Developing liquid chromatography ion mobility mass spectrometry techniques

Stephen J Valentine, Xiaoyun Liu, Manolo D Plasencia, Amy E Hilderbrand, Ruwan T Kurulugama, Stormy L Koeniger and David E Clemmer

When a packet of ions in a buffer gas is exposed to a weak electric field, the ions will separate according to differences in their mobilities through the gas. This separation forms the basis of the analytical method known as ion mobility spectroscopy and is highly efficient, in that it can be carried out in a very short time frame (micro- to milliseconds). Recently, efforts have been made to couple the approach with liquid-phase separations and mass spectrometry in order to create a high-throughput and high-coverage approach for analyzing complex mixtures. This article reviews recent work to develop this approach for proteomics analyses. The instrumentation is described briefly. Several multidimensional data sets obtained upon analyzing complex mixtures are shown in order to illustrate the approach as well as provide a view of the limitations and required future work.


Analytical challenge associated with complex mixtures of proteins

Consider 1 ml of human plasma. It is believed that such a sample could contain thousands of different proteins in measurable quantities [1]. The ability to characterize them all would produce physiologically relevant signatures and has become a significant challenge for an emerging group of biotechnologists who aim to understand the molecular mechanisms of diseases [2-12]. However, the analytical issues that are associated with analyzing plasma in detail are difficult to overstate. For example, proteolytic digestion of a mixture of 10^4 proteins in which an average of 50 fragments for each protein were produced would lead to a mixture of approximately 5 x 10^5 peptides. An ideal separation of this mixture, in which peptides line up and exit the chromatographic column at a rate of one per second (near the maximum rate for mass spectrometry [MS]-based detection and identification approaches, i.e., MS and tandem MS [MS/MS]) would require nearly 1 month of instrument time. Even more daunting is that protein concentrations in plasma differ by more than ten orders of magnitude (from ~10 mg/ml to <1 pg/ml) and some are temporally dependent, varying with diet, age, state of health and so on [1]. With this in mind, it is perhaps not surprising that the number of novel proteins that can be used to characterize physiologic state (i.e., biomarkers) remains relatively low [13,14].

Overview of this review

In this paper, recent work aimed at developing a high-throughput and high-coverage approach for analyzing complex mixtures such as plasma is reviewed. The approach is based on a combination of liquid chromatography (LC) with ion mobility spectrometry (IMS) [15] and MS. The mobility of an ion through a buffer gas depends upon the ion’s charge and collision cross-section with the buffer gas. Since gas densities are much lower than the density of the condensed phase, the separation can be carried out on very short time...
scales (usually in the order of milliseconds). Thus, it is possible to include the mobility separation within a LC and MS analysis to create a 3D-LC-IMS-MS experiment. Here, the following topics are covered:

• The background of IMS separations and the basics of LC-IMS-MS instrumentation
• Example data sets for several selected complex systems, including human plasma
• A discussion of current limitations and future improvements

At this point, LC-IMS-MS is an emerging technology, and although it appears that it offers significant advantages compared with LC/MS alone, much work is required before it will be used routinely—perhaps not unlike the state of high-resolution Fourier transform MS and high-throughput LC/MS/MS approaches a decade ago.

**Early developments associated with multidimensional techniques that incorporate IMS**

Early in its development, IMS was coupled to various separation strategies including gas chromatography (GC), supercritical fluid chromatography (SFC) and LC [16]. Although such work utilized two separation steps, it did not make full use of both dimensions of separation because limitations in electronics required that at least one dimension of the system be operated in a scanning rather than dispersive mode [17]. In the late 1990s, advances in electronics and data acquisition systems enabled the development of the first multiply dispersive method, IMS coupled with time-of-flight (TOF)-MS [18]. Shortly thereafter, IMS-MS measurements were coupled in the same fashion with condensed-phase separations to provide three dimensions of component dispersion [19]. Since this experiment, the multiply dispersive approaches (LC-IMS and LC-IMS-MS) have been used to analyze mixtures of small molecules, tryptic peptides, combinatorial libraries and proteome digests [19–24].

