of such a mixture to be able to measure them all. However, a simple and inexpensive separation technique like FAIMS can be a great aid towards increasing the numbers of molecular species that can be separated in a single analysis. The use of FAIMS results in a 2.5-fold increase in the number of detected peptide-like signals. Moreover, the peak capacity is increased without a corresponding increase in time to make the measurement.

Conclusions

We have evaluated the use of high-field asymmetric waveform ion mobility spectrometry (FAIMS) in proteomics experiments to provide an extra dimension of gas phase peptide separation between the nanoflow liquid chromatography separation and an ion trap mass spectrometer. All our experiments were conducted on the same aliquot of the soluble fraction of a whole-cell yeast lysate (*S. cerevisiae*). Our principle findings are that FAIMS increases the peak capacity by 8–9 for peptides as well as increasing the dynamic range over non-FAIMS by >5-fold. While we find that while using FAIMS the absolute signal intensity decreases, FAIMS eliminates most background noise to such an extent that the dynamic range improves because of the increased detection of low intensity ions.

There is certainly more that can be done to improve this system. However, the ability to increase peak capacity and dynamic range of ion trap mass spectrometer measurements simply by fitting this relatively simple differential mobility device to the atmospheric pressure inlet of a mass spectrometer is certainly of value. Future work will require improving the FAIMS interface to better accommodate nanoflow chromatography and improving transmission of ions through the FAIMS device into the mass spectrometer.

Acknowledgments

The prototype FAIMS interface used in these experiments was provided by ThermoFisher Scientific. We thank Michael Belford, Jean-Jacques Dunyach, Julie Horner, and Julian Philips of ThermoFisher Scientific for their assistance with FAIMS and helpful discussions. The authors would also like to thank Gary Valaskovic of New Objective for helpful discussions and the loan of the nanospray probe for use in the FAIMS interface. We gratefully acknowledge Keqi Tang of Pacific Northwest National Laboratory for helpful discussions. Lastly, we would like to thank the entire MacCoss laboratory for helpful discussions and friendly debates. Support for this work was provided in part by National Institutes of Health grants P41 RR011823 and R01 DK069386 and an unrestricted Basic Research Grant from Merck Research Laboratories.

Reference List

1. Mayya V, Rezaul K, Cong YS, Han D. Mol. Cell Proteomics 2005;4:214–223. [PubMed: 15608339]
2. Blackler AR, Klammer AA, MacCoss MJ, Wu CC. Anal. Chem 2006;78:1337–1344. [PubMed: 16478131]
3. Link AJ, Eng JK, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR III. Nat. Biotechnol 1999;17:676–682. [PubMed: 10404161]
4. Washburn MP, Wolters D, Yates JR III. Nat. Biotechnol 2001;19:242–247. [PubMed: 11231557]

