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Analytical Chemistry Considerations in Plant Metabolomics

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Abstract: Metabolomics is defined as the comprehensive analysis of all the metabolites of a biological system, although there is considerable confusion in terminology. The task of metabolomics is not attainable with the current state of analytical science, and various approaches are used to accommodate this limitation and the associated complexity of the metabolome. Major advances and a totally new approach to analysis will be essential. Meanwhile, a number of techniques, namely, metabolite profiling, metabolite fingerprinting and target analysis are combined to make this task possible. There is an implicit assumption in these approaches that the data are complementary and that the metabolome can be assessed as a summation of individual data. The integrity of metabolomics data depends on state-of-the-art sample handling and measurement techniques. These ideas are developed in this review.

Keywords: Metabolomics, metabolome, metabolite profiling, plants, analysis, extraction

INTRODUCTION

The suffix "omics" relates to a particular scientific approach, and recently a new wave of omic technologies have been established, including transcriptomics and proteomics, in light of the phenomenal success of genomics. Metabolomics is also one of this new breed of omic technologies that is based on comprehensive biochemical and molecular characterizations of an organism,

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tissue or cell type (1). The term "metabolome" was first used by Olivier et al. in 1998 (2) to describe the set of metabolites synthesized by an organism; however, Goodacre (3) has limited the definition to "the quantitative complement of all of the low molecular weight molecules present in cells in a particular physiological or developmental state." Other definitions state that the metabolome of a cell represents the amplification and integration of signals from other functional genomic levels such as the transcriptome and proteome (4), and reflects the current focus on linking the various omic disciplines for a more integrated perspective of biological systems.

Metabolomics represents a major emerging research field, but there has been much confusion in the use of the term (5). The first instance of its use was in 2002 by Fiehn (6), in a fashion analogous to that of proteomics and transcriptomics, whereby *metabolomics* was defined as a comprehensive analysis in which all the metabolites of a biological system were identified and quantified. The related *metabonomics* was defined by Nicholson et al. (7) as the "quantitative measurement of the time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification." Fiehn defines metabonomics as relating to the coordinated channelling of substrates through tightly connected enzyme complexes (8), while Lindon (9) states that metabonomics measures the fingerprint of biochemical perturbations caused by disease, drugs and toxins.

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Ambiguity is compounded since the two terms are often used interchangeably, and additionally metabonomics and metabolomics have been described as subsets of each other (8, 9). Metabonomics and metabolomics have also been distinguished on the basis of the technology platform used for analyses; metabonomics being based on nuclear magnetic resonance (NMR) spectrometric analyses, with metabolomics reliant on mass spectrometric methods (1). This is not a particularly useful distinction and a unified approach is needed as exemplified by the Metabolomics Society, which aims to "bring together, under one roof, scientists from study areas representing metabolomics, metabonomics, metabolite target analysis, metabolic profiling, metabolic fingerprinting or foot printing, metabolic flux analysis, biochemical modelling and related informatics fields."

This review focuses on metabolomics (and metabolome analysis) and its application to plants. The number of metabolites in the plant system is estimated to exceed 200,000 (10) indicative of its complexity and the challenge faced in this field. It is interesting to compare this complexity with that faced in the genome (linear polymer of 4 nucleotides with very similar properties) and the proteome (structure based on 22 primary amino acids). The review addresses critical issues relating to the generation of metabolomic data from an analytical chemistry perspective, and describes how such data are critically dependent on sound analytical practices. Related reviews have detailed advances in methodologies and technologies (11), metabolite profiling (12), applications of mass spectrometry (MS) (13, 14) and NMR spectrometry (15). Recent reviews also focus on metabolomics

and its relevance to systems biology (16–18) and functional genomics (1, 19, 20).

CLASSIFICATION OF METABOLOME ANALYSES

Feihn (6) proposed that metabolome analysis can be conducted using four distinct approaches, namely, metabolite profiling, metabolite fingerprinting, target analysis and metabolomics. The primary goal of metabolomics is the unbiased relative quantification of *every* metabolite in a biological system (21). Thus, metabolomics in the strict sense is an impossible task due to the complexity of the metabolome and the analytical difficulties associated with its measurement. Metabolite profiling, metabolite fingerprinting and, to a lesser extent, target analysis, therefore, represent a compromised approach to the analysis of the metabolome. By their nature they provide a restrictive non-comprehensive view of the metabolome. Villas-Bôas et al. (5) argue that metabolomics should be reserved to describe the area of science rather than the analytical approach. However, progress in this "area of science" can only be achieved by developments in analytical science.

Metabolite profiling involves the identification and quantitation by a particular analytical procedure of a pre-defined set of metabolites of known or unknown identity and belonging to a selected metabolic pathway (5, 6, 11). Metabolite profiling represents the oldest and most established approach and can be considered the precursor for metabolomics. The earliest reports of metabolite profiling came from Baylor College of Medicine in the early 1970s and relate to the analysis of steroids, acids and urinary drug metabolites by GC-MS rather than to plants (for example (22, 23) cited in (1)). The potential of profiling techniques is demonstrated by the discovery of dihydrocaffeoyl polyamines in solanaceous species (24). A more typical example is provided by the analysis of the biophenolic profile of olives using high-performance liquid chromatography (HPLC) with UV detection (25, 26). This example illustrates the dependence of metabolite profiles on detection method in that different profiles are generated at different detection wavelengths. The use of a universal detector may eliminate some of this dependence. For example, evaporative light scattering and refractive index detection approximate universal detectors as does MS. However, some metabolites do not ionize optimally or even at all under fixed conditions in MS. There is a further method dependency (ignoring sample preparation steps) in that the column in both gas chromatography (GC) and liquid chromatography (LC) acts as a filter and not all components injected are necessarily eluted.

Metabolite fingerprinting aims to rapidly classify numerous samples according to relative distances using multivariate statistics (27), and Sumner et al. (1) have proposed that any technology whose output is processed with pattern recognition software and without differentiation of individual

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metabolites should be termed "metabolic fingerprinting." Quantitation is generally not involved. Halket et al. (28) have made a distinction between metabolite profiling and metabolite fingerprinting, where the latter refers to more rapid and general screening methods that are suitable for determining differences and classifying samples. An example of fingerprinting is provided by the LC and NMR of transgenic food crops (29).

Target analysis is constrained exclusively to the qualitative and quantitative analysis of a particular metabolite or metabolites (11). As a result, only a very small fraction of the metabolome is focused upon, signals from all other components being ignored (28). As a requirement, extensive sample preparation and cleanup may be necessary to eliminate interferents, and improve the sensitivity of the analytical procedure for the target analyte(s). Since the identity of the target metabolite(s) is known, general methods incorporate stable isotope-labelled internal standards (28) to aid in quantitative analysis.

Metabolomics in the true sense recognizes that a single genetic alteration may not be limited to one biochemical pathway (6), and so employs a comprehensive analytical approach that is non-selective and universally applicable to identify and quantify metabolites of a biological system. Unlike target analysis and metabolite profiling where the (generic) identities of the metabolites are known, metabolomics faces the challenge of systematically and comprehensively identifying unknown compounds from high-throughput analyses (6). This is distinct from metabolite fingerprinting since identification of individual metabolites is not undertaken in the latter. Because of the complexity of the metabolome, it is generally accepted that a single analytical technique cannot accommodate a comprehensive analysis of the metabolome, and hence multiple technologies are usually employed (30). The question arises as to whether the metabolome can be truly represented as the summation of data from individual analyses.

Metabolomics presents a major challenge to analytical chemistry and bioinformatics. It is logical to question whether metabolomics is truly achievable in its goals without a quantum leap in analytical methodology. Nevertheless, the opportunities of metabolomics and potential for success offer sufficient incentive to drive research in this field. Opportunities for its application include product fingerprinting, particularly for food and beverage markets where product authenticity is critical and problems of adulteration arise (e.g., wine, olive oil) (31). Metabolomics also represents a way to dissect and modify plant metabolism, physiology and development (32). The data may be used to define the level of individual metabolites in order to analyse causes of differences in biochemical networks and determine hidden structure of metabolic regulations (27). The major driving force in metabolomics is, however, the link to functional genomics that refers specifically to the "development and application of global (genome-wide or system-wide) experimental approaches to assess gene function, making use of the information provided by genome sequencing" (5). The aim is to establish a better understanding of the correlation between genes and the functional phenotype of an organism (19). Since the metabolome of a system

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represents the amplification and integration of signals from other functional genomic levels (e.g., transcriptome and proteome) (4) in addition to environmental conditions (34), metabolomics may therefore be considered as a tool for functional genomics, in addition to systems biology. The latter uses a similar approach as functional genomics, but aims to simultaneously monitor all biological processes operating in an integrated system and hence has a more holistic approach than functional genomics (1).