Multidimensional LC-IMS-MS data sets are acquired by nesting rapid analyses within those requiring longer times [18,19]. That is, if the time scales of the multiple separations are substantially different (minutes, milliseconds and microseconds), then each separation can be carried out within individual time windows of the preceding step. For example, within a 20 ms window used to record the IMS separation, one could acquire hundreds to thousands of flight time distributions constituting the entire mass spectrum of the mobility-dispersed ions. Such an approach results in a 3D dispersion of each mixture component such as a peptide from a proteomic digest sample. The hypothetical plots shown in Figure 1 illustrate a particularly useful configuration. If ions are transmitted through the drift tube gently (Figure 1A), then the dispersion results in the indicated positions of the precursor ions. On the other hand, if ions are activated between the IMS and MS separation steps (Figure 1B), then a fragmentation spectrum can be recorded. This approach has been used to generate MS and MS/MS information for ions in parallel [25].

Data generated by LC-IMS-MS measurements are described using a standard nomenclature developed recently [18]. Reported time values for each dispersive measurement are bracketed in their nesting order [18,19]. For example, experimental time values (Figure 1) for data obtained from a LC-IMS-TOF analysis would be listed as $t_R(t_F(t_p))$, where $t_R$, $t_F$, and $t_p$ correspond to the retention time, drift time and flight time of each species, respectively. Often, the ions’ $t_F$ is converted to a mass-to-charge ratio (m/z) using a standard, multipoint calibration. For experiments that utilize two dimensions of LC (e.g., those that couple strong cation exchange [SCX] with LC), the first-dimension elution time (e.g., $t_{SCX}$) or fraction number can also be included in the nomenclature. Thus, a 2D-LC-IMS-MS measurement may report values for data set features as $t_{SCX}(t_R(t_F(t_p)))$ [26].

**Fundamentals & instrumentation**

**Mobility measurements**

Several excellent reviews discuss the fundamentals associated with mobility measurements in detail [27–31]; only a brief description of IMS as it pertains to a multiply dispersive approach is provided here. The time required for an ion to traverse a region filled with an inert buffer gas under
the influence of a weak electric field is related to the mobility \( (K) \) of the ion [32]. Under low-field conditions, the mobility of an ion through the buffer gas is given as \( K = \frac{v_d E}{T} \) (\( v_d \) is the drift velocity of the ion and \( E \) is the electric field) [32]. Often, to permit comparison between different measurements, ion mobilities are reported as reduced mobilities \( (K_0) \) calculated from [32]:

\[
K_0 = \frac{I^2}{t_d} \cdot \frac{1}{L} \times \frac{273.2}{T} \times \frac{P}{760}
\]

Here \( t_d \), \( I \), \( L \), \( P \) and \( T \) correspond to the measured drift time, length of the drift region, the applied drift voltage, and the pressure and temperature of the buffer gas, respectively. IMS measurements are extremely reproducible and any two measurements from the same instrument typically agree to within approximately 1–2%. Ions that adopt compact conformations have higher mobilities than those that exist as extended conformers [28,29]. For ions with similar collision cross-sections, those that exist as higher charge states have higher mobilities as they are influenced by a greater drift force [28,29,33,34].

**LC-IMS-MS instrumentation**

A schematic diagram of one existing LC-IMS-MS instrument constructed to analyze complex mixtures is shown in FIGURE 2. Here, mixture components are separated with LC on a reversed phase \( C_{18} \) column (75 \( \mu \)m x 15 cm). The column has a pulled tip such that eluting species are electrosprayed into the desolvation region of an electrospray ionization (ESI) source. Ions are extracted from the high-pressure source into a high-vacuum region and are focused into an octopole linear ion trap (3D traps [35]) and an electrodynamic ion funnel [36], similar to that developed by Smith and coworkers [37,38] who have also been used). In the trap, ions are accumulated, stored and are periodically pulsed into the drift tube. The drift tube employs a split-field design that utilizes two field regions and is filled with approximately 2.00 Torr He buffer gas [39]. The first field region is approximately 20 cm long and contains equally spaced electrostatic lenses to generate a uniform field (~5 V cm\(^{-1}\)). Ions are separated in this region according to their mobilities (see above). The second field region of the drift tube is approximately 1.2 cm long and is operated under conditions that favor transmission of precursor ions (low-field conditions) or those that favor precursor fragmentation (high-field conditions). During the course of a LC experiment, this field can be modulated between conditions that favor transmission and those that induce fragmentation of mobility-dispersed precursor ions [40]. All ions exiting the drift tube are then focused into the source region of a TOF mass spectrometer for mass analysis.

