

# Plant metabolomics: A new frontier in phytochemical analysis

## AUTHORS:

Fidele Tugizimana<sup>1</sup>  
Lizelle Piater<sup>1</sup>  
Ian Dubery<sup>1</sup>

## AFFILIATION:

<sup>1</sup>Department of Biochemistry,  
University of Johannesburg,  
Johannesburg, South Africa

## CORRESPONDENCE TO:

Ian Dubery

## EMAIL:

idubery@uj.ac.za

## POSTAL ADDRESS:

Department of Biochemistry,  
University of Johannesburg, PO  
Box 524, Auckland Park 2006,  
South Africa

## DATES:

**Received:** 12 Apr. 2012

**Revised:** 10 Sep. 2012

**Accepted:** 01 Nov. 2012

## KEYWORDS:

chromatography; mass spectrometry; metabolite profiling; multivariate data analysis; nuclear magnetic resonance

## HOW TO CITE:

Tugizimana F, Piater L, Dubery I. Plant metabolomics: A new frontier in phytochemical analysis. *S Afr J Sci.* 2013;109(5/6), Art. #0005, 11 pages. <http://dx.doi.org/10.1590/sajs.2013/20120005>

The primary and secondary metabolites found in plant cells are the final recipients of biological information flow. In turn, their levels can influence gene expression and protein stability. Qualitative and quantitative measurements of these metabolites reflect the cellular state under defined conditions, and yield critical insights into the cellular processes that control the biochemical phenotype of the cell, tissue or whole organism. Metabolomics differs from traditional targeted phytochemical analysis in various fundamental aspects; for example, it is a data-driven approach with predictive power that aims to assess all measurable metabolites without any pre-conception or pre-selection. As such, metabolomics is providing new dimensions in the study of systems biology, enabling the in-depth understanding of the intra- and extracellular interactions of plant cells. Metabolomics is also developing into a valuable tool that can be used to monitor and assess gene function, and to characterise post-genomic processes from a broad perspective. Here, we give an overview of the fundamental analytical technologies and subsequent multivariate data analyses involved in plant metabolomics as a research tool to study various aspects of plant biology.

## Introduction

An organism is an expression of its underlying molecular composition that reacts and responds to a variety of intra-, inter- and extracellular stimuli. Whereas genes and proteins are mostly involved in storing and unfolding information needed for actualisation of cellular functional processes, metabolite patterns reveal the actual dynamic cellular environment.<sup>1</sup> In plants, the metabolome is a compilation of all primary and secondary metabolites, and can be regarded as the final recipient of genetic information (Figure 1) where levels of individual metabolites can influence gene expression, protein stability and metabolic fluxes.<sup>2-5</sup> These constituents of the metabolome have a wide range of physiological roles in plants such as participating in basic functions of the living cell, contributing to cellular structural integrity, acting as mobile inter- and intracellular signals, and being involved in passive and active defence responses in plant cells.<sup>2,6,7</sup> Hence, qualitative and quantitative measurements of intracellular metabolites yield critical insights into the cellular processes that control the biochemical phenotype of the cell, tissue or whole organism.<sup>1,8,9</sup>

