Published in final edited form as:

J Mass Spectrom. 2010 December ; 45(12): 1383–1393. doi:10.1002/jms.1850.

Metabolic Profiling of *Escherichia coli* by Ion Mobility-Mass Spectrometry with MALDI Ion Source

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Abstract

Comprehensive metabolome analysis using mass spectrometry (MS) often results in a complex mass spectrum and difficult data analysis resulting from the signals of numerous small molecules in the metabolome. In addition, mass spectrometry alone has difficulty measuring isobars and chiral, conformational, and structural isomers. When a matrix assisted laser desorption ionization source (MALDI) is added, the difficulty and complexity are further increased. Signal interference between analyte signals and matrix ion signals produced by MALDI in the low mass region (<1500 Da) cause detection and or identification of metabolites difficult by mass spectrometry alone.

However, ion mobility spectrometry (IMS) coupled with MS (IM-MS) provides a rapid analytical tool for measuring subtle structural differences in chemicals. IMS separates gas phase ions based on their size-to-charge ratio. This study, for the first time, reports the application of MALDI to the measurement of small molecules in a biological matrix by Ion Mobility-Time of Flight Mass Spectrometry (IM-TOFMS) and demonstrates the advantage of ion-signal dispersion in the second dimension. Qualitative comparisons between metabolic profiling of the *Escherichia coli* metabolome by MALDI-TOFMS, MALDI-IM-TOFMS, and ESI-IM-TOFMS (electrospray ionization) are reported. Results demonstrate that mobility separation prior to mass analysis increases peak-capacity through added dimensionality in measurement. Mobility separation also allows detection of metabolites in the matrix-ion dominated low-mass range ($m/z < 1500$ Da) by separating matrix-signals from non-matrix signals in mobility space.

Keywords

Ion mobility-Mass Spectrometry; Metabolomics; *Escherichia coli*; MALDI; ESI; Peak Capacity

Introduction

1.1 Metabolomics

At present, a variety of analytical and sample preparation techniques are employed to identify metabolites in biological samples. Metabolites are the consortium of endogenously synthesized molecules that participate in or are produced by cellular regulatory processes ¹,

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² of a biological system. Alterations in metabolite levels are directly related to changes in gene expression, translation or post translational modifications of proteins, and or physiological response of an organism to biochemical stressors such as environmental conditions and diseases. The comprehensive study of metabolites (metabolomics) is one of the oldest branches of the biological sciences. Metabolomics has recently emerged as a promising tool to complement genomics, proteomics, and transcriptomics in deciphering complex biological systems ^{3–5}.

A variety of strategies have been used to characterize metabolites ^{6–9} including: 1) Metabolite target analysis: analysis is constrained entirely to a particular substrate or metabolite. 2) Metabolite profiling: analysis focused on a group of metabolites, for example, a class of compounds such as carbohydrates, amino acids, or metabolites associated with a specific pathway. 3) Metabolomics or metabolome profiling: comprehensive analysis of the whole metabolome under a given set of conditions. 4) Metabolic fingerprinting: classification of samples on the basis of biological relevance or origin. 5) Metabonomics: measure of the fingerprint of biochemical perturbations caused by disease, drugs, or toxins. 6) Metabolic foot-printing: used in microbiology for the analysis of metabolites in an extracellular environment such as a growing medium.

1.3 Current analytical methods

Although metabolite profiling has long been used for medical and diagnostic purposes ¹⁰, only recently have efforts been made to develop methods for large scale, intracellular metabolite screening ^{8, 9}. Comprehensive metabolome characterization has been hampered by the lack of efficient instrumental analytical techniques to rapidly analyze complex biological samples. Due to its diverse chemical and physical properties, no single analytical method is capable of adequately visualizing the metabolome. Hence, improved techniques are needed for comprehensive profiling. Selection of a suitable technique is generally a compromise between speed, selectivity, and sensitivity.

A variety of techniques have been used to determine targeted metabolites in the *Escherichia coli* metabolome; these include liquid chromatography-capillary electrophoresis LC-CE, CE-mass spectrometry ¹¹, ion exchange chromatography-reversed phase liquid chromatography-MS ¹², gas chromatography-mass spectrometry, GC-MS ^{13, 14}, two dimensional thin layer chromatography 2D TLC ^{15, 16}; high-performance liquid chromatography, HPLC ¹⁷; enzyme assay, electrospray ionization-MS, ESI-MS ¹⁸; and negative mode MALDI-MS ¹⁹. These techniques have also been used to probe metabolomes such as by GC ^{20–23}; by HPLC ^{17, 24–29}; and by CE ^{30–33}. Spectroscopic methods such as NMR have been investigated for possible application to metabolomics ^{34–39}. Mass spectrometry as a standalone technique for comprehensive metabolite determination has been reported as well. However, because of the resulting complex mass spectrum and the difficulties in separating isomeric and isobaric compounds using MS, a separation method prior to mass analysis is generally used ^{40, 41}.

