I.2 Current Status and Forward Looking Thoughts on LC/MS Metabolomics

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1 Introduction

The metabolome can be viewed as the consequential end products of gene expression and the goal of metabolomics includes the comprehensive evaluation of the metabolome (Trethewey et al. 1999; Fiehn et al. 2000; Trethewey 2001; Oliver et al. 2002; Sumner et al. 2003). Quantitative and qualitative measurements of large numbers of cellular metabolites provide a high-resolution biochemical phenotype of an organism which can be used to monitor and assess gene function (Fiehn et al. 2000) or a system's response (Weckwerth 2003). Although mRNA/transcripts represent a mechanism for information transmission from the genome to the cellular machinery for protein synthesis, mRNA levels do not always correlate well with protein levels (Gygi et al. 1999). Furthermore, once translated a protein may or may not be enzymatically active as post translational modifications, protein sorting, protein–protein interactions, and controlled proteolysis all contribute to the regulation of active enzyme levels. Due to these factors, changes in the transcriptome or the proteome may not always lead to alterations in the metabolic phenotype. In addition, the majority of transcript and protein annotations are currently inferred based on sequence or structural similarity. It is estimated that less than 10% of annotated genes have experimental evidence supporting assigned function and thus, the accuracy of these annotations are of some uncertainty (Somerville and Somerville 1999; Somerville and Dangl 2000). In the absence of functionally annotated database information, transcript or protein profiling often yields limited information. For example, transcriptomics or proteomics often reveal the differential accumulation of a hypothetical or unannotated protein; however, without annotation it is very difficult to infer biological context. Microarray or proteomics experiments may also yield putative or generic protein identifications such as a putative peroxidase or peroxidase-like protein. These generic annotations have limited information as many of these enzymes are promiscuous and/or involved in a large number of different reactions. However, metabolomics has the ability to reveal that the accumulated peroxidase/enzyme is more specifically related to lignification or to another specific biochemistry. Thus, profiling the metabolome may actually provide the most direct and "functional" information of the "omics" technologies.

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The plant metabolome is quite complex with current estimates on the order of 15,000 metabolites within a given species and over 200,000 different metabolites within the plant kingdom (Dixon 2001; Hartman et al. 2005). Due to the chemical complexity of the plant metabolome, it is generally accepted that a single analytical technique will not provide comprehensive visualization of the metabolome, and therefore, multiple technologies are generally employed. The selection of the most suitable technology is generally a compromise between speed, chemical selectivity and instrumental sensitivity. Tools such as nuclear magnetic resonance spectroscopy (NMR) are rapid, highly selective, and non-destructive, but have relatively lower sensitivity. Other methods such as capillary electrophoresis (CE) coupled to laser induced fluorescence (LIF) detection are highly sensitive, but have limited chemical selectivity. Chromatographically coupled mass spectrometry methods such as gas chromatography (GC)/mass spectrometry (MS) and liquid chromatography (LC)/MS offer the best combination of sensitivity and selectivity, and therefore are central to most metabolomics approaches. Mass selective detection provides highly specific chemical information including molecular mass and/or characteristic fragment ion(s) information that are directly related to chemical structure. This information can be utilized for compound identification through spectral matching with data compiled in libraries for authentic compounds or used for de novo structural elucidation. Further, chemically selective MS information can be obtained from extremely small metabolite quantities with limits of detection in the pmole and fmole level for many primary and secondary plant metabolites.

GC/MS has proven capability for profiling large numbers of metabolites with reports covering several hundred to slightly more than a thousand various components (Fiehn et al. 2000; Roessner et al. 2000, 2001; Birkemeyer et al. 2003; Wagner et al. 2003; Broeckling et al. 2005; Schauer et al. 2005; Welthagen et al. 2005). The term component is used because a large number of metabolites often yield more than one derivatized component which are observed in the GC/MS analysis. The achievable range and number of metabolites profiled by GC/MS can be attributed to the high separation efficiencies of long (30−60 m) capillary GC columns (i. e. $N \geq 250{,}000$ for 60 m). These high efficiencies enable the separation of very complex mixtures, and with mass selective detection, qualitative identification of a significant proportion of these compounds is achievable. This makes GC/MS a very efficient and cost effective metabolomics tool. A major prerequisite for GC/MS is sample volatility which is necessary to enable separation in the gas phase. Analytes may be innately volatile or chemically derivatized to yield volatile compounds. Unfortunately, there exist a large number of metabolites which are not amenable to GC/MS even following derivatization. These include compounds such as phenylpropanoid and other natural product glycosides whose labile glycosidic bonds degrade during heating and vaporization. Thus, alternative techniques are necessary and especially so for the study of secondary metabolism.