5. Wolters DA, Washburn MP, Yates JR III. Anal. Chem 2001;73:5683–5690. [PubMed: 11774908]
6. Peng J, Elias JE, Thoreen CC, Licklider LJ, Gygi SP. J. Proteome. Res 2003;2:43–50. [PubMed: 12643542]
7. MacCoss MJ, McDonald WH, Saraf A, Sadygov R, Clark JM, Tasto JJ, Gould KL, Wolters D, Washburn M, Weiss A, Clark JI, Yates JR III. Proc Natl Acad Sci U S A 2002;99:7900–7905. [PubMed: 12060738]
8. Wu CC, MacCoss MJ, Howell KE, Yates JR. Nat. Biotechnol 2003;21:532–538. [PubMed: 12692561]
9. Syka JE, Marto JA, Bai DL, Horning S, Senko MW, Schwartz JC, Ueberheide B, Garcia B, Busby S, Muratore T, Shabanowitz J, Hunt DF. J. Proteome. Res 2004;3:621–626. [PubMed: 15253445]
10. Hardman M, Makarov AA. Anal. Chem 2003;75:1699–1705. [PubMed: 12705605]
11. Wiener MC, Sachs JR, Deyanova EG, Yates NA. Anal. Chem 2004;76:6085–6096. [PubMed: 15481957]
12. Meng F, Wiener MC, Sachs JR, Burns C, Verma P, Paweletz CP, Mazur MT, Deyanova EG, Yates NA, Hendrickson RC. J. Am. Soc. Mass Spectrom 2007;18:226–233. [PubMed: 17070068]
13. Yates NA, Deyanova EG, Geissler W, Wiener MC, Sachs JR, Wong KK, Thornberry NA, Roy RS, Settlage RE, Hendrickson RC. International Journal of Mass Spectrometry 2007;259:174–183.
14. Finney GL, Blackler AR, Hoopmann MR, Canterbury JD, Wu CC, MacCoss MJ. Anal. Chem. 2008
15. Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR, Norton S, Kumar P, Anderle M, Becker CH. Anal. Chem 2003;75:4818–4826. [PubMed: 14674459]
16. Conrads TP, Anderson GA, Veenstra TD, Pasa-Tolic L, Smith RD. Anal. Chem 2000;72:3349–3354. [PubMed: 10939410]
17. Romine MF, Elias DA, Monroe ME, Auberry K, Fang R, Fredrickson JK, Anderson GA, Smith RD, Lipton MS. OMICS 2004;8:239–254. [PubMed: 15669716]
18. May D, Fitzgibbon M, Liu Y, Holzman T, Eng J, Kemp CJ, Whiteaker J, Paulovich A, McIntosh M. J. Proteome. Res 2007;6:2685–2694. [PubMed: 17559252]
19. Buryakov IA, Krylov EV, Nazarov EG, Rasulev U, Kh. Int. J. Mass Spectrom. Ion Proc 1993;128:143–148.
20. Guevremont R, Barnett DA, Purves RW, Vandermey J. Anal. Chem 2000;72:4577–4584. [PubMed: 11028613]
21. Barnett DA, Ells B, Guevremont R, Purves RW. J. Am. Soc. Mass Spectrom 2002;13:1282–1291. [PubMed: 12443018]
22. Barnett DA, Ding L, Ells B, Purves RW, Guevremont R. Rapid Commun. Mass Spectrom 2002;16:676–680. [PubMed: 11921246]
23. Venne K, Bonneil E, Eng K, Thibault P. Anal. Chem 2005;77:2176–2186. [PubMed: 15801752]
24. Klammer AA, MacCoss MJ. J. Proteome. Res 2006;5:695–700. [PubMed: 16512685]
25. Krylov EV. Instruments and Experimental Techniques 1997;40:628–631.
26. Potvin, L.; Baribeau, Y. Waveform generator electronics based on tuned LC circuits. Ionalytics Corporation. 10/529,309[7078678]. 7-18-2006. Ottawa, ON (CA)
27. McDonald WH, Tabb DL, Sadygov RG, MacCoss MJ, Venable J, Graumann J, Johnson JR, Cociorva D, Yates JR III. Rapid Commun.Mass Spectrom 2004;18:2162–2168. [PubMed: 15317041]
28. Horn DM, Zubarev RA, McLafferty FW. J. Am. Soc. Mass Spectrom 2000;11:320–332. [PubMed: 10757168]
29. Hoopmann MR, Finney GL, MacCoss MJ. Anal. Chem. 2007
30. Eng JK, McCormack AL, Yates JR III. J. Am. Soc. Mass Spectrom 1994;5:976–989.
31. Käll L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. Nat. Methods 2007;4:923–925. [PubMed: 17952086]
32. Tabb DL, McDonald WH, Yates JR III. J. Proteome Res 2002;1:21–26. [PubMed: 12643522]
33. Giddings, JC. Unified Separation Science. John Wiley & Sons, Inc.; 1991.
34. Coon JJ, Ueberheide B, Syka JE, Dryhurst DD, Ausio J, Shabanowitz J, Hunt DF. Proc. Natl. Acad. Sci. U.S.A 2005;102:9463–9468. [PubMed: 15983376]
35. Coon JJ, Syka JE, Shabanowitz J, Hunt DF. Biotechniques 2005;38 519, 521, 523.

36. Good DM, Wirtala M, McAlister GC, Coon JJ. Mol. Cell Proteomics 2007;6:1942–1951. [PubMed: 17673454]
37. Swaney DL, McAlister GC, Wirtala M, Schwartz JC, Syka JE, Coon JJ. Anal. Chem 2007;79:477–485. [PubMed: 17222010]
38. Valentine SJ, Counterman AE, Hoaglund CS, Reilly JP, Clemmer DE. J. Am. Soc. Mass Spectrom 1998;9:1213–1216. [PubMed: 9794086]
39. Henderson SC, Valentine SJ, Counterman AE, Clemmer DE. Anal. Chem 1999;71:291–301. [PubMed: 9949724]
40. Koeniger SL, Merenbloom SI, Valentine SJ, Jarrold MF, Udseth HR, Smith RD, Clemmer DE. Anal. Chem 2006;78:4161–4174. [PubMed: 16771547]
41. Merenbloom SI, Koeniger SL, Valentine SJ, Plasencia MD, Clemmer DE. Anal. Chem 2006;78:2802–2809. [PubMed: 16615796]
42. Tang K, Li F, Shvartsburg AA, Strittmatter EF, Smith RD. Anal. Chem 2005;77:6381–6388. [PubMed: 16194103]
43. Hoaglund CS, Valentine SJ, Sporleder CR, Reilly JP, Clemmer DE. Anal. Chem 1998;70:2236–2242. [PubMed: 9624897]
44. Sowell RA, Koeniger SL, Valentine SJ, Moon MH, Clemmer DE. J. Am. Soc. Mass Spectrom 2004;15:1341–1353. [PubMed: 15337515]
45. Taraszka JA, Kurulugama R, Sowell RA, Valentine SJ, Koeniger SL, Arnold RJ, Miller DF, Kaufman TC, Clemmer DE. J. Proteome. Res 2005;4:1223–1237. [PubMed: 16083272]
46. Valentine SJ, Plasencia MD, Liu X, Krishnan M, Naylor S, Udseth HR, Smith RD, Clemmer DE. J. Proteome. Res 2006;5:2977–2984. [PubMed: 17081049]
47. Belov ME, Buschbach MA, Prior DC, Tang K, Smith RD. Anal. Chem 2007;79:2451–2462. [PubMed: 17305309]
48. Tang X, Bruce JE, Hill HH Jr. Rapid Commun. Mass Spectrom 2007;21:1115–1122. [PubMed: 17318922]
49. Shvartsburg AA, Li F, Tang K, Smith RD. Anal. Chem 2006;78:3706–3714. [PubMed: 16737227]
50. Guevremont R, Ding L, Ells B, Barnett DA, Purves RW. J. Am. Soc. Mass Spectrom 2001;12:1320–1330. [PubMed: 11766759]
51. Purves RW, Barnett DA, Ells B, Guevremont R. Rapid Commun. Mass Spectrom 2001;15:1453–1456. [PubMed: 11507759]
52. Guevremont R, Purves R. J. Am. Soc. Mass Spectrom 2005;16:349–362. [PubMed: 15734328]