Ion mobility spectrometry is a gas phase analytical method that separates ions using differences in mobility. The mobility (K) of an ion (the ratio of the ion velocity to the magnitude of the electric field, $\text{cm}^2\text{V}^{-1}\text{s}^{-1}$), is defined by the overall shape and size of the ion subjected to a weak homogenous electric field gradient in a drift gas ^{42–45}. By monitoring ion arrival time also termed “drift time” (travel time from the ion-gate to the detector), both qualitative and quantitative information can be obtained. When interfacing IMS to MS, the complexities arising from mobile phase compositions (as in any LC separation technique) are avoided. Additionally, the separation time of an IMS experiment is in milliseconds, reducing sample analysis time and increasing throughput. When compared to chromatographic separations, the correlation between mobility (size-to-charge) and mass-

to-charge observed in an IM-MS experiment results in lower coverage of the two-dimensional space available in a 2D method. However, gas phase separations by IM-MS provide the rapid analyses required in a metabolomics experiment.

The compatibility of IMS with a variety of ionization sources offers flexibility in the analyses of solid, liquid, or gas phase samples⁴⁶. Because separation in IMS is based on differences in the size-to-charge ratio, IMS enables separation of isomeric and isobaric analytes and provides analyte structural information. Additionally, separation selectivity can be adjusted with the choice of shift reagents (drift gas composition, anions, and cations^{47–49}). Thus, IMS allows rapid analyses of complex matrices such as separation and identification of the diverse metabolites present in a metabolome.

ESI and MALDI are the most common means of creating gas phase ions for mass spectrometric analysis of bio-molecules. Although ESI can be used to ionize any size of molecule, multiple charging, sensitivity to salts and buffers, and incompatibility with automation are major hurdles to rapid, multiple sampling, high throughput metabolomics experiments. Conversely, the popularity of MALDI is due to the advantages it offers over ESI — low spectral complications and a high tolerance for salts and buffers. While MALDI has been used extensively for the ionization of large molecules (peptides^{50–53}, proteins^{54–58}, oligosaccharides^{59–63} and lipids^{64–68}), it has only rarely been used to ionize small molecules^{69–73} due to strong matrix ion interference^{71, 74–76}. A recent publication⁷⁷ explored the applicability of MALDI with matrix suppression as an ionization source for metabolome analysis using a 30-metabolite synthetic cocktail. The authors concluded that “matrix-suppressed laser desorption/ionization mass spectrometry (MSLDI-MS) offers a rapid and high-throughput option for analysis”. The application of negative-mode MALDI-TOFMS to metabolite detection in complex biological samples such as *Escherichia coli* and islets of Langerhans¹⁹ has also been reported. In addition, atmospheric pressure MALDI offers ease in sample handling; however, inefficient ionization and or ion transmission results in reduced peak intensity.

Applications of IM-MS in proteomics and glycomics have demonstrated the strength of the technique for probing complex biological samples^{78–81}. The advantages of combining MALDI with the high analytical speed and separation capability provided by IM-MS inspired the development of MALDI-IM-MS for metabolomics applications. Metabolic profiling by IM-MS using ESI as the ionization source has been recently applied to metabolic profiling of *Escherichia coli*⁸², human blood⁸³, and rat-lymph fluid⁸⁴ metabolomes. MALDI-IM-MS has been demonstrated as a powerful tool for direct sampling⁸⁵ and mapping of the spatial distribution of chemicals in tissue sections⁸⁶ and for probing intra-molecular interactions⁸⁷. This manuscript demonstrates, for the first time, the application of MALDI-IM-MS to metabolome profiling. We qualitatively compare MALDI-TOFMS, MALDI-IM-TOFMS and ESI-IM-TOFMS for rapid and comprehensive profiling of intracellular *Escherichia coli* metabolome.

Materials and Methods

2.1 Protocol for *Escherichia coli* metabolite extraction

High performance liquid chromatography grade solvents (methanol, water, acetonitrile, and acetic acid) were purchased from J. T. Baker (Phillips burgh, NJ). Trifluoroacetic acid (TFA), dihydroxybenzoic acid (DHB) and Luria-Bertani medium were purchased from Sigma-Aldrich Chemical Co. Inc. (Milwaukee, WI, 53201, U.S.A.)

Triplicate cultures of *Escherichia coli* W3110 were grown in 5ml Luria-Bertani medium at 37°C with a 125 rpm shaking to achieve a final optical density of 1.0 at 600nm, OD_{600nm} =

1.0. The cultures were immediately quenched by adding an equal volume (5ml) of -80°C methanol. The samples were then centrifuged at $7500 \times g$, 10 min, at -20°C . The supernatant was decanted. The cell pellet, containing intracellular metabolites, was immediately re-suspended in 0.7 ml of hot (70°C) methanol and incubated at 70°C for 15 min to release the intracellular metabolites. Next, the sample was mixed with 0.7 ml sterile H_2O , vortexing at high speed for 20 s and centrifuged at $12000 \times g$ at 23°C for 1 min. The supernatants were decanted into new tubes and stored at -20°C until they could be analyzed for intracellular metabolites. Sample aliquots of various concentrations (2X, 4X, and 10X) were also prepared using a SpeedVac Plus SC110a (Savant Co.) until the desired volume was attained.