Liquid introduction techniques for mass spectrometry such as electrospray ionization, atmospheric chemical ionization, and photo ionization remove the necessity for chemical derivatization. Thus, aqueous samples can be analyzed with minimal sample processing or even directly from the tissue source (Takats et al. 2004). Further, these techniques allow for the analyses of more labile and larger metabolites, and for the coupling of liquid separation technologies to mass spectrometry. Therefore high performance liquid chromatography (HPLC) and CE are readily coupled to mass spectrometry to yield powerful tools for targeted metabolic profiling and non-targeted metabolomics.

The utility of LC/MS emanates from the coupling of a 'universal' separation technology to a selective and sensitive mass analyzer detector. HPLC is commonly considered a universal separation technique because of its applicability to a broad range of chemical classes with a diversity of physical and chemical properties. For example, HPLC has been utilized for the analysis of ionic compounds, inorganics, volatile organics, polar organics, non-polar organics, lipids, amino acids, carbohydrates, nucleotides, carotenoids, phenylpropanoids, hormones, peptides, proteins, and the list goes on. The major point is that HPLC can be used for many of those compounds commonly analyzed by GC and many more. LC/MS also removes the need for derivatization and thus, complex samples can be analyzed directly or with minimal sample processing. As a result of these favorable properties, it is not surprising that LC/MS and LC coupled to tandem mass spectrometry (LC/MS/MS) have become popular tools for metabolism investigations.

HPLC is performed on various scales utilizing different column sizes. General values are provided in Table 1 for preparative, analytical, micro, capillary and nano-scale modes of HPLC. Generally, preparative scale HPLC is used for compound(s) purification and analytical scale is traditionally used for the quantitative analyses of plant extracts. However, smaller scale technologies (micro, capillary, nano) are now commercially available for quantitative analyses. These smaller scale separations offer significant sensitivity enhancements, and thus reduce the amount of material necessary for analysis. Further, capillary and nano HPLC often offer increased chromatographic resolution. Unfortunately as the separation scale gets smaller it becomes more difficult to reproducibly generate mobile phase gradients and the retention time variance increases. However, this problem is continually decreasing as novel instrumentation and approaches become available.

Table 1. General liquid chromatographic scales

Scale	Column internal diameter	Flow rate
Preparative	2.1–>200 mm	10 mL/min,
Analytical (*conventional*)	2.1–4.6 mm	1.0 mL/min
Micro	1.0 mm	200 µL/min
Capillary	300 µm–1 mm id	4 µL/min
Nano	25–300 µm id	200 nL/min

2 Chromatography Theory

Currently, the chromatographic performance of HPLC, relative to GC and CE, is lower, and there is a significant need for improvement. However, to discuss this issue and possible improvements in detail, several terms must be defined. A number of quantifiers are used to assess chromatographic performance. These include resolution (R_s), selectivity (α), efficiency (N), and peak capacity (n) which are defined below:

1. *Resolution* (R_s) is a quantifier of the degree of separation between mixture components, i. e. two peaks t_a and t_b with peak widths at the base w_a and w_b. A resolution of 1 indicates that two adjacent peaks are baseline resolved. Resolution can also be expressed as a function of the theoretical plate number (N) and selectivity (α) as defined below in Eq. (1):

$$R_s = \frac{2(t_b - t_a)}{w_a + w_b} = \frac{2\Delta t_R}{w_a + w_b} \qquad R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_2}\right) \tag{1}$$