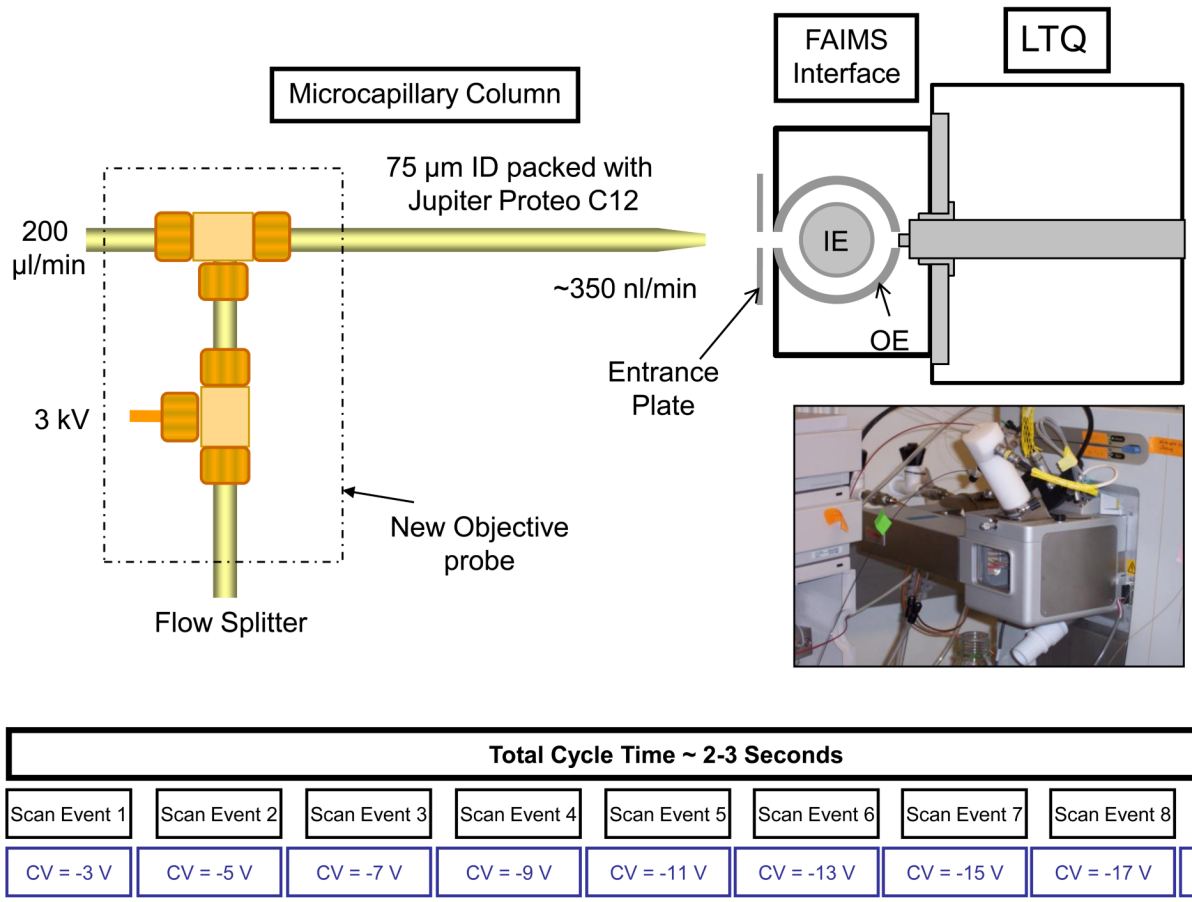

Figure 1.

A schematic of the nanoLC-FAIMS-MS apparatus. The cylindrical inner (IE) and outer (OE) electrodes are mounted perpendicular to the ion flow. Acquisition of mass spectra was synchronized with serially stepped FAIMS compensation voltages (CV). These data were acquired in a continuous cyclic fashion during the entire chromatographic separation.