ANALYTICAL APPROACH

A protocol referred to as MIAMET: Minimum Information About a Metabolomics Experiment (19) has been developed to address the lack of standard procedures for the presentation and exchange of metabolomic data, which currently impedes the dissemination of metabolomic data to the broader research community. The aim of the protocol is to facilitate better understanding of metabolomic data in context of the experimental design, sample preparation, analytical methodology and data interpretation that have been used. The authors note that MIAMET is an evolving concept that should be refined with input from the metabolomics community (19). Related initiatives by the National Institute of Health (NIH, USA) under their New Pathways to Discovery theme relate to human health and advances in medical science rather than plants but also emphasise the importance of metabolomics technology development, and standards for proteomics and metabolomics (34).

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Sample Procurement

Sample procurement represents the initial and most critical step in any analytical procedure and underpins the success of the results obtained. In dynamic living systems, it is imperative that details of the origin of the sample be known, and for plants this will include documentation of species/genotype, variety, age/extent of maturation, plant organ and tissue function, location and information concerning the environmental and growing conditions of the plant. Many of the considerations relevant to environmental sampling (35) are also applicable to metabolomics. Thus, careful experimental design with appropriate replication is essential. King (32) has commented as follows: "One indisputable truism in research is that the results obtained are likely to be only as good as the experimental system used." An extensive sampling protocol for plants has been developed (36) that encompasses both random and selected sampling and aims to standardize sampling strategies and enable comparisons on a regional, national and international scale (36). For metabolomics, MIAMET has been proposed to ensure comprehensive detailing of experimental design including experimental type, experimental factors, experimental description and quality control steps taken (19).

A common approach for sample procurement is the collection of samples from individuals of the same organism under different environmental conditions, or from related organisms of different genotypes under the same environmental conditions (37). Nevertheless, biological variations, arising from quantitative variations in metabolite levels between plants grown under (near) identical conditions, represent the major limitations of the resolution of metabolomic approaches (1). Variance can be minimized by pooling samples; however, this is not always desirable, since sample variation may actually be biologically significant and result from functional differentiation of tissues (1). Similarly, averaging of metabolite data may lead to misunderstandings of biodiversity, physiology, genetics and ecology of plants. Fiehn et al. (38) have found that the biological variability seen between genetically identical plants grown under identical conditions was the largest source of observed variability, and exceeded the variability due to the overall analytical precision. This result was substantiated by Roessner et al. (39), who found that the biological variability associated with potato tuber plants grown side-by-side under identical conditions exceeded the experimental error by a factor of up to 10.

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In an investigation of *Cucurbita maxima* phloem, large differences in phloem composition between individual plants and individual leaves was observed using unsupervised clustering methods and metabolic network computation (27). In fact, distinct vascular exudate profiles were observed for each leaf that were similar to those of leaves from the same plant, but distinct from leaves from other plants harvested at the same developmental stage. Such variation must be considered when designing a sampling regime for plants. Comparison of leaf phloem sap samples obtained from petiole recesses, and leaf disks (25 mg fresh weight) taken from the same leaf, yielded markedly different GC-MS metabolite profiles, particularly with respect to carbohydrates (glucose, fructose), which were comparatively decreased in the former (based on normalization to absolute volume). Futhermore, unknown amino compounds were almost exclusively found in phloem (27). Such analyses are indicative of metabolite compartmentalization, although the number of studies at the sub-cellular level has been limited. For instance, the spatial (and temporal) distribution of sugars and ions was reported in carrot taproot (40) while non-aqueous extraction (41) was used to study compartmentalization of glycolytic intermediates, sugars and sugar alcohols, nucleotides, amino acids and organic acids in the amyloplast, cytosol and vacuole of potato tubers (42).

Sample Pre-treatment and Storage

Sample preparation is a critical step in any analysis, since alteration of sample properties as a result of contamination, loss or metabolism represents errors that must be minimized (35). Highly sophisticated analytical methods will

not compensate for poor sampling and preparative strategies, which ultimately destroy the validity of metabolome data and prevent any real interpretation of plant metabolism and phenotype. Sample preparation is particularly crucial in real/natural product samples where the matrix components are biologically active and the identity of numerous metabolites is unknown. The situation has not changed since the observation in 2001 (6) that "no systematic study has yet been published on metabolomic recoveries and breakdown reactions comparing different techniques of sample preparation, homogenization, and extraction, although true metabolomic approaches must consider these questions with great care."

Sample drying is a preliminary step in many cases where plant samples are analyzed. However, volatile compounds other than water may be lost during (oven) drying of plant samples (43). Freeze drying is often regarded as a more gentle treatment than oven drying and usually prevents the introduction of contaminants (44). Nevertheless, the drying method has been established to effect the concentration of some metabolites (45–48). For instance, the effect of various drying treatments (air-, oven- and freeze-drying) relative to extraction from fresh leaves has been investigated for analyses of birch for biophenols (49). There were significant concentration differences among the methods for the majority of biophenols and, with few exceptions, the concentrations after drying treatments were lower than in extractions of fresh leaves. Differences were attributed, in part, to competing processes: effects of drying temperature on rate of enzyme inactivation and differences in the thermostability of the biophenols. Furthermore, enhanced concentrations of enzymes and substrates encountered during drying processes may facilitate degradation processes. The observed differences may also relate to use of intact versus homogenized leaves, in which destruction of leaf compartmentalization during the crushing of fresh leaves releases hydrolytic enzymes into the cytoplasm. Thus, rapid quenching of all biochemical processes in the sample is necessary to preserve the fidelity of the metabolome.

The goal of sample quenching is to stop all enzymatic reactions rapidly and simultaneously to ensure an instantaneous snapshot of metabolite concentrations (1, 50). The type of quenching method used will depend on the physical composition of the sample. For example, the physiology and morphology of filamentous fungi are distinct compared to that of yeast, and thus different quenching methods must be considered (13). Furthermore, the quenching approach used will also depend on whether intracellular and/or extracellular metabolites are of interest in the analysis, since the metabolic rate of the two classes differ, and generally the half-life of extracellular metabolites is longer than that of intracellular metabolites (13). Obviously, quenching methods will become more painstaking when each class needs to be analyzed in isolation to prevent cell leakage.

Typical approaches to quenching for plant tissues include freezing with liquid nitrogen, freeze drying, and addition of alcohol or acid treatment.

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Jernejc (50) has tested different methods for the extraction of metabolites from *Aspergillus niger*, with determination of the concentrations of (Krebs cycle) metabolites by enzymatic methods. Acid and alkali extraction yielded better recoveries of metabolites compared to that of boiling buffered ethanol. However, acid treatment is generally undesirable and its use should be avoided unless there are positive indicators for its use as, for example, in analyses for anthocyanins where it enhances solute stability and partitioning behavior (51, 52). Freezing with liquid nitrogen was recommended by Fiehn (6) for quenching enzyme activity in plant tissues. A concern with freeze drying was potential loss of metabolites due to irreversible adsorption on cell walls and membranes. The validity of freezing has also been questioned with respect to loss of metabolites (11, 53), and both freezing and freeze drying produced substantial changes in the glucoside composition of leaves (46, 47, 54). Dessication at room temperature and freeze-drying at low temperature, preferably without pre-freezing, were the preferred methods for analysis of biophenols in mature willow leaves. Alternatively, vacuum-drying fresh leaves permitted quantification of both phenolic glycosides and condensed tannins from the same leaf material (54).

Homogenization of fresh or pre-treated samples represents the most vulnerable stage of any analysis with regard to contamination (44). Containers used for grinding or homogenizing must be thoroughly cleaned and surfaces leached prior to use (44) so that contamination is not an issue. Freeze-dry grinding in a freezer mill by impact degradation under liquid nitrogen has been recommended to prevent sample contamination (44). Sample storage is often essential because of time constraints introduced by transport of samples to the laboratory or by sample handling time. Dried plant samples (tea leaves, lucerne, barley) stored for 10–20 years under different conditions including in plastic bottles or paper bags at room temperature and humidity, or at $-20^{\circ}C$ showed no significant change in their inorganic chemical composition (55).