2. *Selectivity* (α), which is also referred to as the separation factor, is a ratio of the retention or capacity factor (k') of two peaks. The capacity factor is a relative retention parameter that has been normalized using the void elution time (t_v) or volume (V_v) and is therefore independent of column geometry – see Eq. (2). The void value is the volume or time of an unretained component. The selectivity parameter provides a quantifier of the relative separation of two components. Selectivity can be altered based on the chemical composition of the stationary phase, stationary phase manufacturer, mobile phase, and pH:

$$\alpha = \frac{k'_2}{k'_1} \qquad k'_1 = \frac{t_1 - t_v}{t_v} = \frac{V_1 - V_2}{V_v} \tag{2}$$

3. *Column efficiency* is usually quantified based upon a column's theoretical plate number (N) which is unitless and a measure of band broadening per unit time – see Eq. (3). This can be practically quantified using retention time (t_R) and peak width. Peak width can be defined at the base (W_b) or at half height ($w_{1/2}$) as they are directly related if one assumes a Gaussian peak shape, i. e. $W_b = 1.698\ w_{1/2} = 4\sigma$ where σ equals the standard deviation of the peak. Alternatively, plate number can be calculated using the column resolution (R) and selectivity (α).
4. *Separation efficiency* is also quantified using a normalized theoretical plate number based on column length, i. e. (N/L) with units of plates/m. The theoretical plate number can be dramatically increased by decreasing the peak width. Plate number and efficiency are also related to particle size (d_p) and column length (L) as described below:

$$N = \left(\frac{t'_R}{\sigma}\right)^2 = 16\left(\frac{t'_R}{W_b}\right)^2 = 5.54\left(\frac{t'_R}{w_{1/2}}\right) = \frac{16R^2}{(1 - \alpha)^2} = \frac{L}{d_p} \tag{3}$$

5. *Peak capacity* (n) is a measure of the maximum number of theoretical peaks resolvable by the chromatographic system based on optimum performance and equal variation in the partitioning of all components in the mixture – see Eq. (4). The peak capacity is a good parameter for estimating the maximum number of compounds resolvable by a given chromatographic system. Ideally this value should approach or exceed the number of compounds that need to be separated, i. e. the number of metabolites:

$$n = \frac{\sqrt{N}}{4R} \ln\left(\frac{t_2}{t_1}\right) + 1 \tag{4}$$

3 Limitations of Current Metabolic Profiling Approaches and Proposed Solutions to Advance Metabolomics

Currently, the major limitation of metabolomics is its inability to comprehensively profile all of the metabolome. This inability is directly related to the chemical complexity of the metabolome, the biological variance inherent in most living organisms, and the dynamic range limitations of most instrumental approaches (Sumner et al. 2003). Many biological responses to altered gene expression or to environmental stimuli result in both quantitative and qualitative changes in metabolite pools. Understanding these responses is most dependent upon the qualitative identification of the altered metabolite. Quantitative measurements are also important, as both temporal and spatial changes in metabolite concentrations are expected; however this information is of little use if it cannot be assigned to a specific metabolite or biological process. Thus, comprehensive qualitative and quantitative analysis of all metabolites within a cell, tissue or organ is the ultimate goal of metabolomics; however, this is still a very ambitious goal and far from a reality for any system. Bino and colleagues (Bino et al. 2004) proposed two major objectives to increase the comprehensive nature of metabolomics. They were:

1. Increase the current capacity for metabolite separation and differentiation (i. e. the number of resolvable components within the complex metabolome mixture) using multi-dimensional separations.
2. Increase the number of identifiable metabolites through the generation of spectral libraries, high resolution accurate mass measurements, and tandem mass spectrometry.

Unfortunately, the separation of complex metabolome mixtures is still quite challenging. Currently, analytical scale HPLC (4.6 × 250 mm) is most commonly used for natural product analyses; however, the upper peak capacities (i. e. theoretical number of maximum peaks resolvable based on optimum performance) of these systems is approximately 300 (Tanaka et al. 2004). Based on this estimate, a maximum of 300 components could be resolved in a best

case scenario; however in practice this value is seldom achieved and more realistic peak capacities are between 100 and 200. Thus, current HPLC technologies are limiting the comprehensive scope of metabolomics. Separation efficiencies can be improved by altering selectivity, increasing column lengths, reducing particle sizes, increasing temperature, and/or alternative column materials. Alternatively, the utilization of multidimensional chromatography offers increased HPLC peak capacities of greater than 1000 to provide more comprehensive coverage of plant natural products (Tanaka et al. 2004). Each of methods to increase HPLC efficiency is discussed below.