α -Cyano-4-hydroxycinnamic acid (CHCA) was the only other matrix against which DHB as matrix was compared to and chosen against based on larger number of peaks detected and lesser extent of clustering and fragmentation observed. The DHB matrix recipe used was selected based on homogeneous crystallization of the dried droplet. Also, metabolite to matrix ratio was adjusted so that larger number of non-matrix peaks is observed upon analysis. For MALDI-IM-TOFMS and MALDI-TOFMS analysis, a matrix solution was prepared by mixing ~20 mg of DHB in 750 μL of solvent (0.1% TFA, 50% acetonitrile). Then 1 μL of matrix-*Escherichia coli* supernatant (9: 1 v/v) was spotted on a MALDI plate and allowed to dry before analysis. For ESI-IM-TOFMS analysis, a 200 μL aliquot of *Escherichia coli* supernatant was mixed with water and acetic acid to make a solution of 47.5% water, 47.5% methanol, and 5 % acetic acid. Because we observed the most MS peaks in the 10X sample, we used it for the MALDI experiments. For the ESI experiments, only the 1X sample was used. With MALDI, samples of lower analyte-to-matrix ratio did not provide observable analyte signals which could be due to lower analyte ionization efficiency compared to matrix ions at lower analyte-to-matrix ratio. After concentration step, total sample consumed in MALDI was comparable to that used in ESI because 1) ESI is a continuous ion source and IMS gate block 99 % of the ions generated by the ESI source, and 2) longer acquisition time is required to acquire acceptable ESI-IM-MS data. The goal of the authors was to acquire best data that could be acquired by the specific instruments and qualitatively compare the respective methods. However, the three methods compared in the manuscript are specific to the instruments used and provides an overall picture of the methods.

2.2 MALDI-TOFMS

A Voyager DE-STR MALDI time-of-flight instrument (Perseptive Biosystems/Applied Biosystems, Foster City, CA) was used for conventional MALDI analysis in reflectron mode with delayed extraction. A nitrogen UV laser (337 nm) at a repetition rate of 3 Hz was employed for ionization. Mass spectra were acquired in positive mode and were the sum of 256 laser shots. Mass spectrum was acquired and processed with Data Explorer (Applied Biosystems, Foster City, CA); it was plotted using Microsoft excel worksheet. The instrument was calibrated externally with a peptide calibration mixture and internally with DHB ions.

2.3 ESI-IM-TOFMS

Experimental details of the *Escherichia coli* metabolome analysis using ESI-IM-TOFMS can be found in detail elsewhere^{82, 88}. The instrument ESI-IM-TOFMS comprises an electrospray ionization source, an ion mobility spectrometer, and a time-of-flight mass spectrometer. Details of the instrument and the data acquisition system are reported in an earlier publication⁸⁹.

2.4 MALDI-IM-TOFMS

The MALDI-IM-TOFMS instrument used in this study consists of a frequency-tripled Nd:YAG 355 nm MALDI source (Model PowerChip PNV, JDS Uniphase) operated at 150 Hz and 2.4 torr helium pressure. The IM-TOFMS instrument designed and constructed at Ionwerks Inc., Houston TX, consists of a 31-cm long periodic-focusing drift cell coupled to an orthogonal two stage reflectron TOFMS with a 60-cm flight path⁹⁰. Instrumental details can be obtained from previous publications^{91, 92}.

The potential difference between the sample plate and the IM tube drives ions into the drift tube. Ions in the IM cell then travel across an electric field gradient created by the successive electrodes of the ion mobility tube. After exiting the drift tube ions are guided into the mass spectrometer by differentially pumped skimmer region electrodes. Typically, the mobility drift times are up to 1 ms; flight times in the mass spectrometer are 25 μ s or less. Because of the difference between ion mobility and flight times, more than 400 interleaved mass spectra can be obtained after every laser pulse depending on the target mass range. This process is repeated over several hundred laser shots, until the data is sufficiently intense to permit analysis. The typical resolving power of the IMS was ~ 50 (drift time in ms / full width at half height in ms) and that of TOFMS was ~ 2500 (m/z in Da / full width at half height in Da) for analytes of m/z values less than ~ 1000 Da). The two-dimensional data thus acquired is best represented as 2-D contour plots of ion intensity as a function of ion mobility drift time (y-axis) and m/z (x-axis). All contour plots were produced using IDL software (Research Systems, Boulder, CO).

