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Separation and mass spectrometry in microbial metabolomics

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Measurements of low molecular weight metabolites have been increasingly incorporated in the characterization of cellular physiology, qualitative studies in functional genomics, and stress response determination. The application of cutting edge analytical technologies to the measurement of metabolites and the changes in metabolite concentrations under defined conditions have helped illuminate the effects of perturbations in pathways of interest, such as the tricarboxylic acid cycle, as well as unbiased characterizations of microbial stress responses as a whole. Owing to the complexity of microbial metabolite extracts and the large number of metabolites therein, advanced and high-throughput separation techniques in gas chromatography, liquid chromatography, and capillary electrophoresis have been coupled to mass spectrometry – usually high-resolution mass spectrometry, but not exclusively – to make these measurements.

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Introduction

Genomics, proteomics, and transcriptomics have all made considerable contributions to the field of functional genomics. However, understanding of the genome, transcriptome, and proteome is not enough to fully characterize cellular function. For example, the proteome cannot be completely predicted from the transcriptome owing to differences in regulatory mechanisms at the protein level (e.g. post-translational modifications). Furthermore, an approach based solely on transcriptomics will also be inadequate, since there are many genes that are not under transcriptional control.

The metabolome, however, is further from gene expression and, thus, more closely reflects the activities of a cell at a functional level. Furthermore, many metabolites are not exclusively involved in a single metabolic pathway, so it is only when the metabolome is characterized as a whole – or all associated metabolic pathways – that the pathway(s) of interest can be identified with a high degree of certainty. The time it takes for the metabolome to reflect a change may vary depending on the perturbation in question, thus the timing of sample acquisition and the methods used to identify and quantitate the metabolome are crucial.

A universal quenching and extraction protocol for microbes does not yet exist; however, a detailed review of such procedures has been written recently [1]. Previously the extraction of phosphorylated compounds has proven difficult, which is problematic because of the importance of phosphorylated compounds in metabolism (e.g. ATP). This difficulty is many-faceted owing to the potential for cleavage of phosphate groups in a highly aqueous environment [2], as a result of the interaction with phospholipids in the cell [3•], and because triphosphates are readily hydrolyzed (enzymatically or non-enzymatically) even after exposure of the cells to organic solvent [4]. Despite this, extraction procedures have been developed to improve the yield of phosphorylated compounds and continue to be improved [2–4].

Owing to the wide range of physiochemical properties and concentration ranges of metabolites there is no one method that can separate, detect, and identify all known metabolites. Mass spectrometry (MS) is a popular tool that, given the complexity of microbial metabolic extracts, requires a chromatographic separation to reduce isobaric interferences (i.e. compounds of the same mass being indistinguishable in the mass spectrometer) and ion suppression (i.e. more easily ionizable species masking the presence of less ionizable species). In this review we discuss several chromatographic techniques that compliment each other because they are able to resolve compounds of differing physiochemical properties.

Metabolomics by GC–MS

Gas chromatography coupled to mass spectrometry (GC–MS) is a very popular tool within the field of metabolomics [5–7,8••]. The majority of the GC–MS metabolite profiling applications are focused on plant metabolomics rather than microbiological or biomedical research. Since all plant metabolites originate from the plant machinery itself [9], interpretation of metabolite profiles in plants are more straightforward than for microorganisms and mammalian cells. However, rapid development in the field of metabolic engineering and recent advances in analytical techniques have resulted a growing focus in microbial metabolomics [1,10•].

While MS provides individual mass spectra that can differentiate between co-eluting metabolites that are chemically diverse, the main advantages of GC over other separation techniques are a high separation efficiency (as highlighted by its ability to distinguish isomeric compounds), ease of use, robustness, and low cost [6,7,8••]. GC–MS is a technique that usually yields extensive and highly reproducible fragmentation because of the standardized use of electron ionization (EI), which has enabled metabolites to be identified by matching their relative retention times, retention or Kovats indices, and mass spectral fragmentation patterns, to known and predicted information available from extensive databases (e.g. the NIST and Wiley database) [5]. It is noteworthy that the large amount of chromatographic and spectral data generated in metabolomics by GC–MS requires special de-convolution and identification software platforms (e.g. AMDIS, AnalyzerPro and LECO ChromaTOF) [11,12].