Typically, improving selectivity is the best approach to improving chromatographic resolution. Selectivity is based upon the chemical or physical interaction properties that are fundamental to the separation process. More precisely, the separation selectivity of specific components can be optimized by the appropriate choice of column materials, mobile phases, and/or manufacturer. Various generic and proprietary materials are available for various chromatographic modes for HPLC. Example modes include ion-exchange, normal-phase, reverse-phase, hydrophilic interaction, and size exclusion chromatography. All HPLC columns are not equal, and different particles, particle sizes, surface modification chemistries, surface coverage, and packing processes vary significantly from manufacturer to manufacturer. These parameters dramatically influence chromatographic performance.

Often selectivity is optimized for a targeted set of analytes as a means of increasing resolution. However, in more complex mixtures associated with global metabolomics-based approaches, improved selectivity for one class of compounds often results in decreased selectivity for others. Thus, techniques (e. g. reverse-phase chromatography) with a broad range of selectivity are most likely to be the best choices for metabolomics.

One of the simplest means of increasing resolution is to increase the number of theoretical plates. Since the plate number is directly proportional to the column length (Eq. (3)), one needs only to increase the column length to increase resolution. However, Eq. (1) tells us that R is proportional to the square root of N. Thus, to achieve a 2× increase in resolution, we would have to square the column length. For example a 250 mm long column would need to be extended to 625 cm (i. e. 25 × 25 cm) for a twofold increase in resolution. Unfortunately, this is not a practical solution as the operating pressure is directly proportional to the column length. Equation (5) defines the relationship between pressure (ΔP), column length (L), analyte diffusion coefficient (D_m), particle size (d_p), mobile-phase viscosity (η), and column permeability (K^o):

$$\Delta P = \left(\frac{L v D_m}{d_p}\right)\frac{\eta}{K^o} \tag{5}$$

If a typical column of 25 cm has an operational pressure of 3000 pounds per square inch (p.s.i.), then a twofold resolution increase obtained by squaring the

column length $(25\ \text{cm})^2$ would require an operational pressure of 75,000 p.s.i. (i. e. 3,000 p.s.i. × 25). Although this illustrates the advantage of very high pressure liquid chromatography which has been achieved by select groups using custom apparatuses (MacNair et al. 1997, 1999; Tolley et al. 2001; Patel et al. 2004; Shen et al. 2005), commercial pumps do not operate at these pressures (most commercial HPLC pumps have a 5,000-p.s.i. limit). Therefore, significant resolution enhancements achieved through longer columns is limited for most researchers. With that said, several companies (i. e. Waters and JASCO) have recently introduced 15,000-p.s.i. HPLC pumps.

Equation (5) reveals that the pressure differential is proportional to the mobile phase viscosity (η). Thus, lowering of the mobile phase viscosity (η) by increasing the temperature can lower the operational pressure and allow the use of longer columns for resolution enhancement (Djordjevic et al. 1998, 1999, 2000). Selectivity is also affected by temperature and additional efficiency can be achieved by heating alone. However, one must ensure analyte thermal stability if elevated temperature separations are to be employed.

Equation (5) also shows that the pressure is a function of the column permeability (K^o). New monolithic columns offer greater permeability and lower pressures, thus allowing for the use of longer columns. The continuous bed stationary phases of these columns consist of porous polymeric materials generated from silica or organic materials such as acrylamide, styrene, acrylate, or methacrylate monomers which result in lower back-pressure than packed particles. The lower back-pressure allows for the use of longer columns and hence greater efficiencies. Several groups have reported on the use of up to 1 m capillary columns (Que and Novotny 2002; Legido-Quigley et al. 2003; Tolstikov et al. 2003; Tanaka et al. 2004) and this technology looks promising.