The authors are not aware of any comparable studies on the effects of long-term storage on plant metabolites. Deep freeze storage, because of the growth of ice crystals, can cause leakage, cross-contamination and loss of sample integrity, which inhibits any subsequent subdivision of the sample (44). This can be remedied by rapid low temperature freezing, however problems may still arise from sample thawing. Häkkinen et al. (56) in their investigation of anthocyanins in berries compared refrigerator, room temperature and microwave methods for thawing the frozen berries. Variability in the apparent quercetin, myricetin and kaempeferol contents were used to gauge the suitability of the thawing method. Microwave thawing produced the most reliable results, and was also deemed the most practical approach for routine analysis.

A comprehensive study of sample treatment (57) investigated the effects of different methods of sample drying and storage, plus extraction solvent and analysis method on the concentrations of 14 individual hydrolysable tannins

and insoluble ellagitannins in birch leaves. Leaf storage at -20°C was generally preferable to storage at room temperature or 4°C. However, as noted by the author "each plant species, with its presumably unique composition, is likely to have a unique combination of ideal conditions for tissue preservation and extraction." Thus, generalizations are dangerous and this makes the challenge of metabolomics a daunting prospect.

Extraction

Most analyses typically follow the traditional approach of sample pretreatment, homogenization and extraction, separation and detection as demonstrated by the isolation of biophenols from a methanolic extract of the leaves of silky oak (*Grevillea robusta*) (58). Isolation of metabolites from the sample matrix is normally essential in any comprehensive analytical scheme, and the aim of sample extraction generally is to obtain an extract that is uniformly enriched in all components of interest and free from interfering matrix components (59). However, in the case of metabolomics, the matrix is effectively eliminated since analysis of all sample components is required. This has interesting ramifications for sample extraction, and certainly challenges traditional analytical approaches for sample handling.

Sample extraction methods vary in their levels of complexity, and can be laborious, requiring exhaustive extraction (34) and pre-concentration steps or comparatively simple, requiring only liquid-liquid extraction or filtration (60). The extent and severity of sample manipulation will determine the precautions that must be taken to prevent (hydrolytic, oxidative, photodegradative, enzymatic) modification of metabolites (21), which results in the presence of artifacts. Such precautions may include the use of inert atmospheres, manipulation in the dark or addition of enzyme inhibitors or anti-oxidants (61). Addition of exogenous reagents should only be used when essential and only if their presence is demonstrated not to interfere. Extraction protocols will also depend upon whether qualitative or quantitative information is required. In the former, exhaustive extraction procedures can be avoided. In the latter, extraction techniques may be more rigorous to ensure quantitative recovery of the metabolites. Any extraction procedure based on current knowledge and technology must be a compromise between complete recovery of some metabolites and avoiding chemical destruction of more labile metabolites.

In metabolite profiling, selective extraction will be employed. Profiling has been used extensively; for example, Broeckling et al. (62) have used GC-MS based profiling to analyse the response of *Medicago truncatula* cell cultures to elicitation with methyl jasmonate, yeast elicitor or ultraviolet light. As a further example, floral scent production in petunia was studied in vivo using targeted metabolomics by solid phase microextraction (SPME) coupled to GC-MS analysis of volatiles (63). Mature flowers released

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predominantly benzenoid compounds with a circadian rhythm. Results indicated that volatiles were not stored during periods of low emission but rather were synthesized de novo. In contrast, target analysis employs specific extraction methods since only a particular compound(s) needs to be extracted. The extraction method can therefore be catered to match the solubility and related chemical properties of the analyte(s), however matrix interferents must be removed. The UV detection of glycoalkaloids necessitated sample cleanup by solid phase extraction or precipitation with ammonia, whereas sample preparation was greatly simplified with mass spectral detection using MS/MS (64).

Extraction for metabolite fingerprinting is significantly less demanding since fingerprinting serves as a screening tool to discriminate between samples of different biological conditions (11). Because fingerprinting methods do not aim to quantify metabolites, and utilize spectroscopic techniques rather than chromatography, sample extraction is rapid and non-selective and crude extracts are analyzed. Samples are distinguished via information from spectra of total compositions of metabolites (65). Gray and Heath (66) in their investigation of *Arabidopsis* metabolome and cold acclimation used simple solvent extraction followed by FTMS to generate a comprehensive list of masses (based on mass-to-charge ratios) that were reflective of individual components or putative metabolites.

Since metabolomics aims to quantify every metabolite in a biological system (21), non-selectivity and universality are essential, and extraction becomes more challenging as a direct result of the chemical diversity of the metabolites that range from ionic inorganic species to hydrophilic carbohydrates, hydrophobic lipids and complex natural products (1). The fact that the majority of plant metabolites remain unidentified further complicates extraction protocols since physicochemical properties of most metabolites are unknown. The structural diversity of metabolites and their effect on physicochemical behavior such as solubility and analyte recovery poses a challenging analytical problem. Additionally, many metabolites (e.g., phenols) are highly sensitive to hydrolysis and oxidation, which further exacerbates sample extraction protocols.

The development and application of a single method suitable for the simultaneous detection of all metabolites in the metabolome is still beyond reach (21). Major advances and a totally new approach to organic analysis are essential before the holy grail of metabolomics is attainable. This will likely involve in-vivo analysis of intact plant materials, and in this context NMR has a distinct advantage over other analytical techniques (67, 68). Meanwhile, a number of metabolite profiling techniques must be combined to make this task possible (21).

The majority of metabolomic investigations have used simple plants and yeasts with defined genomes such as the extensive work on *Arabidopsis*. Complications in extraction arise with increased plant complexity as, for example, with fruits, oily plants, and seeded plants with distinct compartments where

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analyte compartmentalization becomes a consideration. Most recent approaches involving accelerated solvent extraction (ASE), microwave extraction, supercritical fluid extraction (SFE), and SPME incorporate simultaneous variation of multiple parameters rather than optimizing a single variable at a time (69). Extractions can be enhanced by using conventional solvents at temperatures above their atmospheric boiling points, namely pressurized liquid extraction, or by using microwave or sonic extraction (13). Such techniques generally require smaller sample sizes and use less solvent than traditional extraction methods.

Weckwerth et al. (33) have developed a novel extraction protocol whereby hydrophilic and lipophilic metabolites, proteins and RNA were sequentially extracted from the same *Arabidopsis thaliana* sample. Such a method combined with gas chromatography-time of flight mass spectrometry (GC-TOFMS) yielded the detection of 652 metabolites (and 297 proteins and clear RNA bands). Hydrophilic metabolites (sugars, amino acids, organic acids) were extracted using water/methanol solvent, while lipophilic compounds (lipids, chlorophylls and waxes) were partitioned into chloroform. The organic phase was subsequently dried and dissolved in methoxamine hydrochloride (in pyridine), shaken, incubated, and then mixed with N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) to facilitate derivatization of the polar functional groups prior to GC-TOFMS.

Extraction may be combined with various techniques such as hydrolysis and derivatization to aid in the structural elucidation of metabolites and/or facilitate analysis. Acidic (70), alkaline (71) and enzymatic hydrolysis (72) are used and the choice of treatment will depend upon extract composition. For example, specific enzymes may be used to give highly specific cleavage patterns. While derivatization can also aid in analyte identification and overcome the limitations of poor volatility and thermal stability of metabolites (39, 73), difficulties may arise due to the increased molecular mass of the analyte, particularly when mass spectrometry (MS) analysis is to be performed because of the limited operational mass range of mass spectrometers. For derivatization, optimal reaction conditions are often established as a compromise between reaction completeness and maintenance of the integrity of labile compounds (38). A further complication arises from the possible formation of multiple derivatives from a single analyte and artifactual by-products. In a comparative study of five trimethylsilylation reagents, Roessner et al. (39) found that MSTFA not only gave the best results with the broadest range of polar chemical compounds, but produced the least by-products.

Metabolite concentrations in sample extracts are often very low as a result of dilution effects or poor recovery of the particular metabolite. Because of this, sample concentration may be required for metabolite enrichment with the usual approaches including solvent evaporation via freeze drying or in vacuo (27, 39) or use of a rotary evaporator. The former is advocated since thermal degradation of metabolites is prevented. Indeed Loskutov et al. (74)

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compared numerous preparation and extraction methods for the quantitative analysis of safranal in stigmas of *Crocus sativus* L. and found rotary evaporation to have a deleterious impact on safranal recovery. Instrumental methods can also be implemented for sample enrichment such as large volume injection with GC analysis. Enrichment procedures must cope with diverse classes of metabolites ranging from neutral (e.g., carbohydrates), cationic (amines), zwitter-ionic (amino acids, catecholamines) and anionic (carboxylic acids, nucleotides) species. Improved strategies for on-line sample preconcentration of metabolites by capillary electrophoresis (CE) have been developed (75). The three complementary methods, dynamic pH junction, sweeping and dynamic pH junction-sweeping, for electrokinetic focusing of large volumes of sample directly on-capillary have realized up to three orders of magnitude increase in concentration sensitivity.