**1D-LC-IMS-MS measurements**

**Urinary proteome**

One of the first proteomics experiments to be performed using a single dimension (1D) of LC coupled with IMS-MS techniques was the study of a tryptic digest of the human urinary proteome [22]. For these experiments, an instrument that incorporated an octopole collision cell after the drift separation and before the mass analysis was used. Here, two separate LC runs were recorded. The first and second runs utilized collision cell voltages that favored transmission and fragmentation of precursor ions, respectively. Upon completion of the experimental runs, peaks from the fragmentation data set were linked to features from the parent ion data set using \( t_R \) and \( t_D \) delimiters. This MS/MS information was then used to search a protein database to obtain peptide identifications for features observed in the precursor multidimensional data set.

FIGURE 3 shows a 2D \( t_R(t_D) \) plot of the human urinary proteome digest. The data set demonstrates the utility of the gas-phase mobility dispersion; many features that are not resolved along the LC dimension are resolved along the mobility
dimension. Also shown is the base peak ion chromatogram. It is estimated that a 2D peak capacity of approximately 6000–11,000 was achieved for this experiment. FIGURE 4 shows a 2D $t_D$/$t_D$ plot from the urinary proteome analysis obtained using conditions that favored precursor fragmentation after the mobility separation. As aforementioned, related fragments and their precursors have the same drift times, thereby allowing their grouping into MS/MS data sets [25]. Also shown in FIGURE 4 are mass spectra obtained by integrating all bins within three narrow drift time ranges. Identiﬁcations for tryptic peptides corresponding to the proteins xanthine dehydrogenase/oxidase, urmodulin and serum albumin were obtained. A preliminary analysis of the most intense features in the data set yielded 27 peptide assignments corresponding to 13 proteins (FIGURE 3). Although the preliminary work yielded few assignments (largely a result of the extent infor-matics tools), it was one of the ﬁrst demonstrations of a proteomics analysis using LC-IMS-MS techniques.

**Drosophila melanogaster proteome**

Other experiments utilizing a single LC separation step coupled with IMS-MS techniques involved studies of the model organism *Drosophila melanogaster*. The ﬁrst of these compared results obtained from a proteomics analysis of the heads of three different organisms [26]. In all, more than $2.5 \times 10^4$ features corresponding to protonated peptides were observed. Peak intensities were compared for features identiﬁed in all three samples to determine differences in individual protein expression. No statistically signiﬁcant differences for the most intense features were observed, suggesting that low-abundance features (not identiﬁed) may to a large extent contribute to individual characteristics.

Separate, ongoing experiments involving *D. melanogaster* include the characterization of the proteome as a function of embryogenesis (12 time points) [39]. These experiments were performed on an IMS-MS instrument that has been slightly modiﬁed from the version shown in FIGURE 2. To improve ion storage and ESI source transmission capabilities, an electrodynamic ion funnel was incorporated at the front of the drift tube, replacing the linear trap [36,38]. In this instrumental set-up, ions accumulate at the back of the ion funnel and are periodically pulsed into the drift tube to initiate mobility measurements. Overall signal levels are increased by factors of 10–20 using the new instrumentation.

FIGURE 5 shows 2D $t_D(t_D)$ plots of the data collected for an embryo proteome digest at 0–2 h of development. The plots demonstrate the complexity of the samples. Although the drift dimension provides an increase in overall peak capacity, FIGURE 5A shows that many features are indistinguishable when plotting the entire data set, even when employing a large intensity threshold. Typically, data are plotted as 2D base peak plots where only the most abundant features at each $t_D$ and $t_D$ are represented (FIGURE 5B). For these experiments, the estimated peak capacity for the LC-IMS separation is approximately 6000. The overall number of proteins observed in the various time point samples as well as changes in protein expression as a function of embryogenesis are currently under investigation.