A major drawback of GC–MS is that analytes are required to be volatile. Since a large number of metabolites are non-volatile, time-consuming derivatization steps are required [11,13]. Furthermore, thermally labile compounds, such as phosphorylated metabolites, can easily degrade when exposed to the high temperatures in the GC oven. Additionally, metabolites can have varying affinities for derivatizing agents, which could lead to inaccurate quantification unless derivatized chemical standards and data correction strategies are used to normalize for such a bias [14]. One must also be aware of the formation of byproducts from the derivatization procedure [15], as well as the possible conversion of analytes (e.g. arginine to ornithine, cyclization of open-chain sugars, decarboxylation of alpha-ketoacids, etc.) and/or degradation of the final product(s) (e.g. the hydrolysis of trimethylsilyl derivatives), which could lead to misinterpretation of the data generated. A two-step derivatization method (e.g. methoximation followed by silylation), can provide a wider coverage of analyzable metabolites [16].

Although GC–MS is a well-established analytical technique and most of its metabolomics publications focus on applications rather than method development and/or optimization [17,18,19••], the extremely complex nature of biological samples required enhanced separation performance and improved instrumentation, which led to the development of multidimensional separation techniques, such as GC–GC time of flight (TOF)–MS [18,20]. This novel approach presents a dramatically improved peak separation capacity and an increase in sensitivity, while TOF–MS provides a very fast scanning rate and additional sensitivity for improved detection. Furthermore, the application of two different GC columns (i.e. polar/non-polar) can provide greater metabolite detection coverage for metabolomics analysis, via improved selectivity [21–23]. Despite this recent advance, GC–GC–TOF–MS is not yet routinely used in metabolomics partly because of practical problems (e.g. optimization of fraction modulation) and partly because of high instrument cost.

Metabolomics by LC–MS

The combination of GC–MS with liquid chromatography coupled to mass spectrometry (LC–MS) can provide greater coverage of the metabolome [24]. Smilde *et al.* were able to detect a very high number (93%) of the commercially available metabolites of the *in silico* metabolome of *Bacillus subtilis* and *Escherichia coli*. Similar coverage (95–97%) was achieved for the same microorganisms and *Saccharomyces cervisiae* with the application of six different analytical methods [10•]. It must be noted, however, that only around half of the estimated metabolites were commercially available as reference compounds, which confers a higher degree of confidence in the identification of metabolites.

As a standalone technique, LC–MS is still prominent among the technologies currently available to perform metabolic profiling not only because there is no need to derivatize analytes, but also because LC has demonstrated its ability to resolve large numbers of metabolites [25,26]. LC separations that are compatible with electrospray ionization (ESI) are desirable because of the polar and ionizable nature of most metabolites [10•]. Even though ESI is the most commonly used ionization technique with LC, techniques such as APCI [27] and APPI [28] are becoming increasingly popular due to their applicability to less polar metabolites. Additionally, LC is an attractive alternative to GC because it is a particularly versatile separation technique: varying the stationary and mobile phases through approaches like ion pairing (IP) LC–MS [29], hydrophilic interaction liquid chromatography (HILIC) MS [24,30], and reverse phase LC–MS [31–33] allows for the simultaneous quantitative analysis of different classes of important metabolites.

The HILIC–MS obtained metabolic fingerprint of starvation stress response by *E. coli* and *Saccharomyces cervisiae* [34•] is an example of the benefit of LC–MS

because the metabolites did not require derivatization. Upon carbon or nitrogen starvation, the changes in concentration of 68 cellular metabolites were measured. Quantitative changes in metabolic profiling have also been measured with an isotopic dilution strategy [35], where fully isotopically labeled metabolites are used as internal standards. LC–ESI–MS–MS analysis of these pooled samples was utilized to measure glycolytic and tricarboxylic acid cycle intermediates in *Saccharomyces cervisiae* following a glucose pulse. In addition to quantification, isotopic labeling has also been used to assign molecular formulae to single compounds [36•].