Plate number and efficiency are also related to particle size (d_p) and column length (L) as shown in Eq. (3). This equation shows that decreasing the particle size increases the theoretical plate number/efficiency (MacNair et al. 1997, 1999; Tolley et al. 2001; Shen et al. 2005). However, Eq. (5) shows again that pressure increases with smaller particle size. Fortunately, new commercial ultra-high pressure liquid chromatography pumps (UPLC) are now available from multiple manufacturers that allow the use of smaller particles in the range of 1−2 μm. These instruments offer substantial resolution enhancements with plate numbers on the order of several hundred thousand and peak capacities in excess of 400 (Wilson et al. 2005). In addition to increased resolution, UPLC also offers higher speed separations as the optimum flow velocity has a significantly broader range which allows for increased flow rates without significant loss of resolution (Wilson et al. 2005). Estimates of up to ninefold increases in flow rates without significant loss of resolution have been suggested (Wilson et al. 2005). It is important to note that ultra-high pressure separations result in increased frictional heating; however this can be reduced by down-scaling the chromatography dimensions with the heating being negligible in columns of less than 1 mm (MacNair et al. 1997).

4 Future Directions and Forward-Looking Thoughts

Although several of the above principles can be used to achieve enhanced chromatographic resolution, the resolution enhancements are still far from that which is needed for very complex metabolomics mixtures. To separate these mixtures, peak capacities of thousands to tens of thousands are necessary. Currently, only multidimensional chromatographic methods offer peak capacities of this magnitude (Mondello et al. 2002; Evans and Jorgenson 2004). Multidimensional chromatography utilizes combinations of two or more separation mechanisms with different selectivity, e. g. ion-exchange and reverse-phase or capillary electrophoresis and reverse-phase LC. These systems offer enhanced resolution due to the utilization of multiple columns with independent chemistries which expands the selectivity of the method. Recall that selectivity improvements can dramatically improve resolution. The maximum peak capacity of a multidimensional system is the product of the two or more individual separation dimensions. For example, a realistic system that has a peak capacity in the first dimension (n_y) of 150 and the peak capacity in the second dimension (n_z) of 50, then the total maximum peak capacity of the multidimensional system is $n_y \times n_z = 150 \times 50 = 7500$. If one considers that an individual metabolome consists of 15,000 metabolites, then one recognizes that this is a considerable increase in comprehensive coverage.

Multidimensional LC-LC separations have been capitalized upon in the area of proteomics and are often referred to as multidimensional protein identification technology (i. e. MUDPIT; Washburn et al. 2001; Wolters et al. 2001); however multidimensional separations have only recently been pursued for metabolomics using GC×GC/time-of-flight (TOF)-MS (Welthagen et al. 2005). Unfortunately, these complex separations will come with increased analysis times, but I believe they will be worth the additional temporal costs.

The above discussion focuses on homogenous multidimensional separations (i. e. LC×LC/MS or GC×GC/MS, but multidimensional LC×GC separations are possible. In fact, the combination of these technologies is commonly referred to as unified chromatography (Chester and Parcher 2001; Chester and Pinkston 2002; Wells et al. 2002, 2003; Luo et al. 2003) and often associated with supercritical fluid chromatography (Chester and Parcher 2001; Chester and Pinkston 2002; Mondello et al. 2002; Wells et al. 2002, 2003; Luo et al. 2003). Although this technology is conceptually exciting, it is still somewhat empirically limited. Another possible LC×GC approach would be to couple HPLC with ion mobility mass (IMS) TOF-MS spectrometry (Verbeck et al. 2002; Guevremont 2004; Liu et al. 2004; Shvartsburg et al. 2005). In this configuration, analytes are ionized as they elute from the HPLC and an electrostatic field propels the analyte ions through a gas field maintained at elevated, atmospheric, or sub ambient pressures. Ions of different size and geometric structure traverse the gas field at different rates dependent upon their charge and collisional cross section therefore allowing separation. The LC-IMS method has been demonstrated for proteomics (Lee et al. 2002; Matz et al. 2002; Liu et al.

2004) and more recently applied to metabolite analyses (Kapron et al. 2005). Extension of this concept to metabolomics will surely occur.