**2D-LC-IMS-MS measurements**

**Experimental set-up**

Due to the gas-phase, mobility separation is a post-ionization event, and it is possible to couple any number of condensed-phase separations steps with IMS-MS analyses without sacriﬁcing ion signals. Thus, beneﬁts in overall peak capacity and component resolution associated with multiple dimensions of LC can be availed by IMS-MS techniques. Several 2D-LC strategies have recently been combined with IMS-MS measurements. The ﬁrst and more developed of these involves the use of ofﬁne fractionation using SCX chromatography with reversed phase LC-IMS-MS analysis. Although not described here, it is noted that several other methods employing multi-dimensional condensed-phase separations coupled with IMS-MS are under investigation. Such experiments include the use of size-exclusion and afﬁnity chromatography techniques to increase the resolution and sensitivity of the multiply dispersive measurements. Here, recent developments in studies utilizing an SCX-LC-IMS-MS approach for proteomics analysis are described.
D. melanogaster mapping studies

The proteomes of two developmental stages of D. melanogaster (adult head and embryo) have been analyzed using 2D-LC-IMS-MS methods [43]. Experimental data were compiled into high-dimensional proteome maps containing the $t_{{SCX}}$, $t_{{P}}$, $m/z$ and $m/z$ values for each feature observed in the data sets, as well as their intensity. Additionally, peptide and protein assignments obtained from database searches were included in the maps. In total, 780 and 660 proteins were identified in the adult head and the embryo, respectively. A comparison between the two maps showed that 307 of these were in common between the two samples. Total numbers of proteins for each sample as well as their overlap as a function of gene ontology (GO) cellular component are shown in Figure 6. Differences in the numbers of proteins for specific cellular components have been discussed. For example, it was suggested that the observance of an increased number of mitochondrial proteins in the adult head is corroborated by findings from studies suggesting mitochondrial densities are higher in neurons than other types of cells [42]. Other explanations for such observations were also discussed.

Human plasma experiments

With the advances in LC-IMS-MS methods mentioned above, an ambitious undertaking to characterize the human plasma proteome using LC-IMS-MS methods is currently underway. Initial experiments have utilized a 2D-LC approach to analyze a whole-plasma digest (i.e., abundant proteins were not removed). In all, ten SCX fractions were analyzed using a short, 21-min LC gradient. Overall, 2–7 $\times$ 10^4 features were observed for each LC run and 598 unique peptides corresponding to 399 proteins have been identified from a partial analysis of the data [36].

It is instructive to compare such results with those obtained for plasma using state-of-the-art LC/MS/MS instrumentation. In head-to-head comparisons, the number of resolved and identified features from LC-IMS-MS experiments was observed to be more than ten- and twofold higher, respectively. The 2D-LC-IMS-MS analysis of plasma may also be compared with the work reported by Smith and coworkers, who recently used LC/MS/MS and 2D-LC/MS/MS techniques to identify over 1600 proteins in a plasma sample—a significant achievement [43]. For their experiments, a single SCX-LC/MS/MS analysis yielded approximately 600 protein identifications requiring a 75-h experiment (15 SCX fractions with 5-h LC runs). Due to the scanning nature of the MS instrument, multiple runs were required to obtain missed assignments. A partial analysis of the 2D-LC-IMS-MS approach described here allowed the identification of approximately 400 proteins from a 3 h 20 min experiment. Clearly, the approach holds great promise with regard to experimental throughput, which is increasingly important for profiling efforts. That is, many experiments may be required to address normal protein variabilities.

Another advantage of the 2D-LC-IMS-MS approach for proteomics analysis (particularly useful for plasma) is that features associated with low-abundance species can be observed in the presence of coeluting higher abundance species as they are often separated by the mobility dispersion. This leads to an effective increase in the experimental dynamic range. Figure 7a shows a 2D $t_{{D}}(m/z)$ plot obtained over a 1 min $t_{{P}}$ range for the analysis of a single SCX plasma fraction. To demonstrate the utility of the mobility dispersion, a mass spectrum obtained by integrating all bins for a narrow drift time range is shown in Figure 7b. Here, a feature that is buried in the noise in the total mass spectrum (obtained by integrating all bins over the entire drift range) is clearly resolved. The MS/MS data obtained for this feature provided an identification for the peptide MLVNFILR27 from the protein interleukin-26 (Figure 7c). Such assignments are indicative of the high sensitivity of the LC-IMS-MS technique, as cytokines have nominal plasma concentrations in the...
pg/ml range. Thus, it is possible to obtain assignments for peptides from proteins of vastly different plasma concentrations (a $10^8$–$10^9$ concentration range). Capabilities such as these should significantly enhance plasma profiling efforts by allowing the monitoring of very low-abundance species.