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INSTRUMENTAL ANALYSIS

Sumner et al. (1) have suggested that a comprehensive metabolomic analysis for plants should include multiple metabolic pathways in both primary and secondary metabolism. The magnitude of the task can be illustrated by examining one class of compounds, namely, the flavonoids as a subset of the phenylpropanoids. Out of a total of 5,000 different flavonoids, 300 different glycosides of quercetin alone have been identified (59). Similarly, in grapes of *Vitis vinifera*, more than 200 different aglycones conjugated to glucose exist (76). Analyte diversity is not the only complication as the metabolome extends over an estimated 7–9 magnitudes of concentration (pmol-mmol) (11). Thus, a successful analytical approach in metabolomics must be capable of accurately measuring numerous known and unknown compounds that span a diverse chemical spectrum and a large dynamic concentration range. It is apparent that no single technique can satisfy these requirements and meet the diverse challenges of metabolomics, so most studies employ several complementary techniques.

The different strategies for metabolome analysis: namely, metabolite profiling, metabolite fingerprinting, target analysis and metabolomics (6) differ in their identification, quantitation and data generation requirements. Because of this, it is well recognized that each of the strategies will incorporate different instrumental approaches. Hence, fingerprinting approaches employ high-throughput methods compared to the more detailed analyses performed in metabolite profiling situations. With respect to instrumentation, metabolite profiling methods rely on hyphenated techniques including gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-mass spectrometry (CE-MS). Alternatively, direct injection MS, NMR, Raman and Fourier Transform Infrared spectrometry facilitate metabolite fingerprinting (28), since preliminary metabolite separation using chromatography is not

necessary. Metabolite fingerprinting does not aim to establish the identity of individual metabolites, but rather to interpret spectral information to determine whether signals arise due to experimental conditions or to some interfering signals (65).

In targeted analysis, instrumental methods can be specifically tailored to complement the chemical structure and properties of the analytes. Knowledge of analyte identity is also advantageous in that appropriate internal standards can be selected, aiding in quantitation processes. In particular, the use of labelled internal standards is advocated, especially when novel extraction techniques are used as, for example, headspace-SPME (HS-SPME) (69).

Metabolomics is non-selective and universally applicable (21) but currently employs the same techniques as encountered in metabolite profiling. MS represents the most universal, sensitive and versatile method for the detection of metabolites (21). Determination of both absolute concentrations and relative amounts of metabolites has advantages and disadvantages (39). Relative values may be appropriate for fingerprinting (24, 38) but absolute concentrations are always desirable in metabolomics.

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Results are expressed in a variety of ways. Quantitative data may be expressed relative to external standards (21) or internal standards (33) where absolute concentrations are essential as in a study of long-distance transport or the calculation of fluxes along a biochemical pathway. In other cases metabolite levels are quantitatively studied but with reference to a control metabolome as in a comparison of a plant grown in stressed conditions and unstressed conditions. The results may be expressed relative to fresh (33, 39) or dry weight (24–26), the latter avoiding problems associated with metabolite dilution due to plant tissue growth. Metabolite levels may also be expressed as metabolite equivalents, and this is particularly useful when the identity of metabolites is unknown (77, 78). On the downside, this method assumes the linear response of unknown metabolites compared to the reference metabolite.

Two orthogonal dimensions of chemical characterization relative to an authentic compound have been established as the minimum quality standard for metabolite identification and profiling (19). There is also a need for a suite of purified or synthetic reference compounds to be available to all metabolomic researchers for analysis by complementary techniques in order to establish comprehensive, publicly available reference databases or libraries. Data comparison would also be facilitated through a collection of standard authentic reference mixtures of extracts from homozygous plant lines, in addition to plant reference material. Neilson and Oliver (4) have extended these requirements to include the definition of appropriate data standards; the development of standard analytical methods, and the development of mass spectral libraries for standard analytical methods. To this list we would add the need for chemometrics and informatics and skills in their application and interpretation.

Suitably configured databases are the initial output of metabolomics. It is beyond the scope of this review to address the tools used for data analysis

(79, 80) although this field is critical in metabolomics. The interested reader is referred to several recent reviews in this area for further information (80–82). Metabolic networks and systems biology are developed from these databases using a variety of algorithms for metabolic network reconstruction coupled to suitable modelling algorithms (18). Database construction involves the application of sophisticated biostatistical techniques due to the complexity of datasets from metabolomics (83). These techniques fit into the area of pattern recognition methods (9), and these can be separated into two categories as supervised and unsupervised. The latter include principal component analysis (PCA) and clustering methods such as hierarchical cluster analysis and are used to establish whether any intrinsic clustering exists in a data set. Supervised methods include soft independent modelling of class analogy, K-nearest neighbor analysis and neural networks and optimize the separation between two or more sample classes using class information given for a training set of sample data. Methods such as PCA permit a simplified view of the variation in metabolomics data by reducing its dimensionality by 2D or 3D mapping, thereby facilitating visualization of inherent patterns in the data set. Where a priori information exists about the data, it can be used in an unsupervised data analysis with weighted PCA (WPCA) (84). The two models give different views of the data in which the WPCA model accounts for the non-uniform experimental error and focuses more on the natural variation in the data.

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Multidimensional Approaches

Biologically meaningful interpretations of metabolomic data are often limited by poor spatial and temporal resolution of the acquired data sets (27), and one way to remedy this is to limit the complexity of the cell types being studied. Thus, much work has been conducted on probing the metabolome of the model plant *Arabidopsis thaliana* (75, 85) for which the complete genome sequence and functional gene annotations are publicly available. An alternative approach is to implement multidimensional systems that may be classified in various ways. For example, based on the type of displacement used, such systems are either simultaneous or sequential (86). The latter is achieved using two or more separation processes, which occur in different media, under different conditions, for example LC-GC. Such processes may be conducted either on-line or off-line. In an on-line approach, fractions eluting from the LC are directly transferred to the GC second dimension. Coupling of the LC and GC dimensions must be suitably optimized, and precautions must be taken to address the comparatively large volume of the LC fractions (87). In an off-line LC-GC analysis, fractions from the LC would be manually collected and reinjected (with or without additional sample treatment) into the GC. Contrary to this, simultaneous displacements are much more rigid, in that the displacements take place in the same media,

under the same conditions, such as in the same or similar solvent, at the same temperature and pressure. Multidimensional approaches can also be performed in an off-line, multiplexed or parallel approach (30). Examples of such approaches include fraction collection with subsequent separative analysis, or fractions of the same sample being analyzed by a series of parallel, independent separative systems.

A multidimensional chromatographic system comprises the coupling of two or more separative mechanisms in order to substantially increase total separation power, or *peak capacity* of the systems (88). Increased peak capacity ultimately results in greater resolution of analytes, yet also affords numerous advantages including decreased sample preparation and cleanup requirements for the removal of interferents. Multidimensional separations may also be classified as hyphenated or comprehensive, depending upon the method of effluent transfer between coupled dimensions (89). In comprehensive systems, the second dimension faithfully samples all peaks eluting from the first dimension, and the resulting peak capacity is the product of the peak capacities of the individual dimension (88), so long as the separative mechanisms of the individual dimensions are orthogonal (i.e., not correlated) (90). In hyphenated multidimensional systems, peak capacities are additive, since only a specific retention region or heartcut is subjected to further separation by the second dimension. With respect to terminology, hyphenated systems are denoted using the hyphen symbol (e.g., LC-GC), whereas comprehensive multidimensional approaches use the multiplex symbol (e.g., GC × GC).

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To achieve maximal gains in peak capacity, systems with independently high peak capacities should be coupled (88). This is necessary since according to the statistical model of overlap (SMO), a maximum of only 37% of a column's peak capacity can actually be analyzed to facilitate adequate peak resolution in a one-dimensional system for a purely random analysis (91). This significant redundancy in peak capacity thus necessitates the use of columns with considerably inflated peak capacities for multidimensional approaches to be valuable. However, maximized peak capacity does not guarantee optimal analyte resolution, since resolution of analytes will also depend on the compatibility of the coupled dimensions for the analytes of interest. This can best be explained by the concepts of sample dimensionality and system dimensionality (92).