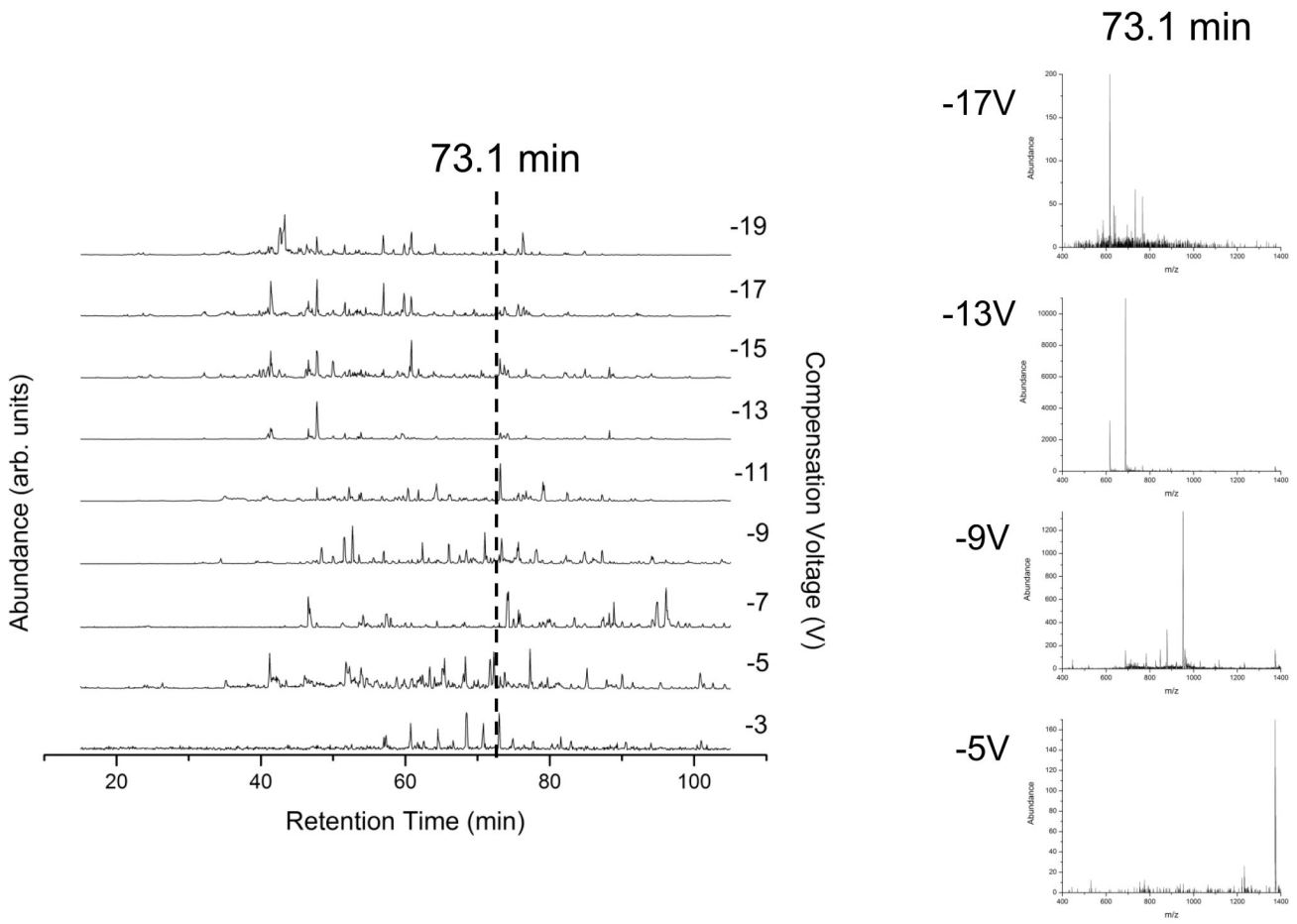

Figure 2.

Example base-peak chromatograms from a single nanoLC-FAIMS-MS experiment. The extracted compensation voltage (CV) chromatograms at nine different CV were plotted from a single 90-minute nano-LC separation. Mass spectra at selected CV at retention time *RT* ~ 73.1 minutes are plotted on the right. Generally speaking, we find that the most intense peptide signals appear at CV values between −9 and −15 V, however detectable signals are present at values of CV where signal level is very low, e.g. 5–10 counts relative intensity. FAIMS helps separate peptides of very different abundances that elute at the same time, helping to increase the measurement dynamic range.