Although the results for plasma analysis are quite promising, a note of caution is necessary: probability-based scoring algorithms such as that used by MASCOT for the present studies are prone to contain false-positive assignments. Thus, it becomes necessary to visually inspect the MS/MS data for questionable assignments. Also noted is that the ability to resolve low-abundance peptides such as the one described above requires high on-column sensitivities (attomole range). Previously, attomole detection limits have been reported for ion mobility studies using direct infusion of peptide mixtures [21]. Since these early experiments, ion signals have been improved by over one order of magnitude. Thus, it is likely that the LC-IMS-MS method has the requisite sensitivity to observe very low-abundance species. In related studies on human plasma, other low-abundance proteins have been observed, including those with multiple tryptic peptide assignments [UNPUBLISHED DATA].

The data from the 2D-LC-IMS-MS analysis of plasma have also been compiled into an initial proteome map. The map contains the position delineators and intensities for all observed features as well as the peptide and protein information for identified species. The creation of the map is the first step towards the use of LC-IMS-MS data for comparative proteomics and individual profiling studies. The current status and future developments envisioned for LC-IMS-MS mapping techniques are described below.

**Current limitations**

**IMS-MS peak capacity and resolution**

Although the 2D peak capacity of LC-IMS is quite high (see above), IMS and MS measurements are correlated, leading to a smaller IMS contribution (five- to tenfold for the current instrument) to overall 3D peak capacity. While an order of magnitude improvement with no significant sacrifice to signal is quite good, peak capacity advantages associated with IMS are more apparent for the parallel collision-induced dissociation (CID) method (i.e., the fragmentation of mobility-dispersed precursors). Here, the correlation is broken as the parallel fragmentation allows the use of the entire drift range (peak capacities of $\sim 25$ for the experiments described above). Improving the peak capacity further will require improvements in IMS resolution (discussed below).

One of the problems associated with peptide identification results from the relatively low resolution of the current mobility separation. Since many features are not completely resolved in the drift dimension, MS/MS spectra may contain undesired, overlapping fragment peaks. Such features may hinder peptide assignments, especially where there is a significant difference in precursor abundance. This problem can be addressed using two different approaches. First, more of the information from the LC separation can be utilized to better correlate fragments with precursors. Such information may include peptide retention time persistence. That is, only fragments that persist in $t_d$ as long as the precursor will be used in a MS/MS query. Currently, only a match to retention and drift time is required. Similarly, peak intensities may also be used; MS/MS data sets for given precursors will not contain fragments that are in disproportionate abundance. While such methods utilize data processing algorithms, instrumental approaches also exist. For example, the resolution of the instrument can be increased by incorporating a longer drift tube. Recently, the resolving power has been increased by factors of 2–3 without significant loss in ion transmission through the drift region [UNPUBLISHED DATA]. This is accomplished using an ion funnel at the back of the drift tube similar to the method reported by Smith and coworkers [36].

**Infomatics**

Currently, the rate of data generation from a single LC-IMS-MS proteomics experiment is quite large (0.2–1 Gbyte/min). Thus, for a 21-min LC separation, a 21 Gbyte raw data file can be generated. Such a generation rate not only requires a large capacity for data storage but also sophisticated algorithms for data processing. Successful implementation of the instrumental improvements described above will exacerbate the computing hardware and software needs.

Figure 5: 2D $t_d(t_f)$ datasets for a LC-IMS-MS analysis of a Drosophila melanogaster embryo digest. The data were taken at the 0–2 h time point of embryogenesis. (A) was obtained by integrating the total ion signal for each $t_f$ and $t_d$ value. (B) is a 2D base peak plot obtained by plotting the most intense features for each $t_f$ and $t_d$ value.

IMS: Ion mobility spectrometry; LC: Liquid chromatography; MS: Mass spectrometry.
Over the last 2 years, significant progress has been achieved with regard to data analysis and should be noted here. To appreciate such progress, it is necessary to describe the data analysis process from sample collection to data set feature identification. Currently, the overall process is being carried out on a 24-node, dual central processing unit (CPU; Opteron, 2.4 GHz) computer cluster. After the collection and transfer to the cluster of a LC-IMS-MS data set, algorithms create 3D data arrays containing features observed for parent ion or CID conditions. A second set of algorithms finds the positions and intensities of features observed in the parent ion and CID data arrays. Next, fragments are linked to their precursors via their mobilities and MS/MS data sets are created for protein database searches. Finally, results from protein database searches are grouped with features in the 3D parent ion array to create initial analytical maps.