Metabolomics by CE–MS

Capillary electrophoresis (CE) offers several potential advantages over GC and LC for the analysis of complex mixtures of metabolites, including even higher separation efficiencies, extremely small sample injection volumes (nL range), rapid method development, and low reagent costs. CE–MS is commonly utilized with electrospray ionization (ESI) and, like LC–MS, can also be used for structural elucidation via tandem mass spectrometry [37].

CE may be used to perform highly efficient separations of a wide range of sample types, and can even be used to separate intact microorganisms as well [38]. The simplest and most commonly applied mode of CE is capillary zone electrophoresis (CZE), which has been used to separate charged analytes from the lysate of microorganisms [3•,39,40]. The incorporation of additives, such as surfactants, into the separation buffer can be used to separate neutral and charged compounds via Micellar Electrokinetic Chromatography (MEKC) [41]. Both APCI [27] and APPI [28] have been coupled with CE to measure less polar compounds, and the latter has also been coupled to MEKC separations, which are not normally compatible with ESI [28]. Chiral compounds can also be separated by adding cyclodextrins to the separation buffer [42]. Both MEKC and the chiral method have been used as quality control assays to determine the level of impurities that have arisen from the production of amino acids by fermentation [43].

Recently, a comprehensive and quantitative survey of anionic and cationic metabolites from *E. coli* was conducted via CE–MS [3•]. From the results obtained, 375 charged hydrophilic intermediates in primary metabolism were identified, of which 198 were quantified, further cementing the notion that CE–MS can make a major contribution to the field of microbial metabolomics. CE–MS has also been used in functional genomics studies on various microorganisms, including response to antibiotics [44], salt [45], and cadmium stress [40].

The main limitation of CE is the lack of sensitivity due to small sample injection volumes, especially when coupled to MS. The sample can be further diluted by a sheath liquid. However, the combination of a reduced sheath flow rate and the employment of online sample preconcentration procedures, such as pH-mediated stacking and transient isotachophoresis [46], can achieve sensitivities similar to that of current LC–MS protocols. Furthermore, the dilution effect brought about by the sheath flow can be avoided entirely by using a sheathless interface [47].

Current CE–MS protocols that utilize an acidic electrolyte, such as formic acid, appear to be among the best approaches for measuring cationic species, as they can yield highly efficient separations, with extremely good sensitivity [3•,39,40]. The measurement of anions appears to be more difficult, primarily because a reversal of the electroosmotic flow (EOF) is often required to achieve highly efficient separations [3•]. Furthermore, this separation strategy requires the use of rather expensive coated fused silica capillaries. In 2006, however, Harada *et al.* published a method that utilized conventional CE separation with an EOF and a supplementary pressure gradient to achieve highly efficient separations of coenzyme-As, organic acids, nucleotides and sugar phosphates [48•]. A similar approach was also used to measure sugar phosphates, including regioisomers such as hexose-6-phosphate and hexose-1-phosphate, from the lysate of *E. coli* [37].

The characterization of unknown metabolites is one of the biggest challenges that face metabolomics today. The current strategy used by most laboratories is to utilize the relative migration times of chemical standards, together with mass spectral information, for the identification of unknown metabolites. Such a strategy requires a large chemical standards library, which can be extremely costly. To circumvent this problem, a computer-simulated algorithm was developed to predict the CE migration profile of charged analytes [49•]. Physicochemical properties such as mobility and acid dissociation constants were used for the simulation. There was excellent agreement between experimental and simulated relative migration times, with an average error of $<2\%$. Thus, prediction simulations would ensure the characterization of metabolites that are not readily synthesized or commercially available [49•].