Sample dimensionality describes the intrinsic nature of the sample, and is defined as the number of individual variables that must be specified to identify components in a sample (92). For example, in a mix containing only straight-chain alkanes, the sample dimensionality would be based exclusively upon carbon number, thus yielding a dimensionality of 1 (89). System dimensionality simply refers to the number of coupled separation stages or dimensions used in the multidimensional system (88, 92). For example, a single column gas chromatograph would have a dimensionality of 1, while a liquid chromatograph coupled to a gas chromatograph (LC-GC) system has a

dimensionality of 2. Optimum resolution of sample components arises when sample and system dimensionalities are matched. This is demonstrated in Figure 1.

Based on the recommendations of Sumner et al. (1) that metabolomic investigations should at a minimum include carbohydrates, amino acids, organic acids, lipids/fatty acids, vitamins, phenylpropanoids, terpenoids, alkaloids and glucosinolates, and assuming that all of these compound classes could be extracted simultaneously, it is feasible then that the metabolome has a minimum sample dimensionality of 9. Minimum requirements for metabolome resolution are thus a multidimensional system with nine orthogonal dimensions. Certainly, such a system is not available and is difficult to conceive. Since pure metabolomics is currently not achievable because of such extensive sample dimensionality, metabolite profiling techniques, where sample dimensionality can be controlled, combined with multidimensional systems with inherently high peak capacities, offer the best opportunities for analyte resolution, and represent the most powerful means for metabolome analysis.

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Hyphenated Techniques in Plant Metabolome Analysis

Before considering hyphenated techniques we need to examine the merits of the various single-dimensional techniques that are commonly coupled. These

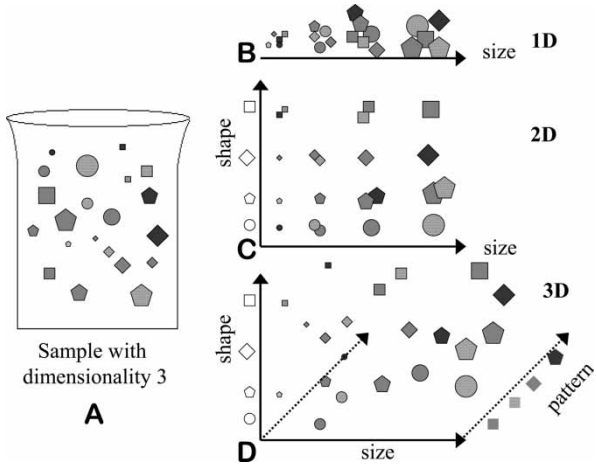

Figure 1. A schematic illustration comparing the peak capacities of chromatographic systems with different dimensionality. A shows the one dimensional chromatographic trace, and B represents the equivalent one-dimensional peak capacity. C and D show the comparative gains in peak capacity achieved using a hyphenated two-dimensional system and a comprehensive two-dimensional system, respectively. Adapted from (89).

techniques can be categorized as chromatographic or spectrometric methods and include GC, LC plus CE with IR, NMR and MS. The single-dimensional chromatographic techniques are not widely used in metabolomics but NMR and IR have been used because of their ability to provide comprehensive spectral data without the need for preliminary separation of sample components. However, these spectra are complex multivariate datasets and their analysis requires chemometrics for high-throughput metabolic fingerprinting (93). Near infrared spectra in the region from 10,000 to 4,000 cm^{-1} arise from overtone and combination-band absorptions of CH, OH and NH groups. Fourier transform IR spectrometry (FTIR) is used for the mid-IR (MIR) region from 4,000 to 400 cm^{-1} where fundamental vibration modes occur. MIR spectrometry provides much greater chemical information content that enabled classification of control and salt-treated tomato fruits with respect to their metabolic fingerprints (94). Discriminatory regions within the spectra were identified with the aid of genetic algorithms (94, 95). MIR performed at least as well as NIR for modelling grass composition in a comparison of MIR and NIR for the study of forage grasses (96, 97). No significant advantage was achieved by combining data from the two spectral regions (98).

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NMR is a high-throughput, non-targeted fingerprinting technique that permits the study of any molecule that contains an active nuclide (i.e., odd atomic number or odd mass number such as ^{1}H or ^{13}C, respectively). Conventional NMR traditionally provides mainly qualitative data, although this restriction is more imagined than real (99). Various nuclides are used as in the solid state and liquid state ^{1}H and ^{13}C NMR spectrometry of mango pulp and juice (100). Spectral assignment enabled the identification of several organic acids, amino acids and minor components in mango while metabolite compositional changes during ripening were followed through changes in the spectra. However, ^{1}H NMR remains the most widely employed because of its ubiquitous presence in organic compounds, high sensitivity and high isotopic natural abundance although other nuclides including ^{13}C provide useful data. ^{14}N is susceptible to efficient quadrupolar relaxation resulting in broad signals for most nitrogenous metabolites (101) while ^{15}N gives much narrower and more useful signals, but its low abundance means that plants must be fortified with ^{15}N-labelled substrates.

The potential of NMR spectroscopy as a tool for probing the operation of metabolic networks is largely untapped (15). Most NMR studies focus on assigning the spectra of tissue extracts, a process that typically identifies 20–40 metabolites in an unfractionated extract (15) such as orange (31) and apple juice (102). For example, soluble sugars, organic and amino acids plus some secondary metabolites were profiled by 1-D ^{1}H NMR in fruit, roots and leaves (103). Quantitative data were validated by comparison with enzymatic or HPLC analyses. Acquisition times of 15–25 minutes were required to quantify 14–17 metabolites depending on the species and tissues. The data can be used to identify marker compounds that discriminate

between samples as in the identification of pulpwash addition to orange juice (31).

Other studies have compared the overall metabolic composition of plant material, and assessed the impact of stress conditions or genetic modification on the plant metabolome. Metabolite profiles of natural and genetically modified potato tubers were compared by NMR and HPLC (104). With the latter, 40 peaks were readily evidenced in chromatograms, of while 20 were identified while structural assignments were made for approximately 30 compounds by NMR. Compositional differences between modified and non-modified crops of two varieties were assessed at the level of whole profiles (by PCA) and individual compounds (by ANOVA).

NMR is limited by relatively poor sensitivity (500 ng for structural elucidation of compound with MW ca. 300; cf. pg range for MS) (105) to detection and analysis of the more abundant metabolites although sensitivity can be improved by use of cryogenic probes, longer analysis times and application of higher magnetic fields ($\geq$11.7 tesla; a proton resonance frequency of 500 MHz). Thus, high field ^{1}H NMR in conjunction with chemometric analysis was used to assess the effects of xenobiotics on endogenous metabolite levels in *Crotalaria cobalticola* plant cells (106). Extraneous data that resulted from spectrometer variation were removed by data filtering. The filtered data revealed key changes in biochemical status caused as a result of exposure to cobalt. Branched-chain amino acids, succinate and secondary metabolite precursors phenylalanine and tyrosine were all higher in the control samples, while choline, glutamate, alanine and lactate were higher in the dosed samples.

Two-dimensional techniques are now commonly used as in a detailed assignment of ^{1}H NMR spectra of lyophilised lettuce leaves (61). Aqueous and organic solvent extracts were examined by 1D and 2D ^{1}H, ^{13}C and ^{31}P to fulfil the detailed spectral assignments to metabolites belonging to different classes such as carbohydrates, polyols, organic and amino acids (water-soluble fraction) and sterols, carotenoids, hydrocarbons, fatty acids and other lipids (organic-solvent-soluble fraction). The 600 MHz ^{1}H NMR spectrum of tomato juice is reproduced in Figure 2 (107). The spectrum contains several hundred signals corresponding to many tomato metabolites but is dominated by resonances of D-glucose and D-fructose, the major components. However, signals from minor components are detectable due to the high dynamic range of NMR (Figure 2, inset). An unambiguous spectral assignment of chemical shifts to specific compounds was accomplished by 2D spectra. The validity of NMR data including 2D spectra were verified by comparison with HPLC data (60).