Results and Discussion

3.1 Ions produced by ESI and MALDI sources

Typically, with MALDI as the ionization source and DHB as the matrix, singly charged analyte ions are generated as $\{M(H_2O)_n + H\}^+$, $\{M(H_2O)_n + Na\}^+$ and/or $\{M(H_2O)_n + K\}^+$. Adduct ions of analytes and DHB are also observed. The most common ions observed in ESI with water, methanol, and acetic acid as the ESI solvent are $\{M(H_2O)_n + H\}^+$, $\{M(H_2O)_n + Na\}^+$, $\{M(CH_3OH)_n + H\}^+$, $\{M(CH_3COOH)_n + H\}^+$, $\{M(H_2O)_n + K\}^+$, and $\{M(H_2O)_n + NH_4\}^+$ where M is an analyte. Ions with any combination of the analyte and solvent are also generated. Multiply charged ions, usually protonated and sodiated, are typically produced from analytes with masses greater than 1000 Da (e.g., proteins, peptides, lipids, and carbohydrates).

3.1.1 Analyses Comparison between Instruments—The comparison results reported here are intended primarily to present the major trends observed between MALDI-TOFMS, MALDI-IM-TOFMS and ESI-IM-TOFMS. Because three different instruments were used, the comparisons are specific to the instruments; the experimental conditions and results depend on, and vary with, the respective instrument strength. All instruments were operated at conditions of minimum fragmentation: Using standards such as methamphetamine the potentials on the sample plate-first IMS drift tube electrode-last IMS drift tube electrode-IM-MS interface lenses were adjusted to minimize fragmentation.

3.2 Comparison: MALDI-TOFMS Versus MALDI-IM-TOFMS (Background)

Figures 1a and 1b show ion intensity (y-axis) versus mass-to-charge ratio (x-axis) plots in the m/z range of 50-2000 Da obtained by MALDI-TOFMS for the background (DHB matrix + blank *Escherichia coli* extract) and the sample (DHB matrix + *Escherichia coli* extract) respectively. Figures 2-A1 and B1 show acquired data contour MALDI-IM-TOFMS plots for the background and sample (m/z range: 50-2000 Da), respectively, with drift times in μ s on the y-axis and m/z on the x-axis. In an IM-MS raw data set, the electronic noise signals

(dark-counts) are dispersed throughout the 2D IM-MS analytical space along with the analyte signals. To view the analyte signals, as shown in any IM-MS plot reported in the manuscript, noise signals are removed by increasing the ion-count threshold and averaging data-bins. In the absence of suitable software a “stare and compare” strategy was used prior to creating peak lists to eliminate peaks due to noise. After removal of noise signals, only those signals with ion-counts greater than five were included in the peak list. However, the possibility of discarding low intensity analyte peaks increases with smoothing and averaging and with exclusion of low ion-count data points. In the case of MS data processing, signals above the noise level were included in the peak-lists via threshold adjustment.

Approximately fifty background ions were observed with MALDI-TOFMS, of which 80% had m/z values < 500 Da. With MALDI-IM-TOFMS ~250 background ions were detected, only ~40% had m/z values < 500 Da. A roughly four fold increase in the number of background ions was observed with MALDI-IM-TOFMS as compared to MALDI-TOFMS.

MALDI-TOFMS produced high intensity matrix peaks at m/z values of 109 (DHB-COOH)⁺, 136 (DHB - H₂O)⁺, 137 (DHB - H₂O + H)⁺, 154 (DHB)⁺, 155 (DHB+H)⁺, 173 (DHB + H₂O + H)⁺, 177 (DHB + Na)⁺, 245 [(DHB - COOH)⁺ + DHB], 263 [DHB - COOH)⁺ + (DHB - H₂O + H)⁺], 272 2(DHB - H₂O)⁺, 273 [(DHB - H₂O) + (DHB - H₂O + H)⁺], 290 [(DHB - H₂O)⁺ + DHB], 291 [(DHB - H₂O) + (DHB + H⁺)], 449 [(DHB + Na)⁺ + 2(DHB - H₂O)], and other combinations, most of them with m/z values below 500 Da. With MALDI-IM-TOFMS: 1) matrix cluster ions of m/z value greater than 500 Da were predominant, 2) isomeric and or isobaric forms of matrix ions were produced, and 3) the same mass ions formed by fragmentation had different drift times than those formed at the ionization source.