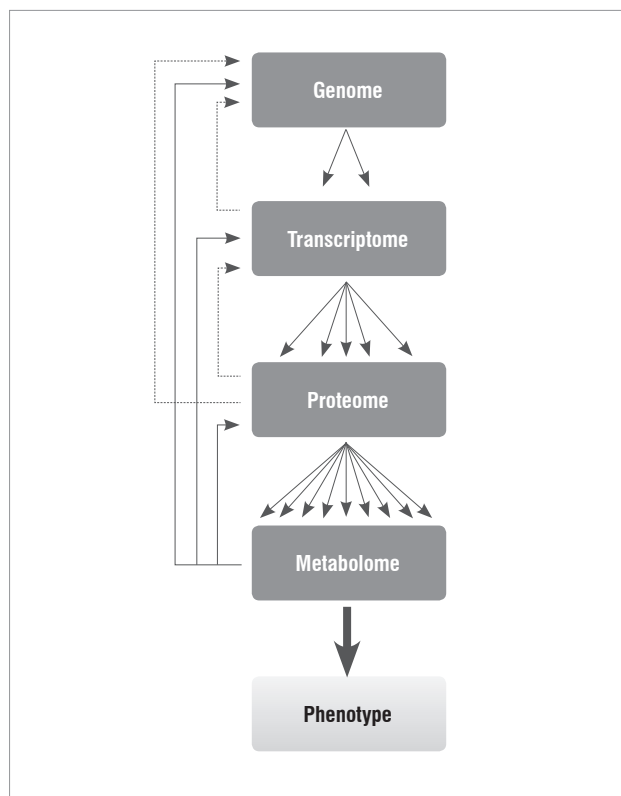
## Metabolomics and systems biology

Metabolomics developed from metabolic profiling and is the most recent of the '-omics' approaches to emerge. The word 'metabolome' was first suggested in 1998 by Stephen Oliver (University of Manchester, UK) to designate the set of all low molecular mass compounds synthesised by an organism. In 2002, Oliver Fiehn (Max Plank Institute, Golm, Germany) introduced the word 'metabolomics' to designate a comprehensive analysis in which all the metabolites of an organism were supposed to be identified and quantified.<sup>4</sup> Metabolomics is generally defined as a holistic qualitative and quantitative analysis of all metabolites present within a biological system under specific conditions.<sup>1,3,8,9</sup> It differs from the classical or traditional targeted phytochemical analysis in various fundamental aspects, such as being a data-driven approach with predictive power that aims to assess all measurable metabolites without any pre-conception or pre-selection. In order to attain this goal, advanced analytical tools that provide high degrees of sensitivity, selectivity and reproducibility are required.<sup>5,9-11</sup>

Metabolomics has become a valuable tool for advancing our understanding of primary and secondary metabolism in plants and is revolutionising the field of plant biology.<sup>12</sup> It is viewed as a complementary technique to other functional genomics approaches such as transcriptomics and proteomics. Furthermore, metabolomics is a cornerstone in the integration of the '-omics' technologies that contribute to a systems biology overview.<sup>13</sup> As such, it assists in providing a holistic understanding of the organisation principle of cellular functions at different levels, and in providing ways of monitoring all biological processes operating as an integrated system.<sup>1,4,6,14</sup> Moreover, metabolomics as a post-genomics tool is often regarded as offering distinct advantages when compared to other '-omics' technologies. This point of view is based on the fact that changes in the transcriptome or proteome do not always correlate to biochemical phenotypes.<sup>1,11,15,16</sup>

Metabolomic approaches, on the other hand, monitor the ultimate products of gene expression – the metabolites – thus providing a phenotypic assessment of a biological system. Metabolites are organic compounds that may not be directly encoded in the genome, and their biosynthesis often involves a diversity of enzymes. Furthermore, metabolites are stoichiometrically interrelated, which results in more complex metabolic networks that do not exist in the case of transcripts or proteins. Thus, metabolomic strategies may actually offer the most valuable and functional information that is crucial in systems biology studies.<sup>1,4,6,13,17</sup>

All metabolomic analyses are a snapshot (or a point-in-time chemistry) of a biological system (cell, tissue or whole organism), showing which metabolites are present and their levels at a given time point and under specific physiological conditions.<sup>3,9,11,14,18</sup> Different metabolomic strategies have thus been developed for different applications, and are presented in Table 1.<sup>9,18-20</sup>



**Figure 1:** Biological information flow from genome to metabolome. The metabolome is complementary to the transcriptome and proteome, captures the functional or physiological state of the cell, and provides a link between genotypes and phenotypes. Altered gene expression is ultimately reflected in changes in the pattern and/or concentration of metabolites. There are an estimated 200 000 plant metabolites and many remain unknown.<sup>4</sup>

### The workflow for plant metabolomic analysis

Plant metabolomes can be very diverse. Because of the complexity and divergent physicochemical properties of the cellular metabolome, a combination of two or more metabolomic strategies (outlined in Table 1) may be considered to achieve a comprehensive coverage of the plant metabolome. Furthermore, in any metabolomic approach, a broad metabolic picture is achieved through the combination of multiparallel

and complementary analytical systems, including the use of various extraction protocols.<sup>5,6,9,18,21</sup>