Metabolite fingerprinting of crude plant extracts of *Arabidopsis thaliana* ecotypes utilized proton NMR and multivariate analysis (108). Comparison by principal component analysis using SIMCA-P allowed the generation of residual NMR spectra of the compounds that contributed significantly to the differences between samples. PCA is the most common approach to extracting

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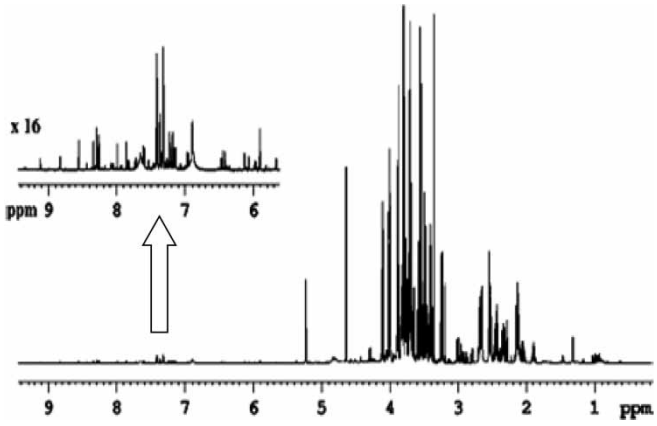

Figure 2. ^{1}H NMR spectrum of tomato juice with inset an expanded view of the aromatic region of the spectrum. Adapted from Sobolev et al. (107).

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useful data from complex overlapping NMR spectra. Many factors affect the spectra and hence the robustness of the PCA. Inconsistency of the peak position is generally detrimental to multivariate analysis and careful control of experimental variables is essential. This includes sample preparation and control of extract pH and solvent composition (109, 110). Chemical shifts of some compounds are affected by molecular association as in the case of caffeine, which can self-associate and form complexes with biophenols necessitating mathematical alignment of peaks (109, 111, 112). Resolution and the ability to differentiate metabolite signals are degraded by broadened NMR spectral lines. Linewidths are related to molecular dynamics resulting from sample viscosity, sample heterogeneity, binding of small molecules, macromolecules and compartmentalization. These are inherent problems with tissue and cell samples where minimal sample preparation is employed as is the norm for NMR studies.

Fourier transform ion cyclotron mass spectrometry (FTMS) has the highest resolving power of all mass analysers. This resolution facilitates analysis of extremely complex mixtures without resorting to chromatography. FTMS was used to identify metabolites associated with the development and ripening of strawberries (113). Extracts were analyzed by electrospray ionisation (ESI) and atmospheric pressure chemical ionization (APCI) in positive and negative ion mode generating more than 400 unique monoisotopic masses. This paper demonstrated that more than 1,000 metabolites could be monitored in a simple and rapid procedure. However, widespread adoption of FTMS is currently restricted by the high cost of instrumentation.

Hyphenated systems in which the spectral capacity is combined with spatial resolving power currently provide the best opportunities for success in metabolomics. Some of these techniques have now matured and are

regarded as standard laboratory apparatus. These include GC-MS, HPLC with photodiode array detection and LC-MS. Interfacing of HPLC with NMR (105) and HPLC with MS and NMR are less mature but have still yielded valuable multidimensional data (114–116). Indeed, each of these could be considered as mature techniques when compared with sample preparation procedures, which remain as the most error prone steps.

GC-MS typically using single quadrupole mass analyzers allows the de novo identification of (semi)volatile small molecule plant metabolites that are thermally stable (85, 117), as demonstrated by the detection of 130 volatile metabolites in the headspace of inoculated "Fortress" onion bulbs (118). The separation of very complex mixtures can be attributed to the high separation efficiencies of open tubular GC columns ($N > 250{,}000$ for 60 m). When interfaced with time-of-flight mass analyzers (34) that combine greater m/z accuracies with high scan speeds (up to 500 scans s^{-1} versus 1–6 spectra s^{-1} with quadrupole analyzers) that support ultrafast GC-MS (119) further enhancement in resolution is provided. Scan speed is important as it introduces peak skewing if too slow. Thus, the spectral resolution and deconvolution capabilities of MS improve analyte resolution and differentiation such that more than 400 compounds can be resolved in a single analysis.

As various papers (118, 120) demonstrate, GC-MS is ideally suited to profiling volatile metabolites although less volatile species are also amenable to this technique. Successful GC analysis of less volatile metabolites frequently involves derivatization (121), which may complicate chromatograms as many metabolites yield more than one derivative, observed in the GC-MS. Silylation has replaced esterification as the most popular derivatives for organic acids (28). Enolization reactions (61) are circumvented by oximation of keto groups, although the syn- and anti-isomers of the oximes can sometimes partially separate. Halket et al. provide further insight into derivatization reactions and the possibility of artifacts including reactions such as the conversion of arginine to ornithine during silylation (28). Artifacts may arise at any stage of an analysis including the detection phase, as in the case of peaks arising from column bleed or hydrolyzation of MSTFA derivatizing reagent in GC-TOFMS (33).

GC-MS and derivatization applied to metabolic profiling allowed simultaneous determination of carbohydrates, sugar alcohols, organic acids, sterols and amino acids to assess the effects of pesticide applications on plants (122). In this application, the chromatograms were extremely complex due to the absence of any sample pre-fractionation. Chromatographic profiles of polar metabolites following fractionation allowed identification of 150 compounds in potato tubers (39) and over 300 metabolites in *Arabidopsis thaliana* leaf (38) in a single extract. The success of GC-MS is shown by the paper of Roessner et al. (39), who demonstrated that in vitro and field grown tubers showed a number of biochemical differences contrary to previous beliefs. Figure 3 shows the GC-MS total ion mass chromatogram of a potato tuber extract with a representative expansion, demonstrating the

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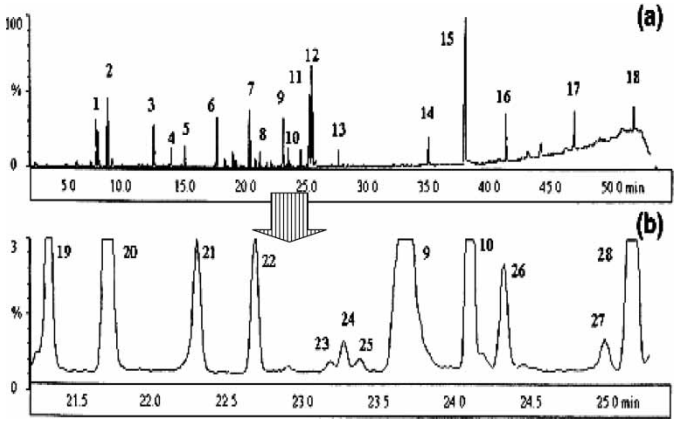

Figure 3. GC-MS total ion chromatogram of a tuber extract from *Solanum tuberosum* L cv. Desirée. Figure (a) complete chromatogram, 4.0–50.0 minutes; Figure (b) expansion of the chromatogram in (a) for the region 21.5–25.0 minutes. Peaks can be identified as follows: 1, glyceraldehyde MEOX1 TMS; 2, heptanoic acid TMS (time reference); 3, phosphoric acid TMS; 4, nonanoic acid TMS (time reference); 5, unknown; 6, malic acid TMS; 7, ribitol TMS (quantitative internal standard); 8, undecanoic acid TMS (time reference); 9, asparagine, *N,N,O*-TMS; 10, tridecanoic acid TMS (time reference); 11, glucose MEOX1 TMS; 12, citric acid TMS; 13, pentadecanoic acid TMS (time reference); 14, nonadecanoic acid TMS (time reference); 15, sucrose TMS; 16 tricosanoic acid TMS (time reference); 17, heptacosanoic acid TMS (time reference); 18, hentriacontanoic acid TMS (time reference); 19, glutamic acid *N,O,O*-TMS; 20, pyroglutamic acid, *N,O*-TMS; 21, glutamine, *N,N,N,O*-TMS; 22, phenylalanine, *N,O*-TMS; 23, glucoheptonic acid TMS; 24, ribonic acid TMS; 25, unknown; 26, unknown; 27, mannitol TMS; 28, quinic acid TMS. Derivatives are per-trimethylsilylated unless otherwise indicated. Adapted from (39).

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sample complexity in terms of numbers of component and the range of concentrations.

Pre-fractionation is clearly undesirable for metabolomics but the lack of such treatment in sample processing complicates data output. Derivatized chloroform methanol extracts allowed identification and quantitation of sugars, sugar alcohols, organic and amino acids in apricots (114) and secondary metabolites in potato tubers (73, 123) in the polar phase and fatty acids and alcohols, sterols and aliphatics in the non-polar phase of leaf tissue extracts (124). In most cases the majority of analytes have not been identified as in the GC-MS profiling of fusarium head blight resistant and susceptible wheat cultivars (125). More than 700 compounds were detected, with only 55 of these tentatively identified. Factor analysis discriminated between the resistant and susceptible cultivars.