For example, ions at m/z 137 (DHB - H₂O + H)⁺ were separated in the IM-MS space with drift times of 373 and 408 μ s. This could be due to: 1) isomeric and or isobaric forms of DHB produced isomeric and or isobaric (DHB - H₂O + H)⁺ ions, or 2) one ion was produced at the source while another resulted from fragmentation of a larger ion. Analysis of the data showed that the (DHB - H₂O + H)⁺ ion at the higher drift time (408 μ s) was produced by fragmentation of the clusters [(DHB - COOH)⁺ + DHB] at m/z 245 Da and [DHB - COOH)⁺ + (DHB - H₂O + H)⁺] at m/z 263, because all three peaks (263, 245 and 137) had the same drift times. In addition isobaric DHB ions at m/z 137 further fragmented to produce the (DHB - COOH)⁺ ions at m/z 109 with drift times of their parent ions. The (DHB - H₂O + H)⁺ ion with a lower drift time (373 μ s) did not show a parent ion. This could be because: 1) the ion was formed at the source, or 2) because complete fragmentation of the parent-ion occurred at the IM-MS interface. However, the smaller drift time suggests that the ion was produced at the source. Although matrix ions were produced by both MALDI-TOFMS and MALDI-IM-TOFMS, matrix ions with m/z values of < 500Da were predominant in MALDI-TOFMS whereas ions of m/z values across the m/z range were generated in MALDI-IM-TOFMS (Figures 1b and 2-A1). This suggests that all different species of ions produced at the MALDI source undergo fragmentation at the source chamber-mass spectrometer interface whereas when ions traverse the ion mobility cell placed between the source chamber and the TOFMS, the identity of the ion produced at the source is preserved and fragmentation is minimized. Conversely, it might also be attributed to differences in laser power and source pressure between the two instruments. Though high-vacuum conditions are more conducive to ionization of large analyte species, fragmentation of matrix-analyte cluster ions produced under MALDI-MS conditions can be explained if the cluster ions produced are more energetic than the ones produced under MALDI-IMS-MS conditions where soft collisions with neutral gas molecules lower the energy of the ions produced.

3.3 Comparison: MALDI-TOFMS Vs. MALDI-IM-TOFMS (*Escherichia coli*)

Approximately 1000 ions (including background ions) were detected using MALDI-IM-TOFMS (Figure 2-B2). As observed with the matrix analysis, a four-fold increase in the total number of ions was observed when the same sample was analyzed by MALDI-IM-TOFMS. Approximately 80% of the ions had an m/z value > 500 Da.

Enlarged contour plots (drift time vs. m/z) in the mass range of 650–850 Da (Figures 2-A2 and 2-B2) clearly demonstrate ion signal dispersion in the second dimension, which allows separation of analyte signals from noise and or matrix signals based on their unique mobility-mass values. For example, an ion with an m/z value of 712 Da was observed in both the background and *Escherichia coli* extracts. However, the drift time of the background ion was 498 μ s while that of the *Escherichia coli* sample was 520 μ s. Similarly, the ion at the m/z value of 725 Da had mobility values of 558 (background) and 517 (*Escherichia coli* sample) and were thus differentiated by IMS. Based on the peak-lists generated by the instrument software for the mass range of 650–850 Da [$m/z \pm 0.2$ Da, mobility ± 5 μ s], a total of ~60 ions from the background and ~225 ions from the *Escherichia coli* sample were detected; fifteen of the ions detected in the *Escherichia coli* sample were from the background. Thus ~200 metabolites were detected in this mass range. In addition, several isomeric and or isobaric metabolites observed from the *Escherichia coli* sample were also separated by IMS. For example, metabolites at m/z value 699 Da had mobilities of 549 and 519 μ s. Similarly metabolites at the m/z value 729 Da had mobilities of 559 and 512 μ s. Figure 3 shows a background spectrum (pink-white contours and bold 1D curves) superimposed over a sample spectrum (blue-white contours and dotted 1D curves) in the m/z range of 700–800 Da showing the separation of several isobaric and or isomeric ions and the dispersion of matrix and sample signals in the 2D IM-MS. Development of software to extract analyte signals from background + analyte signals would ease and speed data interpretation.

Laser intensity also affected the identity and number of ions detected both in the background and the sample. Figure 4 shows the increase in number of metabolic features detected between high and low laser intensity against number of background signals at high laser power. A ten-fold increase in the number of metabolic features detected was observed in the m/z range of 50–500 Da and a five-fold increase in the 500–1000 Da m/z range. Thus with an increase in laser power more analytes over a wider m/z range were detected. However, the increase in the number of analyte signals with higher laser power could also be due to increased ion fragmentation.

3.4 Comparison: ESI-IM-TOFMS Vs. MALDI-IM-TOFMS (*Escherichia coli*)

Figure 5 is a 2D ESI-IM-TOFMS contour plot of ions observed during analysis of the *Escherichia coli* sample. On average (analysis of three samples) 500–550 ions were detected by ESI-IM-TOFMS of which ~75% of the ions were of m/z values less than 500 Da. The expanded contour plot (Figure 5) in the m/z range of 150–250 Da shows detection of ~150 ions in the *Escherichia coli* metabolome. Comparison of the number of ions observed in the mass range of 50–2000 Da shows that ~1000 ions were detected by MALDI-IM-TOFMS, ~500 by ESI-IM-TOFMS and ~250 by MALDI-TOFMS from the *Escherichia coli* extract. However, in the mass range of 50–300 Da the maximum number of ions were observed by ESI-IM-TOFMS (~375) followed by MALDI-IM-TOFMS (~75) and MALDI-TOFMS (~60). A bar chart (Figure 6) shows the distribution of the number of ions detected in both background and sample by MALDI-TOFMS, MALDI-IM-TOFMS, and ESI-IM-TOFMS. This shows that the choice of ionization source used in IM-MS metabolomic experiments dictates the number and identity of the features detected. In addition, the capabilities of the instruments used (such as IM-MS resolving power) also contribute to the above observation.