A metabolomic analysis comprises three main experimental stages: (1) preparation of the sample, (2) acquisition of the data using analytical methods and (3) data mining using chemometric methods followed by compound identification. These steps are crucially interrelated, and as illustrated in Figure 2, may each consist of a series of sub-steps. The resulting analysed data from the various experimental phases form the basis for meaningful biochemical interpretation.<sup>15,22</sup>

Sample preparation is a critical step in transforming the sample into a solution that can be analysed to make a vital contribution in defining the array of metabolite classes to be covered. This step involves a series of different experimental stages: selection and harvesting of samples, drying or enzyme quenching procedures, extraction of metabolites and preparation of the samples for analysis. The selection of plant material depends mainly on the biological question that the researcher seeks to investigate.<sup>22,23</sup> Throughout this step, care must be taken to avoid the introduction of any form of unwanted variability that would significantly affect the outcome of the analysis. Sample degradation (thermal, oxidative or enzymological) and contamination are major factors leading to variations during this step.<sup>8,10,24</sup> Various enzyme quenching methods include drying, treatment with acid, use of enzyme inhibitors or high concentrations of organic solvents.<sup>4,22</sup>

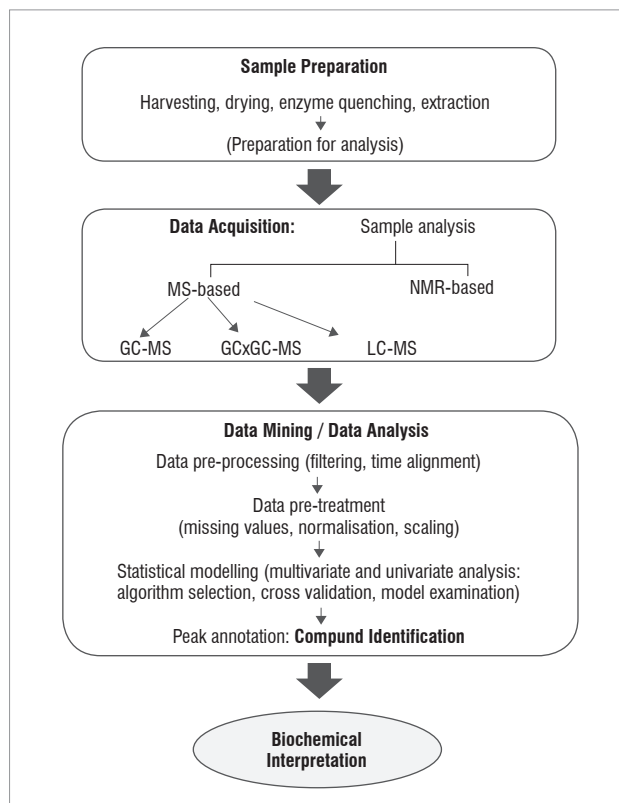
Plant metabolites are structurally diverse, forming a highly complex spectrum of compounds of different size, solubility, volatility, polarity, quantity and stability.<sup>1,19,25-27</sup> Any extraction method would certainly produce an inherently multidimensional sample arising from the chemical and physical differences of the constituents.<sup>28,29</sup> Several methods may be employed to extract metabolites; the choice of method depends on a variety of factors, such as the physicochemical properties of the target metabolites, the biochemical composition of the system under investigation and the properties of the solvent to be used. Some of the common extraction methods include solvent extraction, supercritical fluid extraction, sonication and solid phase extraction. However, no comprehensive extraction technique exists for the recovery of all classes of compounds with high reproducibility and robustness. Thus, for a comprehensive coverage of different classes of metabolites, extraction methods may be used in combination.<sup>5,10,18,19,22</sup>

Data acquisition (sample analysis) follows the sample preparation step and requires advanced analytical techniques as the ultracomplexity of samples for metabolomic analysis makes it impossible to technologically separate, quantify and identify every metabolite within a biological sample.<sup>1,9</sup> A range of analytical platforms are employed in metabolomic studies (separately or in combination), and each platform has its own advantages and limitations, either in selectivity or sensitivity (Table 2). The choice of the analytical platform depends mainly on the