The unambiguous identification of metabolites and the ability to assign an indicator of the reliability of a compound identification are key steps in

GC-MS. This is facilitated by use of retention indices (RI), although most manufacturers' data systems do not handle RI values well. The development of the Automated Mass Spectral Deconvolution and Identification System (AMDIS) (126), which is available for download, overcomes these limitations. The first step in the process is the deconvolution of overlapping mass spectra due to unresolved metabolites or background ions. The deconvoluted spectra are then compared with mass spectral libraries and a match is assigned together with a reliability indicator. AMDIS software has been applied to plant metabolites (27). The deconvolution process is time-consuming and other approaches are used including a strategy for rapid comparison of non-processed mass spectral data files (127). This method permits rapid comparison of large sets of GC-MS data, thereby applying time-consuming deconvolution only to parts of the chromatograms that contribute to explain the differences between the samples.

There are several commercially available spectral libraries (28). The NIST spectral library offers spectra that have been studied in detail by professional evaluators before inclusion. Collections of mass spectra comprising frequently observed identified and non-identified metabolites are an effective means to pool identification efforts. A platform for mass spectral and retention time index libraries that will facilitate this process has been described (128).

Large, thermolabile molecules such as oligosaccharides and sugar nucleotides are not amenable to GC analysis and are more suited to analysis by HPLC. This has traditionally been achieved without derivatization, although there are examples where derivatization has enhanced signals from soft ionization techniques (129). Current standard analytical columns limit the comprehensive scope of metabolomics as a result of practical peak capacities of only 100–200 (30, 130). Column efficiency is described by the number of theoretical plates and typical values for HPLC columns are 10,000–20,000 compared with values for GC columns that routinely exceed 100,000 (131). This is related to slow solute diffusion and high viscosity of liquids in HPLC plus high pressure drops. Newer column developments include the application of C18 monolithic silica capillary columns. These columns coupled to ion trap mass spectrometry detection have been applied to probing the metabolome of *Arabidopsis thaliana* (132).

An ion trap mass spectrometer was used due to its enhanced sensitivity in full-scan mode compared to quadrupole, triple quadrupole or QTOF instruments. This system outperformed commercially available mass spectral deconvolution software. The introduction of ultra-performance LC ($>$10 kpsi) (UPLC) has offered increased resolution capabilities coupled with greater speed of analysis. The term ultra-performance emphasizes the fact that performance in such systems is a factor of particle size (e.g., 1.7 μm) and column dimensions (e.g., length 250 mm; 75–320 μm i.d.) rather than pressure. Columns for UPLC are operated at flow rates of 200 nL to 100 μL per minutes, which is well matched to the requirements of the usual mass spectral detectors. The use of cutting-edge UPLC performance

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has detected in excess of 5,000 different compounds from a metabolomics sample (133). Nevertheless, the spectral resolution of MS was essential and LC-ESI has revolutionized the applications of HPLC, although light-scattering detectors (134) remain largely untested.

Reversed phase systems remain popular particularly for many secondary metabolites as in the profiling of isoflavone conjugates in root extracts of lupine species with LC-ESI-MSn systems (135). Results obtained with triple quadrupole and ion trap analyzers were compared and limited, but useful structural data were elucidated from the nature and intensity of daughter ions in the mass spectra. The merits of RPLC-MS are demonstrated by the detection of 188 compounds in an extract of *Psoralea corylifolia* fractionated by ion exchange chromatography (136). Fractionated extracts were analyzed by APCI/MS and matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF/MS). The results demonstrate the power of integrating these techniques and also its suitability for smaller molecules. Nevertheless, the usual restrictions apply to column types such that reversed phase systems are not amenable to the analysis of highly polar carbohydrates and related compounds. Such compounds are generally analyzed using ion-exchange chromatography with mobile phases composed of high concentrations of involatile inorganic salts, hence precluding hyphenation with MS due to reduced ionization efficiency. In an alternative approach, hydrophilic interaction liquid chromatography (HILIC) coupled to electrospray ion trap MS has been applied to the analysis of highly polar plant metabolites (21). ESI enabled the detection of both positively and negatively charged metabolites including oligosaccharides, glycosides, amino sugars, amino acids, and sugar nucleotides, when coupled to a quadrupole ion trap mass spectrometer using continuous polarity switching. The benefit of polarity switching in ESI MS is that particular analytes will appear in greater abundance in either the positive or negative ion mode, depending upon their structure. For example, analytes that are prone to be positively ionized (e.g., amino acids, amino acid sugars) will show greater abundance in the positive ion mode (21). Adduct formation in positive mode ESI is common, and often such adducts represent the base peak in the mass spectrum rather than the molecular ion $[M + H]^+$. Knowledge of common adducts, such as $[M + Na]^+$, $[M + NH_4]^+$, and $[M + K]^+$, can aid in the identification process, especially when compared to corresponding negative ion spectra.

One of the major instrumental challenges in metabolomics is the dynamic range. For example, dynamic ranges of most analytical mass spectrometers are 10^4–10^6 for individual components, but significant reduction of this range is common in the presence of excessive amounts of some metabolites. The sample matrix may limit the dynamic range of most analytical mass spectrometric methods and interfere with the ability to profile secondary metabolites such as flavonoids. This is particularly notable in the presence of high levels of primary metabolites such as sugars and is caused by electrospray ion suppression when several compounds co-elute (132). Enhanced chromatographic

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resolution is an effective approach to overcome ionization suppression (132), although stable isotope dilution-based comparative quantification is a more common method of overcoming this problem (137). It is important to consider the possibility that incorporation of foreign isotopes may lead to a kinetic isotope effect and to various degrees of anomalous cell physiology (138). In other cases, isotope effects may also influence the chromatography or detection. For instance, [C-13]-methylation-based stable isotope dilution for comparative quantification of flavonoids contrasts to the equivalent deuterium-labeling methylation method, which has an adverse isotope effect on reverse phase chromatography (137).

The development of better solutions for processing of data remains as a major challenge in LC-MS (139) including peak alignment (140, 141). A variety of approaches have been used (83, 142). Differential LC-MS analysis of metabolomics data was facilitated by a freely available software package (139). This software is a toolbox containing methods for all data processing stages preceding differential analysis: spectral filtering, peak detection, alignment and normalization. The authors implemented a new recursive peak search algorithm and a secondary peak picking method for improving already aligned results, as well as a normalization tool that uses multiple internal standards. Visualization tools facilitated comparative viewing of data across multiple samples.

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Spectral libraries for LC-MS are less developed than the corresponding GC libraries. ESI and APCI spectra generally comprise pseudomolecular ions depending on the chemical properties of the analyte, nature of the matrix and solvent composition. Thus, the situation in LC-MS is usually much more complex. Figure 4 compares the EI mass spectrum of curcumin (typical of GC-MS output) with the ESI spectrum. The latter is less useful for mass spectral library searching. The collisionally induced dissociation (CID) product ion mass spectrum (Figure 4C) provides more detail but libraries for CID spectra are still in their infancy. Source CID can be achieved in a single quadrupole instrument, but there is no ion selection as in tandem mass spectrometers so that source CID is applied to all ions in the system. CID in a triple quadruple analyzer represents true tandem mass spectrometry (MS/MS) in that the different phases of the process are spatially separated. Temporal separation of the processes is employed in quadrupole ion trap instruments to produce MS/MS^n spectra (21, 64).