The fact that more ions were detected in the m/z range of 50–300 Da suggests that charge competition between the matrix and metabolites prevents efficient ionization of metabolites in this m/z range. Although multiple charging is a common phenomenon observed via ESI ionization of large molecules, particularly peptides, lipids, oligosaccharides, and proteins (> 1000 Da), multiple charging is rarely observed with low mass molecules such as metabolites in the m/z range of 50–600 Da). Further research into a suitable matrix for metabolites or development of non-matrix MALDI for metabolomics will significantly contribute to metabolomics studies.

3.5 Peak capacity: ESI-IM-TOFMS Vs. MALDI-IM-TOFMS (*Escherichia coli*)

With tandem separation techniques such as IM-MS, CE-MS, LC-MS, the maximum number of peaks that can be fit in a two dimensional space is defined as the peak capacity (Φ) of the tandem method⁹³. Thus peak capacity is a measure of the separation potential of the technique and depends on the resolution of the individual systems and differences in their separation mechanisms (orthogonality). Thus a 2D separation technique will have a very high Φ value if the fundamental separation mechanisms of the individual high resolution systems are completely orthogonal^{94, 95}. For example the peak capacity of LC-MS, where LC separations occur due to differences in the partition coefficient of molecules between mobile phase and stationary phase and MS separation, is based on the m/z ratios of ions, is quite high due to completely different mechanism of separations involved in the individual analytical methods⁹¹. With IM-MS as a tandem technique where separation occurs due to the differences in the Ω/z values (size-to-charge) and m/z values (mass-to-charge), a plot of Ω/z versus m/z generally follows a linear trend and therefore exhibits a smaller Φ than LC-MS. However, IM-MS experiments allow separation of comparable number of metabolic features (10^3)^{96–98} in the observed limited orthogonality. In addition, because ion mobility is a post-ionization gas phase separation method, temporal separation occurs in milliseconds resulting in very high peak production rates (Φs^{-1}). Compared to LC-MS, IM-MS has at least three times greater Φs^{-1} . An estimation of the peak capacity in IM-MS can be calculated as follows:

$$\Phi = \bar{R}_{(IMS)} * \bar{R}_{(MS)} * \text{fraction of orthogonality}$$

Where, $\bar{R}_{(IMS)}$ is the average resolving power of the ion mobility spectrometer, and $\bar{R}_{(MS)}$ is the average resolution of the mass spectrometer in an m/z range. For example, in the mass range of 50–500 Da for the *Escherichia coli* sample analyzed, the minimum and maximum deviation in drift time along a vertical plane parallel to the y-axis defines the fraction of orthogonality as shown by double sided arrows in Figures 7a and 7b. Ions detected by MALDI-IM-TOFMS from the *Escherichia coli* sample are shown in Figure 2 and that detected by ESI-IM-TOFMS in Figure 5. The theoretical trend lines shown in Figures 7a and 7b is the center line of the shaded area along which a constant drift time deviation should be observed. The trend line obtained for the experimental data is depicted as bold lines in Figures 7a and 7b. The length of the 2D space is defined by the m/z range and double sided arrow defines the drift time spread in the given m/z range. For MALDI-IM-TOFMS data, with a drift time spread of ~106 μs the total % deviation measures to be ~12 % [$106/(518+512/2)$] of the total 2D space (Figure 7a, shaded space in the X-Y quadrant). This gives an average deviation in drift time of $\pm 6\%$ along the theoretical trend line shown as dotted line in Figure 7a. With average IMS resolving power of 50, average MS resolution of 2500, and $\pm 6\%$ deviation, the estimated peak capacity is estimated to be ~7500. The peak capacity of the MS as a stand-alone technique can be estimated as follows:

- For the m/z range of 50–500 the average m/z value is $(50+500)/2$

- With resolution of 2500, the peak width at m/z 275 is 0.11 Da (275/2500)
- Therefore, number of peaks that can fit in the m/z range of 450 Da (500-50) is 4090 (450/0.11). This shows that ion mobility spectrometry at least doubles the peak capacity of the mass spectrometer as a stand-alone technique (~4090).