Data mining

Metabolomics data sets are complex owing to the large amounts of data that are generated in three dimensions (i.e. the mass spectra of individual components, their retention times and their intensities). The main obstacle is the extraction of all the useful information that is present in the raw data. While peak de-convolution algorithms have been developed, automated peak picking and integration still remains an important challenge. This is complicated by the wide range of peak intensities, peak asymmetry, and spectral interferences (which is connected to the resolving power of a mass analyzer and

the abundance of an analyte), all of which can lead to incorrect assignments of differences [50]. Current software packages that can perform data extraction from normalized mass spectra and total ion chromatograms include Agilent MassHunter MFE, Bruker Metabolite Detect, ACD/Labs IntelliXtract/CODA and NIST AMDIS.

Extensive libraries are required to identify all the mined data. There are well-established spectral libraries for GC–MS that contain up to 606,000 spectra (Palisade 600k Ed.). However, in the case of LC–MS and CE–MS, it is more difficult to obtain a standardized spectral library owing to a high instrument dependent variability and variable source conditions. Thus, laboratories are normally responsible for building up their own spectral libraries.

The final step to complete a metabolomics study is the application of relevant statistical tests in order to determine metabolic differences or target components that are characteristic of a sample and/or conditions [50]. The use of post-data collection statistical methods can greatly minimize variations in analytical observations such as retention time, mass accuracy, and signal intensity [51]. The most rigorous statistical method is multivariate data analysis [52].

Application of metabolomics tools for isotopomer-based flux analysis

Metabolic flux analysis via ^{13}C labeling is a high-throughput technology to quantitatively track metabolic pathways and determine overall enzyme function in cells. Metabolic fluxes are the functional output of the combined transcriptome, proteome, and metabolome. These data bridge contemporary functional analyses of the cellular phenotype [53,54]. The essence of ^{13}C-based metabolic flux analysis is the precise measurement of the labeling patterns of targeted metabolites from tracer experiments to determine the complex metabolic network [53,55]. Traditionally, isotopomer analysis of metabolites is mainly carried out by the measurement of proteinogenic amino acids via nuclear magnetic resonance (NMR) spectroscopy [56–58] or GC–MS [13,59,60,61•,62,63].

Recently, high-resolution and highly sensitive mass spectrometers have been used to precisely measure the labeling pattern of both amino acids and metabolites in central metabolic pathways at nanomolar concentrations. LC–MS–MS has been used for the determination of intracellular amino acids to profile metabolic flux changes during fed-batch cultivation [64•]. CE coupled to time-of-flight MS (CE–TOF–MS) has been applied to measure the isotopomer distribution of 13 unstable metabolites in central metabolism, including some unstable phosphorylated molecules such as 3-P-glycerate, phospho*eno*/pyruvate, and ribose-5-P [65•]. Direct infusion via ESI coupled to Fourier transform ion cyclotron resonance MS (FT-ICR MS) was able to measure the metabolite isotopomer distribution in a biomass hydrolysate of *Desulfovibrio vulgaris* Hildenborough, unveiling an unusual citrate synthase activity [66,67]. Labeling measurements of metabolites other than amino acids should enable the flux analysis of more complicated metabolic networks (such as mammalian cells) and therefore improve the accuracy of flux determination.

Conclusions

As metabolic quenching and extraction protocols are improved, the vast array of separation techniques that can be coupled to MS in the pursuit of metabolomic analysis will have to be further developed. Presently there is no one technique that seems capable of easily resolving the hundreds of metabolites present in microbial extracts. Although there are academic endeavors to accomplish exactly that, the most comprehensive metabolite coverage has come from the combination of using multiple and overlapping separations that compliment each other in their ability to resolve compounds of differing physiochemical properties. Furthermore, metabolome quantitation is complicated by the lack of comprehensive and automated data mining software, the need to perform statistical analyses to minimize the effects of variations in analytical observations, and may miss the mark entirely when drawing conclusions about cellular activity and function without the application of flux analysis to the results. In a rather short period, however, the field of metabolomics has blossomed from a seemingly impossible undertaking to a fruitful laboratory practice that allows us to address scientific questions that were previously inaccessible.

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