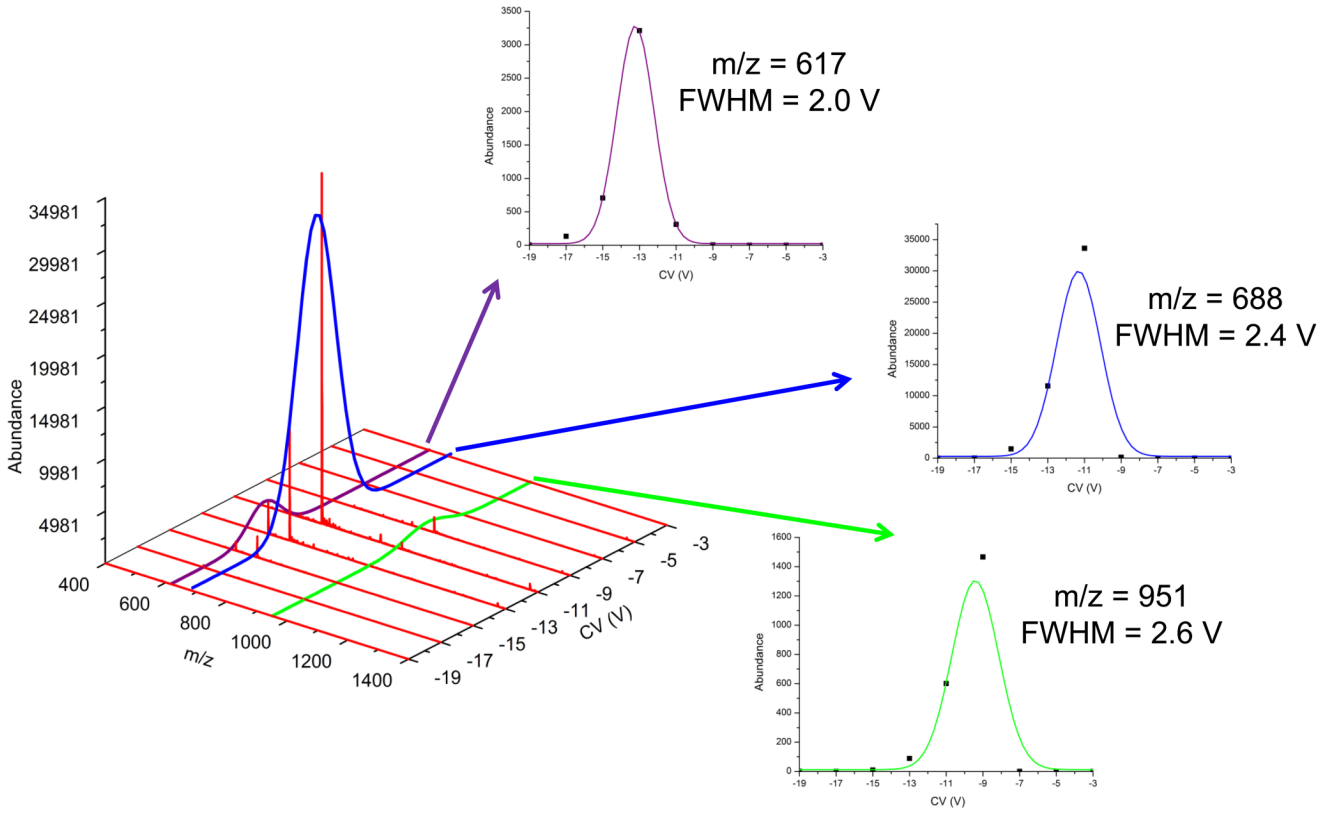

Figure 3.

Evaluation of FAIMS peak capacity using example data at a single retention time. A 3-dimensional plot of mass spectra versus CV, at retention time *RT* = 73.1 min (the same as shown in Figure 2). The purple, blue, and green lines are Gaussian fits to the peak values at m/z = 617, 688, and 951, respectively, three of the more prominent peaks in the plot. This 3-dimensional plot of the 4-dimensional data (retention time, m/z, CV, abundance) provides an estimate of the peak capacity of FAIMS-based separation. At right are shown projections of the m/z planes corresponding to each Gaussian fit, with the same purple, blue, and green lines again representing the same Gaussian fits as in the left panel. The widths (FWHM) of these Gaussians are 2.0, 2.4, and 2.6 V, respectively.