With the recent automation of the processes described above, the data analysis bottleneck has been significantly reduced. For a recent study of human plasma involving a SCX-LC-IMS-MS approach, the time required for feature identification was roughly equivalent to the experimental run time [UNPUBLISHED DATA]. Although the reduction in analysis time is quite significant (several months' work previously), it is noted that improvements are being pursued. Such improvements include parallelizing processing algorithms, increasing the fidelity of peak picking algorithms, as well as optimizing protein database searching on the cluster. A goal would be to reach the same level of throughput on the cluster for data generated simultaneously from multiple IMS-MS instruments.

The enhancement in data analysis has allowed the allocation of more resources for another critical informatics component - knowledge assembly. Aspects of this work include the construction and curating of LC-IMS-MS proteome maps (see below) and the comparison of data sets from disease samples against such maps to determine molecular markers indicative of physiologic state. The recent study of human plasma has resulted in the construction of an initial plasma proteome map (see below) [UNPUBLISHED DATA]. Preliminary informatics work involves the use of commercial software as well as code developed in-house to determine protein and peptide correlations across the multiple LC runs comprising the emerging map.
This work is also focusing on comparisons between data sets from different individuals as well as comparisons of single individuals against the growing plasma map. Such developmental work is beginning to lay the foundation for more elaborate approaches aimed at biomarker discovery.

Future directions

Instrumentation development: increasing LC-IMS-MS ion signals & IMS resolution

Although the addition of the ion funnel has dramatically improved the overall ion signal, instrumentation improvements are envisioned to increase the current signal by approximately tenfold. Instrumentation improvements will focus on the ion storage capacity as well as the ion transmission efficiency in the first drift region. The trapping efficiency (currently ~10%) may be increased by incorporating the novel ‘hour-glass’ ion funnel design described by Smith and coworkers [36]. Such a device affords greater ion storage capacity than that utilized for the studies described here. Increased ion transmission efficiency will be achieved using field-focusing strategies, as described previously [40,44-46].

Other instrumentation development efforts will improve the resolving power of the mobility separation. The resolving power is given approximately as:

\[
\frac{I}{\Delta m} \left( \frac{LEze}{16k_B T \ln 2} \right)^{\frac{1}{2}}
\]

where \(E, z, e \) and \(k_B \) correspond to the drift electric field, ion charge state, elementary charge and Boltzmann’s constant, respectively [47]. A number of groups have pioneered high-resolution IMS measurements [16,48-52]. Initially, high-resolution IMS measurements were achieved by using high pressures as such conditions allowed the use of higher voltages without creating a discharge in the buffer gas. Recently, relatively high-resolution measurements for peptides have been reported using low-pressure IMS instrumentation [36]. This is accomplished by substantially lengthening the drift tube and using an electrodynamic ion funnel to collapse the diffuse ion cloud near the exit aperture. There are two distinct advantages for using a low-pressure, high-resolution drift tube. First, a low-pressure mobility region is easily coupled to ion trapping devices necessary for high sensitivity measurements [35-36]. The second advantage is that CID experiments...

Figure 7. (A) 2D \( t_d/m/z \) plot for data collected from a LC-IMS-MS analysis of a single SCX fraction of a human plasma digest. The data set was obtained at a retention time of 20.4 min. (B) A parent ion mass spectrum obtained by integrating all drift bins for given \( m/z \) values over the drift range indicated by the dashed line box is shown. The feature corresponding with the precursor ion that has been identified by protein database searches as the ion MLVNFLR\(^{2+}\) from the protein interleukin-26 is indicated. (C) The MS/MS spectrum for the MLVNFLR\(^{2+}\) ion indicating fragment assignments is also shown.

IMS: Ion mobility spectrometry; LC: Liquid chromatography; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; m/z: Mass-to-charge ratio.
can be performed within the mobility region, such as those demonstrated with the split-field drift tube [39]. Recently, an extended (~1 m), low-pressure drift tube employing the split-field design for parallel CID has been used to perform LC-IMS-MS experiments for human plasma [UNPUBLISHED DATA].