Similar calculations for the data set obtained by ESI-IM-TOFMS (Figure 5) the peak capacity with average IMS resolving power of 80, average MS resolution of 1000, and ± 20 % deviation in arrival times, the estimated peak capacity is estimated to be ~16000. With a Φ of MS alone $\cong 1650$, IMS increases the peak capacity by a factor of ten when ESI is the ionization source. It is interesting to note that the fraction of orthogonality in the ESI-IM-TOFMS measurements was greater than that observed in MALDI-IM-TOFMS by a factor of three. This resulted in two-fold increase in peak capacity in ESI-IM-TOFMS, even when the MS resolution was three-times lower than that in MALDI-IM-TOFMS. The increase in spread in case of ESI can be attributed to: 1) efficient ionization of metabolites in the m/z range of 50–500 Da via ESI than by MALDI, and 2) difference in ionization process itself between ESI and MALDI where ions of diverse identities (adducts, multiply charged) can be produced via ESI. Conventionally, in chromatography where separation is followed by detection, the peak capacity is defined as number of peaks that can be fitted into a chromatogram provided the neighboring peaks are separated from each other by 4σ ^{99,100}. Factors such as column efficiency, solute properties, and detector sensitivity affect the width of the peak and thus the peak capacity of the separation method. In tandem separation methods the peak capacity is also dependent on the extent of difference between individual separation mechanisms; the “fraction of orthogonality” or the two dimensional space of detectability. Based on the experimental results described above it can be stipulated that when separation is a post-ionization event the types of ions generated by an ionization source also contribute towards defining the “two dimensional space of detectability” and thus the peak capacity of the method. However, it should be noted that overestimation of peak capacity is possible due to the non-homogeneous spread of peaks in the two dimensional space, as can be observed in Figure 7.

3.6 Tentative Metabolite Identification

From the contour plots of background, ESI-*Escherichia coli* and MALDI-*Escherichia coli* lists of detected ions were generated. Tables 1 and 2 (Supplementary information) were generated to compile various known metabolites that were detected in the *Escherichia coli* metabolome based on 1) common ion formation modes observed in ESI and MALDI, 2) metabolites listed in the CCDB database¹⁰¹ and 3) m/z values of peaks detected (rounded to unit resolution). Metabolite assignments were tentative and in no way suggest the identity of the features detected. They could be even unknown metabolites, not listed in the database. Validated identification of metabolites by IM-MS would at least require identification of mobility selected ions through fragmentation pattern recognition and or accurate m/z and unique mobility matching of experimental data to one obtained for known metabolites. Via the reported instrumentation, though the fragmentation of ions can be imparted at the IM-MS interface, selective and controlled fragmentation was not possible making the data interpretation very complicated. A double gate ion mobility spectrometer coupled to TOFMS instead of a single gate IMS, as in the current instrumental design, would have allowed fragmentation of mobility selected ions and thus definite metabolite feature identification. Alternatively, placement of a device such as an ion-trap capable of ion collection, selection, and fragmentation would have also facilitated definite identification of features detected. Unambiguous identification through accurate m/z and unique mobility matching of metabolite features detected to one obtained for known metabolites require creation of a database using commercially obtainable standards which is not currently available but is under development. However, validated identification via this approach

would be limited by the resolving power of the IM-MS and the availability of metabolite standards; and will not serve the purpose in cases of unknown or new metabolites. Fragmentation and or clustering of the ions during their stay in the IM-MS and or during ionization will also complicate the identification attempt.

For ESI generated ions, tentative identification was made as $(M + H)^+$, $(M + H_3O)^+$, $(M + H_5O_2)^+$, $(M + CH_4OH)^+$, $(M + CH_4COOH)^+$, $(M + Na)^+$, $(M + K)^+$, $(M + H_2O + Na)^+$, $(M + H_2O + K)^+$, and /or $(M + NH_4)^+$. For MALDI generated ions, identification was made as $(M + H)^+$, $(M + Na)^+$, and $(M + K)^+$. Tentative assignments of various peaks detected could not be made through our limited possibilities considered for assignment. Peaks which were not identified could be fragment ions of larger molecules, other adducts/dimers/trimers or unknown features. Table 1 lists the m/z values and name of metabolites present in an intracellular *Escherichia coli* metabolome based on CCDB database¹⁰¹ in columns 1 and 2. The m/z values of most common possible ionic forms of metabolites that can be observed in ESI and MALDI are listed in columns 3–12. Columns 3–12 represent the metabolites as $(M + H)^+$, $(M + NH_4)^+$, $(M + H_3O)^+$, $(M + Na)^+$, $(M + K)^+$, $(M + H_5O_2)^+$, $(M + H_2O + Na)^+$, $(M + H_2O + K)^+$, $(M + MeOH_2)^+$, $(M + CH_3COOH_2)^+$ ions respectively. Table 2 is a list of ions detected in an intracellular *Escherichia coli* metabolome by MALDI-IM-TOFMS and ESI-IM-TOFMS. The m/z values and drift times of ions detected by MALDI-IM-TOFMS are listed in columns 1 and 2. The m/z values and drift times of ions detected by ESI-IM-TOFMS are listed in columns 3 and 4. Columns 5 and 6 list the m/z values and tentative identities of common ions, detected between MALDI-IM-TOFMS and ESI-IM-TOFMS methods. An overlap of ~70 ions was observed for ions detected in the m/z range of 60–600 Da by MALDI (~250) and ESI (~450). Common ions detected between MALDI-IM-TOFMS and ESI-IM-TOFMS methods were tentatively identified as usually being organic acids, carbohydrates, amino acids, fatty acids, nitrogenous compounds, and nucleic materials.