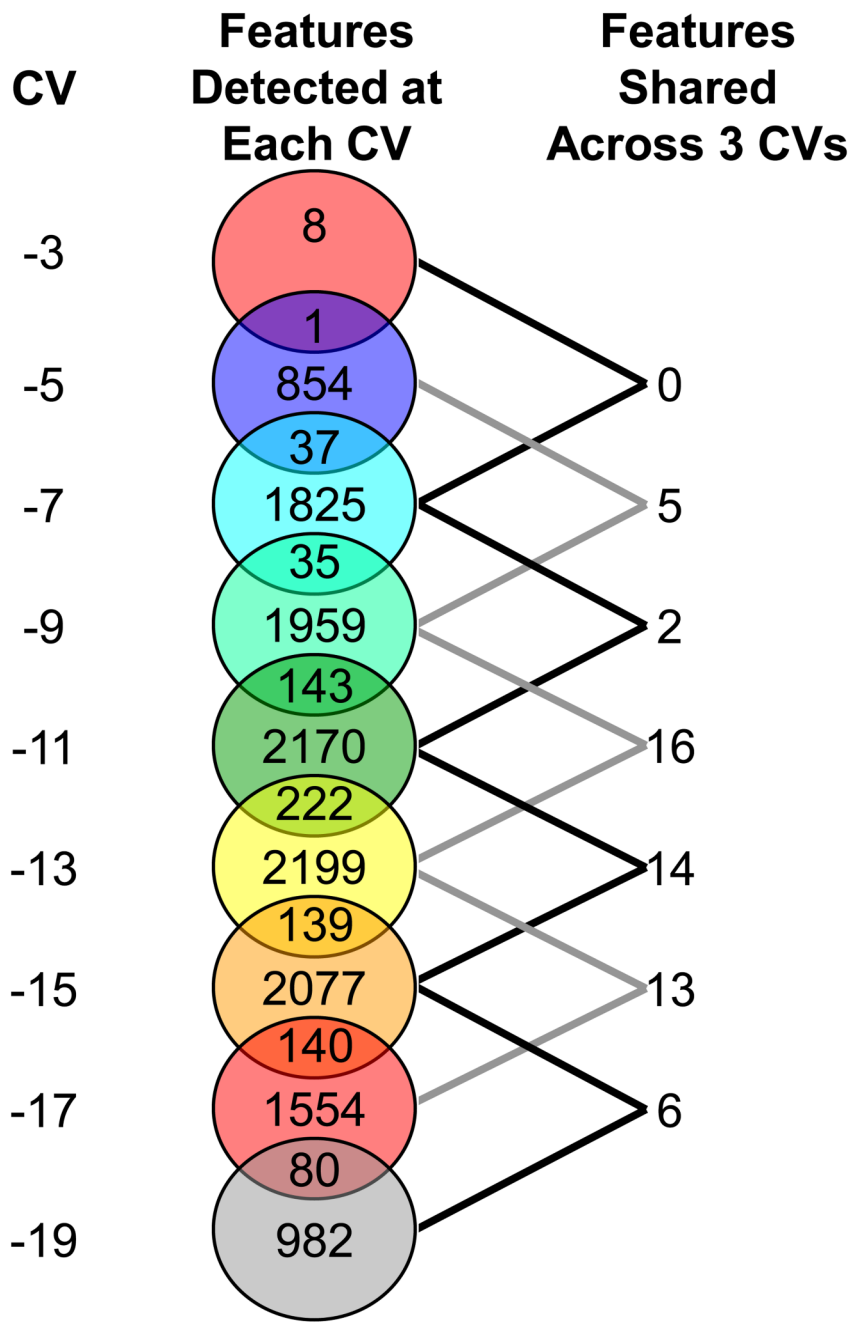

Figure 4.
Overlap of detected signals between CV chromatograms. The number of chromatographically persistent features detected at single CVs and adjacent CVs is listed in the ovals. The numbers at the right are the numbers of features common to three adjacent CVs. There were no signals common to four CVs. A vast majority of the features were only detected at a single CV.