The above text discusses multidimensional chromatographic approaches in an on-line context. However, multidimensional approaches can also be pursued in an off-line, multiplexed, or parallel approach. For example, fractions can be collected off-line using a separate HPLC. The fractions can then be concentrated and reinjected onto an on-line LC/MS system. Alternately, fractions of the same samples could be injected onto a series of parallel systems using different methods (i. e. GC/MS, LC/MS, or various selective modes of each performed with different column selectivities). This is our current approach. For example, samples are fractionated and/or enriched and then the polar and lipophilic fractions are analyzed by GC/MS. In addition methanolic extracts are analyzed for phenolic/saponin content. An interesting concept would be to design a multiplexed system, with multiple chromatographic-mass spectrometry systems operating in an integrated manner. For example, a multiplexed chip system with each chip having a slightly different selectivity and independent mass analyzer could be designed to increase the comprehensive coverage. Such a system with on-line enrichment could also be used to address dynamic range limitations that currently exist for specific compound classes such as phytohormones.

If higher resolution chromatography is obtained, mass analyzers must also be employed with compatible scans speeds to record data for compounds eluting in very short temporal periods. It is expected that LC peak widths of 1–5 s will be routine in the very near future. For accurate quantification, it is commonly accepted that the sampling rate should be sufficient to capture 10 data points across the eluting peak with higher sampling rates being beneficial. Thus, sampling rates should be less than 0.1 s or greater than 10 Hz. This is achievable with current TOF-MS analyzers. It is worth mentioning that quadrupole based mass analyzers, including traps, can approach these speeds; however, TOF mass spectrometers equipped with delayed extraction and ion-reflectrons also offer improved mass accuracy over quadrupoles.

Improvements in the accuracy of the mass analyzer can further enhance metabolite differentiation, elemental composition determination, identification, and allow for the profiling of greater numbers of metabolites. Mass accuracy is directly related to the mass resolution or the ability of the mass analyzer to resolve compounds of different m/z values. Mass resolution is defined in Eq. (6) and is a function of mass (M) divided by the peak width (ΔM) which is most commonly defined at half-height:

$$R_m = \frac{M}{\Delta M} \tag{6}$$

Often, LC/MS is performed with ion-traps or quadrupole mass analyzers that yield mass accuracies in the range of 1.0–0.1 Da. Unfortunately, many metabolites have similar nominal masses which can not be differentiated at this level of mass accuracy. For example, the important natural products genistein and

medicarpin have similar nominal masses of 270, but have different accurate masses of 270.2390 ($C_{15}H_{10}O_5$) and 270.2830 ($C_{16}H_{14}O_4$) respectively due to different chemical compositions. If one could measure their mass with sufficient accuracy, then one could differentiate these compounds in the mass domain even if they could not be physically separated in the chromatographic domain. This mass differentiation can be achieved at a mass resolution ($M/\Delta M$) greater than 6136. Compounds with closer accurate masses such as rutin ($C_{27}H_{30}O_{16}$ = 610.5180) and hesperidin ($C_{28}H_{34}O_{15}$ = 610.5620) would require a higher mass resolution of 13,864 for their differentiation. Mass resolutions on the order of 10,000 can be achieved with modern TOF-MS analyzers, and resolutions in excess of 100,000 with sub-part-per-million mass accuracies (i. e. less than 0.001 at m/z of 1,000 Da) are achievable with Fourier transform ion cyclotron mass spectrometry (FTMS). Newer technologies, such as Thermo Electron Corporation's Orbitraps are currently surfacing that also offer high-resolution solutions. Although high resolution accurate mass measurements have great advantages, this technology is still rather costly.

Interestingly, a significant argument can be made that accurate mass measurements significantly reduce the need for ultra-high resolution separations due to the enhanced separation in the mass domain. However if the chromatography step is omitted or compressed significantly, then ion suppression, competitive ionization, and other matrix affects become increasingly more problematic. I personally believe that both improved chromatographic resolution and accurate mass measurements offer the best solution and that the combination of these techniques will provide greater comprehension and confidence in our ability to profile the metabolome. Further, I also believe that the needed magnitude of enhancements in chromatographic resolution can only be achieved with multidimensional approaches.

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