CE-MS provides a viable alternative to both GC-MS and LC-MS. It is particularly suited to quantification of primary metabolites without chemical derivatizations. Its application has been demonstrated by the measurement of 88 metabolites involved in primary pathways such as glycolysis and the tricarboxylic acid cycle (143). Moreover, various modes of CE provide versatility and both neutral and ionic analytes are amenable to high-resolution separations (144). However, the technique suffers from poor concentration sensitivity underlining the importance of developments in on-line

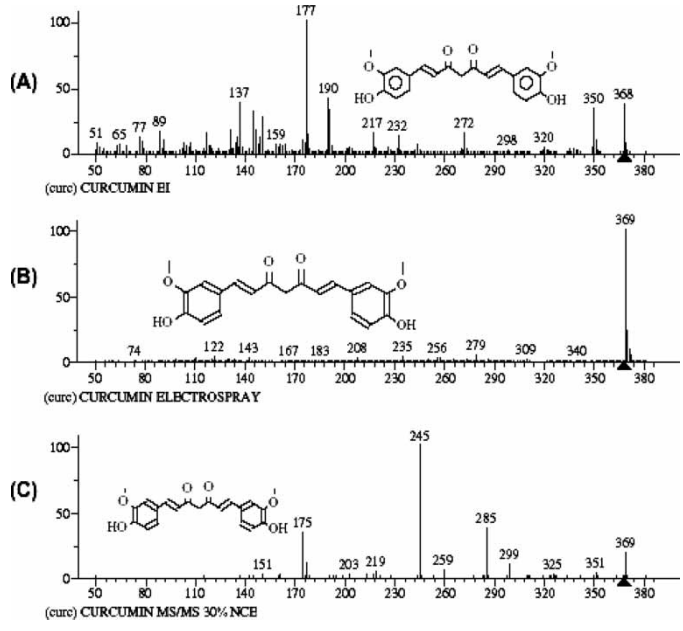

Figure 4. Comparison of (A) EI and (B) ESI mass spectra of curcumin (M = 368) taken from the NIST02 database, showing $[M + H]^+ = 369$. The product ion mass spectrum obtained by collisionally induced dissociation (CID) using an LCQ DECA quadrupole ion trap mass spectrometer is shown in (C). Reproduced from (28) with permission.

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pre-concentration procedures (75). Laser-induced fluorescence detection following suitable derivatization is another solution to increase concentration sensitivity and selectivity (144).

LC-MS-MS and LC-NMR are complementary techniques that facilitate identification of metabolites as in the characterization of nine major flavonoids in the flesh of transgenic ripe tomatoes (145). An exciting development is that of micro-fabricated bioanalytical devices (also referred to as laboratory-on-a-chip) (146). Development and implementation of these microfluidic-based bioanalytical tools involves both established and evolving technologies but is yet to achieve practical applications in metabolomics.

Comprehensive Approaches Used in Plant Metabolome Analysis

Comprehensive chromatographic technologies offer enhanced resolution capabilities as a direct result of their significantly inflated peak capacities derived from column coupling. Comprehensive techniques include comprehensive two-dimensional gas chromatography (GC × GC) and comprehensive two-dimensional liquid chromatography (LC × LC), in addition to

unified chromatography. The latter refers to the heterogenous coupling of LC, GC and supercritical fluid chromatography (SFC) (147), and although these technologies are conceptually exciting, they are still somewhat empirically limited (148). This review therefore focuses on LC × LC and GC × GC.

LC techniques are characterized by a wider variety of column stationary phases of truly distinct selectivities (149) in comparison to GC, wherein retention is generally dependent on solvent vapor pressure. Because of this, a greater number of orthogonal column combinations are possible in LC × LC: however, coupling can be problematic as a result of mobile phase immiscibility, precipitation of buffer salts and incompatibility of stationary phases (149, 150). LC × LC (and its hyphenated non-comprehensive counterpart, LC-LC) is most commonly employed in proteomic investigations (151–153) but has also found a niche application in food analysis. Mondello et al. (154) have developed an LC × LC method (with APCI-MS detection) for the analysis of triacylglycerides (TAGs) in rice oil samples using a silver-ion microbore column in the first dimension (D1), and a reversed phase monolithic column in the second dimension (D2). Separation of TAGs is thus based on the degree of unsaturation and partition number, respectively. The use of a micro-bore column in D1 facilitated the injection of a small sample volume (~20 μL) onto the fast D2, making the transfer of incompatible solvents from D1 to D2 achievable without peak shape deterioration or loss of resolution. A similar approach with respect to column dimensions (and mobile phase differences) has been employed by Dugo et al. (155), whereby a microbore normal phase D1 column was coupled with a monolithic reversed phase 2D column for the analysis of the oxygen heterocyclic fraction of cold pressed lemon oil. Both approaches (154, 155) represent innovative, simple and fully automated LC × LC technologies. Most importantly, they are suited to the analysis of a wide range of analyte polarities, and show much promise for future metabolome analyses.

The first report of GC × GC (with TOFMS) applied to metabolite profiling focused on the analysis of metabolites in human infant urine (156) and not plants. However, a subsequent paper (157) developed a GC × GC-TOFMS method for the analysis of amino acid and organic acid trimethylsilyl derivatives, which was applied to rye grass metabolites. Separation using GC × GC was significantly improved compared to 1D GC, which would have yielded considerable peak overlap, as evidenced by the presence of metabolites with the same first dimension retention times (^1t_R). Comparison of pre- and post-harvest physiology of perennial rye grass demonstrated the suitability of GC × GC-TOFMS for describing the dynamic nature of the system being studied. Contour plots differed markedly between the samples with malic acid dominant in the post-harvest samples. Based on their results, the authors deemed GC × GC-TOFMS to be a rapid and powerful technique for comprehensive metabolite analysis, for target analysis and pattern recognition, and fingerprint studies for investigating cause and effect relationships (157).

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GC × GC-TOFMS has also been applied to the analysis of complex (derivatized) metabolite profiles from mouse spleen (158). In comparison to single dimension GC-TOFMS, mass spectral quality and sensitivity was greatly improved using GC × GC-TOFMS as a direct result of the increased resolution, separation and zone compression capabilities of GC × GC analysis. In fact, GC × GC-TOFMS facilitated detection of 1,200 compounds compared to only 500 compounds detected using GC-TOFMS. Additionally, analytical purity, which refers to the combination of chromatographic resolving power and mass spectral deconvolution power, was significantly improved in the former with purities of better than 0.2, compared to up to 2.5 in the latter, with purity values of zero representing optimal conditions.

Sumner (30) has stated that complex GC × GC-TOFMS systems will come with increased analysis times. It is important to clarify that temporal analysis time using GC × GC is not increased compared to single-dimension GC analysis, but rather post-run data processing times are greatly extended in the former as a result of the significantly larger data files that are acquired. Nevertheless, data file sizes may be reduced by scanning at lower spectral acquisition rates, combined with faster analysis times. Indeed Shellie et al. (159) estimated that data files should not exceed 125 Mb when a spectral acquisition rate of 100 Hz is used over a mass range 415 m/z together with an analysis time of ~37 minutes. This compares favorably to a 570 Mb data file generated using a spectral acquisition rate of 200 Hz over the same mass range but with a 60-minute analysis time.

The quantitative capabilities of TOFMS are superior to those of quadrupole MS (qMS) as a result of mass spectral skewing and limited peak data density in the qMS instrument. Nevertheless, GC × GC-qMS is still able to provide valuable qualitative information, and recently, semi-quantitative (160) and quantitative (161) data have been reported in some studies. The simplicity of GC × GC-qMS with respect to data interpretation and manipulation compared to the more sophisticated GC × GC-TOFMS has favored a recent increase in its application. Instrumental strategies have been employed to increase qMS scan rates that are necessary for fast GC × GC separations, including the use of reduced mass scanning ranges (162) and unique truncated libraries to accommodate these reduced ranges (163). Similarly, selected ion monitoring (SIM), whereby the mass spectrometer is set to scan over a very small mass range (typically one mass unit, or a few selected mass ions) has been used with GC × GC-qMS (160). Such approaches demonstrate the viability of GC × GC-qMS for metabolomic applications of limited mass range.

CONCLUDING REMARKS

The goal of metabolomics to comprehensively profile the metabolome is currently not achievable. This is directly related to the chemical complexity

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and diversity of the metabolome and the inadequacies of current analytical instruments to cater to such intricacies. An important instrumental limitation is the dynamic range and the diverse concentration ranges of metabolites. Although spectroscopic techniques provide useful data, multidimensional techniques are desirable. An important consideration is the issue of enhanced physical separation by exploiting improved chromatographic resolution versus spectroscopic resolving power. Consider the case of MS in which mass accuracies achievable with ion trap and quadrupole mass analyzers as commonly used in LC-MS are 0.1–1.0 Da, whereas Fourier transform ion cyclotron mass analyzers deliver accuracies of 0.001 at m/z of 1,000 Da. Mass resolution is defined as the function of mass divided by peak width at half height. TOF mass analysers are capable of producing mass resolutions on the order of 10,000 while mass resolutions exceeding 100,000 are achievable with Fourier transform ion cyclotron mass analyzers. For example, resolution of rutin (610.5180) and hesperidin (610.5620) requires a mass resolution capability of approximately 14,000, which cannot be achieved with either ion trap or quadrupole analysers but it is feasible with TOF analysers. This suggests that chromatographic resolution is no longer a prerequisite for metabolomics. Unfortunately, matrix effects such as ion suppression and competitive ionization are significant if the chromatographic step is omitted.

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