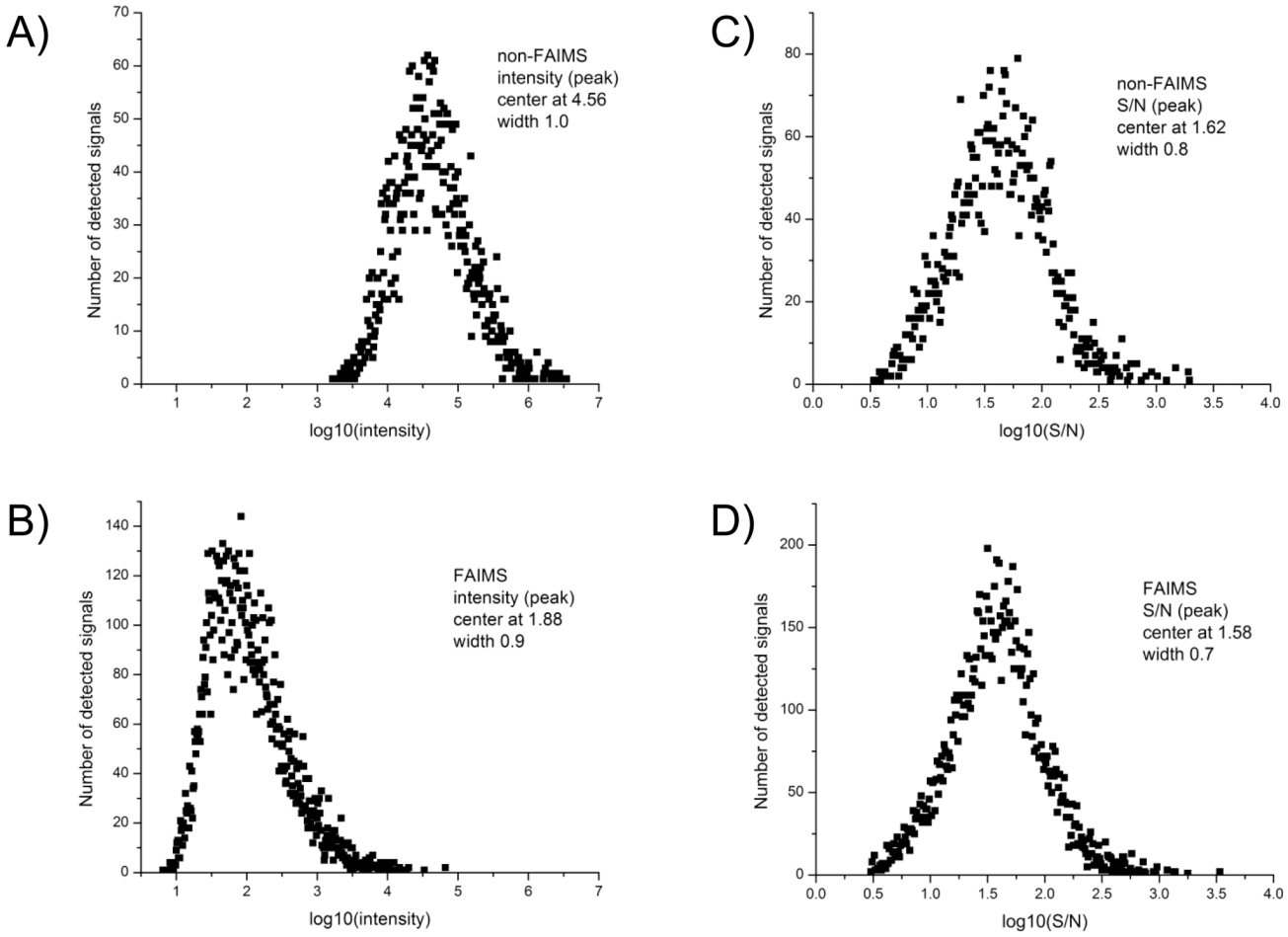

Figure 5.

Comparison of nanoLC-FAIMS-MS and nanoLC-MS dynamic range and signal-to-noise ratio, after filtering the data with our in-house software program MScout. The number of detected molecular species was plotted versus the absolute signal and signal-to-noise for both FAIMS and non-FAIMS experiments. While the absolute signal is significantly decreased in the FAIMS experiments, the full range over which signals were detected is increased by almost an order of magnitude. Additionally, the S/N distributions for FAIMS and non-FAIMS data are very similar, indicating minimal loss in useable signal. Note also the difference in the vertical scales between FAIMS and non-FAIMS experiments, indicating many more detected signals in the FAIMS experiments.