**Developing LC-IMS/IMS-MS technology**

The advantages of the combination of a post-ionization, rapid gas-phase separation with LC/MS methods have been described above in terms of increased peak capacity, dynamic range and sensitivity, as well as the ability to generate a high-resolution proteome map. An extension to this approach is to couple multiple gas-phase separation strategies similar to achievements with condensed-phase separations [53]. Use of a second dimension of mobility separation requires the alteration of an ion’s mobility, which can only be accomplished by a structural transition, or a change in buffer gas composition or temperature. Since mobility measurements are carried out at high pressures, it is possible to induce highly controlled structural transformations for biomolecular ions. For example, studies have shown that ion structures can be varied by changing the energy used to inject into a drift tube [58–59], or by changing the buffer gas temperature [60–62]. Recently, it has also been shown that structures vary depending upon ion storage time in traps (prior to injection into a drift tube) [63,64]. FIGURE 8 shows structural transformations for several charge states of electrosprayed cytochrome c ions stored in a 3D trap for long time periods [65].

Although various methods exist for altering ion mobilities (above), several are preferred for IMS/IMS instrumentation development due to their simplicity as well as their amenability for coupling with existing instrumentation. For example, a simple approach would be to alter the structures of ions (similar to transformations demonstrated in FIGURE 8) after a first mobility separation step simply by injecting them with varying energies into a second drift tube. Another approach can be developed using separate but uninterrupted mobility regions such as that demonstrated with the split-field drift tube design shown in FIGURE 2. As discussed above, when the fields in the second region of the drift tube are increased substantially, ions are collisionally heated and undergo fragmentation. Interestingly, the mobilities of ions can be influenced using a field regime between that used to induce fragmentation and that used for the low-field mobility separation. The nature of the mobility change as ions leave the low-field mobility region is not fully understood at this point; however, such field alterations may provide another means for IMS/IMS separations. FIGURE 9 shows a hypothetical mobility device based on a double split-field drift tube design. The combination allows for several experimental analyses. First, ions separated according to their low-field mobilities in the first drift tube may be transmitted as precursors or induced to undergo fragmentation at the back of the drift tube and, in the second drift tube, the precursors may be subjected to a high-field mobility separation or the precursor fragments from the first separation may be subjected to a low-field mobility separation. At the back of the second drift tube, the twice dispersed precursors or the fragments may be transmitted or again induced to undergo CID. Thus, such experiments may provide a vast array of information such as IMS(low-field)-IMS(high-field)-MS/MS analysis of precursors as well as MS/MS/MS information for mobility-dispersed fragments.

**Proteome mapping**

A goal of proteomics studies is to develop a proteome map, a searchable database of component identifications that can be used in comparative studies. As mentioned above, the multiply dispersive approach allows the construction of a higher resolution map than those afforded by traditional LC/MS methods. An advantage of the multidimensional separations described here is
that the extra feature delineator ($t_d$'s from IMS and IMS/IMS measurements) can be used to aid identification efforts and should facilitate comparisons across multiple data sets increasing the accuracy of the map. Additionally, the higher throughput afforded by the LC-IMS-MS measurements allows the generation of a greater number of data sets that, upon comparison, should also increase the confidence of mapped features. That is, higher peptide assignment confidence is garnered for identical features observed multiple times; similarly, the observation of multiple peptides from a single protein across various data sets increases the confidence associated with the protein assignment.

Conclusions
Although relatively new, the use of multidimensional LC-IMS-MS techniques to analyze complex mixtures is proceeding at a remarkable pace. For example, the first multiply dispersive LC-IMS-MS analysis of a complex mixture of peptides was carried out in 2001 [19]. Over the next few years, the analysis was extended to complex proteome mixtures [21,22,26,36,40,41]. Recent comparisons with LC/MS approaches have shown advantages with regard to resolution, dynamic range and experimental throughput. Here, it is also noted that improvements (described above) are envisioned to significantly increase the overall peak capacity and sensitivity of these methods. Altogether, LC-IMS-MS techniques are poised to move from the arena of fringe technologies to that of mainstream proteomics tools.