Conclusions

Complex biological samples can be profiled with ion mobility spectrometry (IMS) and mass spectrometry (MS) using a matrix assisted laser desorption ionization source (MALDI). We have shown that gas phase separation using IMS prior to analysis with mass spectrometry reduces interference from matrix ions generated by MALDI. Dispersion of analyte signals in the mobility space allows detection of low molecular weight metabolites ($m/z < 1500$) by MALDI. However, with MALDI, the number of metabolites detected in the m/z range of 50–300 Da was lower than with ESI, suggesting charge competition and or suppression by the matrix. The maximum number of ions detected was in the mass range of 300–1500 Da by MALDI-IM-TOFMS and 50–300 Da by ESI-IM-TOFMS. In addition to the mass information obtained for the metabolites by MS, the size information provided by IMS facilitated the separation of isomeric and or isobaric metabolites and the separation of background ions from metabolites. An IM-MS metabolite database and or instrumental designs to allow ion collection, selection, and fragmentation are needed for unambiguous identification of features through accurate m/z and unique mobility matching and or through fragment data interpretation.

Ion mobility coupled to MS at least doubles the peak capacity of MS as an analytical method with MALDI as the ionization source. In addition, using the ESI-IM-TOFMS technique, peak capacity was increased ten-fold in this study as compared to mass spectrometry alone. Ions of diverse identities formed by ESI through adduct formation and multiple charging can be the source of the increased IM-MS orthogonality observed in ESI over MALDI. This work demonstrates the potential of MALDI-IM-MS as a promising analytical technique for the field of metabolomics. The advantages of MALDI as ion an source — high throughput

via automation and multiple sampling, high salt and buffer tolerance, and direct tissue imaging capability —combined with the development of non-interfering matrix and non-matrix MALDI alternatives would contribute significantly to the field of metabolomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by grant from National Institute of Health- Road Map Grant # R21-DK070274, National Institute on Drug Abuse Intramural program for funding to A.S. Woods laboratory, and NIH for NIDA contracts # N44DA-3-7727 and HHSN271200477384C granted to Ionwerks for instrument design and construction.

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Figure 1. 1a-Mass spectrum of background ions (DHB matrix + blank *E. coli* extract) and 1b-sample ions (DHB matrix + *E. coli* intracellular extract) acquired by MALDI-TOFMS in the m/z range of 50-2000 Da

Figure 2. Two dimensional representation of MALDI-IM-MS data

A1-background ions (DHB matrix + blank *E. coli* extrat) , A2-Zoomed in the m/z range 650-850 Da, B1- sample ions (DHB matrix + *E. coli* extract), and B2- Zoomed in the m/z range 650–850 Da. On the x-axis is shown the mass-to-charge ratio of the ions in Da and the y-axis represents the drift times of ions in μs .

Figure 3. Superimposed MALDI-IM-MS spectra in the m/z range of 700–800 Da of 1) background signals (DHB matrix + blank *E. coli* extract): pink-white contours; bold 1D curves and 2) sample signals (DHB matrix + *E. coli* extract): blue-white contours; dotted 1D curves showing the separation of several isobaric and/or isomeric ions and dispersion of matrix and sample signals in the 2D IM-MS space

Figure 4. An IM-MS plot (drift time (μ s) Vs. m/z) showing the peaks detected upon MALDI-IM-MS analysis of Blank (Matrix + Blank *E. Coli* extract) and Sample (Matrix + *E. Coli* extract) Separation of metabolome signals from matrix in the ion mobility space is achieved allowing detection of small molecules by MALDI as ion source.

Figure 5. ESI-IM-MS contour diagram of intracellular *E. coli* metabolome

Drift time of an ion in microseconds is plotted against the mass to charge ratio of the ion. The spectrum on the top covers m/z range of 60-600 Da. The bottom spectrum is a close up view in the m/z range of 150-250 Da.

Figure 6. Distribution of number of ions detected in background (matrix + blank extract) and sample (*E. coli* + matrix) by MALDI-MS, MALDI-IM-MS and ESI-IM-MS is illustrated
On the x-axis is the m/z range in which ions were detected and y-axis represents the number of ions detected in each method.

Figure 7. Illustration of the two dimensional space (shaded area in the X-Y quadrant) occupied by ions detected from intracellular *E. coli* extract by IM-MS with MALDI (7a) and ESI (7b) as the ionization source

The theoretical trend line (dotted line) is the centre line of the shaded area along which a constant drift time deviation should be observed. Trend line for the experimental data is depicted as bold line. The length of the 2D space is defined by the m/z range and double sided arrow defines the drift time spread in the given m/z range.