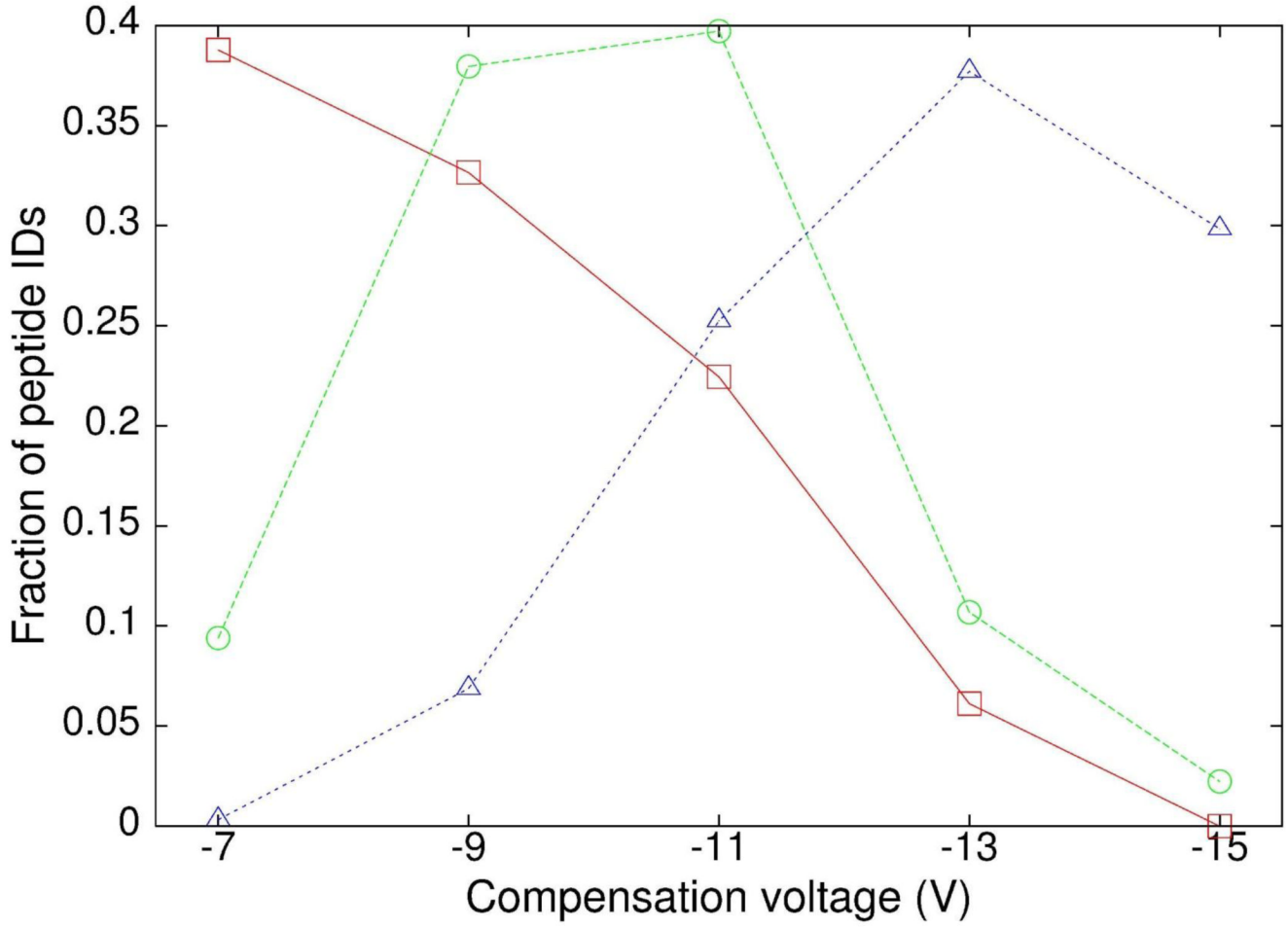

Figure 6.
Distribution of charge states in a FAIMS experiment. The 1+ peptides are shown in red (squares), the 2+ peptides are shown in green (circles), and the 3+ peptides are shown in blue (triangles). The more negative the CV, the higher the charge-state transmitted through the FAIMS electrodes.

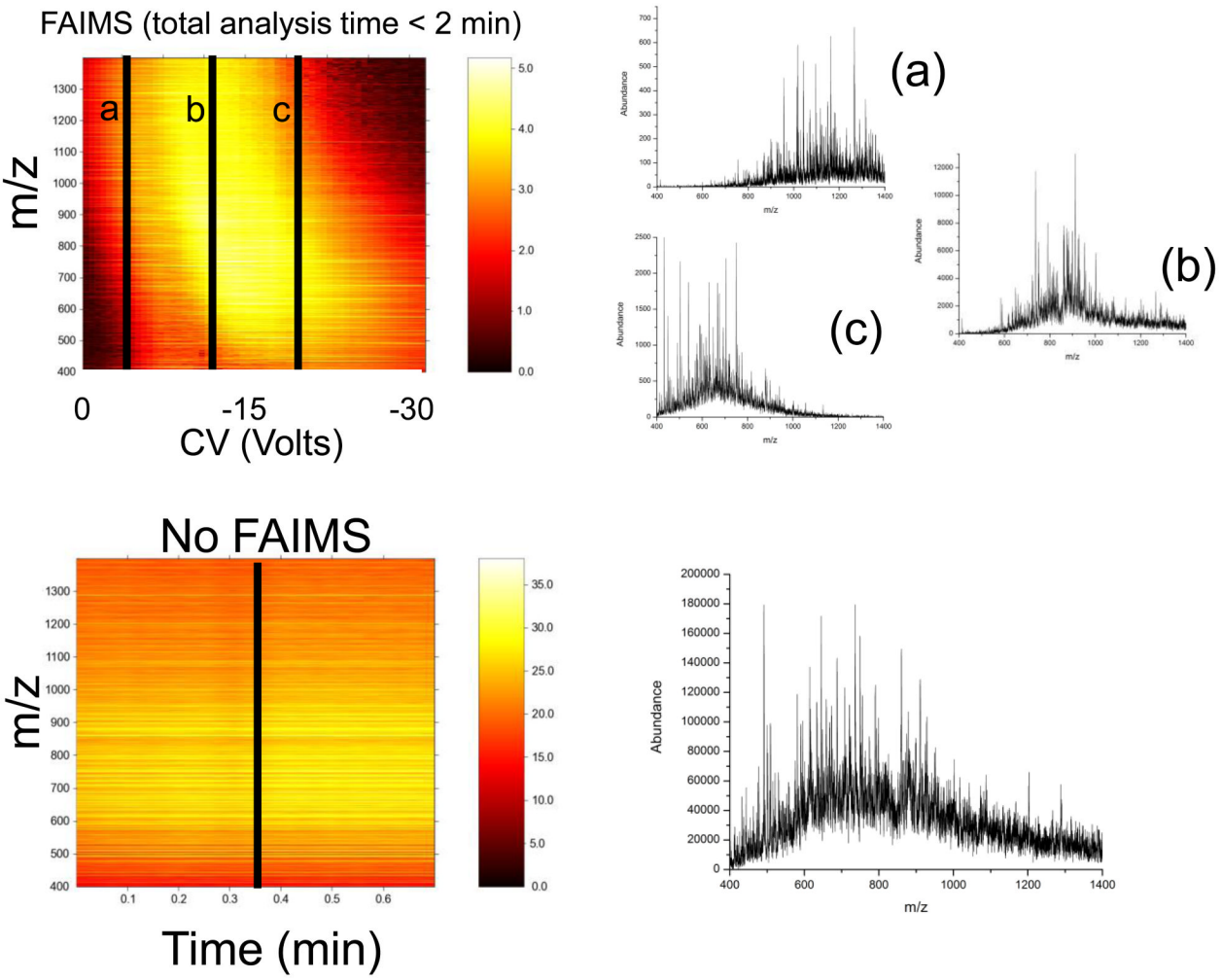

Figure 7.

FAIMS-MS without liquid chromatography. A peptide digest was infused directly into the mass spectrometer while scanning CV. The same experiment was repeated without FAIMS. Spectra corresponding to the black lines in the heat maps are shown on the right. Many more signals were detectable above background with FAIMS than without. With a total analysis time of < 2 minutes, this experiment indicates potential for FAIMS-MS as a high-throughput peptide separation technique.