Expert commentary & five-year view
Over the last few years, improvements in IMS-MS instrumentation have made it possible to perform LC-IMS-MS proteomics analyses. While only a relatively few numbers of studies have been carried out to date, the advantages in experimental throughput, resolution and dynamic range discussed in this paper should prompt greater efforts in technique development. It is conceivable that where there are only a few research groups promoting and developing IMS technology for complex biologic mixture analysis today [19-26,36,45,65-68], the number may increase by more than one order of magnitude over the next 5 years. It is also conceivable that commercial instrumentation utilizing IMS in a dispersive fashion may be marketed during this time frame. Such an event would likely lead to more rapid growth in the numbers of groups using LC-IMS-MS techniques for proteomics studies.

Although the robustness of LC-IMS-MS instrumentation has been demonstrated by many of the experiments described above, the most pressing issues limiting its use are related to the present state of informatics tools. Innovative data processing and mapping algorithms are currently under development and it is now possible to go from data collection to proteome map generation within a single day. Present resources allow the simultaneous analysis of multiple data sets on the time scale required for data collection. Although such efforts are beginning to bear fruit, development will continue throughout a 5-year time frame. A final aspect of the informatics tools required for these studies is the ability to determine statistically significant differences across multiple data sets. Although to date little has been accomplished involving comparison algorithms for IMS-MS data, initial development has begun and will accelerate over the next 5 years. With these informatics tools in place, results from multiply dispersive studies will be ready for a knowledge assembly informatics piece, similar to current proteomics efforts. Here, work will focus on extracting correlations that illuminate molecular signatures and roles associated with disease onset and progression.

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Figure 9. Schematic diagram of a hypothetical mobility region for an IMS/IMS instrument. The mobility region comprises two split-field drift tubes for efficient separation and fragmentation of ions. IMS: Ion mobility spectrometry.
Key issues

- Multidimensional liquid chromatography (LC) ion mobility spectrometry (IMS) mass spectrometry (MS) technology provides advantages in resolution, throughput and dynamic range for the study of complex mixtures.
- Proteome mapping studies are facilitated by LC-IMS-MS techniques as higher resolution, high-confidence maps can be generated.
- Limitations in LC-IMS-MS technology related with feature identification can be reduced with improvements in IMS resolving power as well as improvements in software. Informatics limitations require more computing power and algorithm development for parallel processing.
- Future work may focus on the development of instrumentation that will execute 2D-IMS/IMS separations. Such strategies, coupled with condensed-phase separation steps, should provide unprecedented resolution of complex mixture components and lead to the development of high-confidence maps.

References

Papers of special note have been highlighted as:
• of interest
  •• of considerable interest

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Developing LC-ion mobility-MS techniques


Affiliations

- Stephen J Valentine
  Predictive Physiology & Medicine, 1424 W. Adams Hill, Bloomington, IN 47403, USA
  Tel.: +1 812 856 3987
  Fax: +1 812 855 8300
  stvalent@indiana.edu

- Xiaoyun Liu
  Indiana University, Department of Chemistry,
  800 E. Kirkwood Ave. Bloomington,
  IN 47405, USA
  Tel.: +1 812 856 3987
  Fax: +1 812 855 8300
  xil3@indiana.edu

- Manolo D Plasencia
  Indiana University, Department of Chemistry,
  800 E. Kirkwood Ave. Bloomington,
  IN 47405, USA
  Tel.: +1 812 856 3987
  Fax: +1 812 855 8300
  mplasenc@indiana.edu

- Amy E Hilderbrand
  Indiana University, Department of Chemistry,
  800 E. Kirkwood Ave. Bloomington,
  IN 47405, USA
  Tel.: +1 812 856 3987
  Fax: +1 812 855 8300
  ahilder@indiana.edu

- Rwan T Karuligama
  Indiana University, Department of Chemistry,
  800 E. Kirkwood Ave. Bloomington,
  IN 47405, USA
  Tel.: +1 812 856 3987
  Fax: +1 812 855 8300
  rkarulig@indiana.edu

- Storny L Koeniger
  Indiana University, Department of Chemistry,
  800 E. Kirkwood Ave. Bloomington,
  IN 47405, USA
  Tel.: +1 812 856 3987
  Fax: +1 812 855 8300
  skoeniger@indiana.edu

- David E Clemmer
  Indiana University, Department of Chemistry,
  800 E. Kirkwood Ave. Bloomington,
  IN 47405, USA
  Tel.: +1 812 855 8259
  Fax: +1 812 855 8300
  clemmer@indiana.edu

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