**Table 1:** Strategies for metabolomic analysis

Term	Description
Metabolomics	Holistic quantification and identification of all metabolites within an organism or a biological system, under a given set of conditions. This state is currently unrealisable, with any single or combination of metabolomic approaches.
Metabonomics	This term is normally used in non-plant systems and generally refers to the quantitative detection of endogenous metabolites that are dynamically altered within a living system in response to pathophysiological stimuli or genetic modification. Tissues and biofluids are commonly used for these analyses.
Metabolic/Metabolite profiling	The identification and quantification of metabolites related through their metabolic pathway(s) or similarities in their chemistry.
Targeted metabolite analysis or metabolite target analysis	Qualitative and quantitative analysis of one or a few pre-defined metabolites related to a specific metabolic reaction. Such an approach relies on optimised metabolite extraction, separation and detection.
Metabolite fingerprinting	Rapid and high-throughput methods where global metabolite profiles are obtained from crude samples or simple cellular extracts. In general, metabolites are neither quantified nor identified.
Metabolite footprinting	The measurement of metabolites secreted from the intracellular complement of an organism (or biological system) into its extracellular medium or matrix. This approach is commonly used in microbial metabolomics.

study at hand, taking into consideration the class of compounds, their chemical and physical properties, and their concentration levels. As such, the techniques most often used are nuclear magnetic resonance spectroscopy<sup>3,5,30</sup> and chromatography coupled to MS.<sup>9,31</sup>



MS, mass spectrometry; NMR, nuclear magnetic resonance; GC, gas chromatography; LC, liquid chromatography.

**Figure 2:** Flowchart for plant metabolomic studies. The three main steps of a metabolomic analysis are sample preparation, data acquisition and data mining. A data handling pipeline is established from data acquisition to data mining. These three steps are interrelated and lead to the biochemical interpretations.<sup>22</sup>

## Analytical platforms employed in metabolomics

A range of analytical technologies may be used in metabolomics, including gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS), liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-electrochemistry-mass spectrometry (LC-EC-MS), nuclear magnetic resonance (NMR) spectroscopy, LC-NMR, direct infusion mass spectrometry (DIMS), and Fourier-transform infrared (FT-IR)- and Raman spectroscopies.<sup>8,10,14,31,32</sup> Of these, chromatography-mass spectrometry (GC-MS and LC-MS) and NMR are the most widely applied.<sup>4,5,30,31</sup>

The use of NMR-based fingerprinting marked the beginning of metabolomics as a tool in biochemistry and phytochemical analysis.<sup>19</sup> It is an unbiased, rapid, non-destructive technique that requires little sample preparation and thus lessens the chance of sample loss or the introduction of variability into the preparation.<sup>30</sup> In an NMR analysis, there is no analyte separation process involved (as is the case in chromatographic analyses); NMR can thus provide selectivity without separation, is independent of analyte polarity, and does not require sample derivatisation prior to analysis. The magnetic properties of paramagnetic (e.g. <sup>1</sup>H and <sup>13</sup>C) nuclei allow for a powerful tool for observing the environments of such nuclei bonded all over a molecular skeleton. When samples in a deuterated solvent are placed in a strong magnetic field and irradiated with a radio frequency, the absorption of energy allows the nuclei to be promoted from low-energy to high-energy

spin states. The subsequent emission of radiation during the relaxation process generates the resonances or signals recorded on an NMR spectrum as 'chemical shifts', representing frequencies from all NMR-visible nuclei in the sample, relative to that of a reference proton present in a reference compound. Thus, an NMR spectrum of a multicomponent extract is the result of the superposition of the collective spectra of all NMR-visible individual compounds present in the sample under study. Hence, an NMR analysis would generally give a global view of all the metabolites (primary and secondary) in a sample, provided that they are NMR detectable.<sup>5,30</sup> In NMR spectroscopy, the signal intensity for all compounds is dependent on the molar concentrations and reproducibility is high, even though the sensitivity is relatively low (micromolar range) and more sample is required.<sup>3,5</sup> Following data acquisition, it is necessary to apply solvent suppression techniques, baseline or background correction, and integration and data normalisation methods.<sup>30</sup>

**Table 2:** Some standard techniques used in metabolomic analysis. In general, one technology is not sufficient for the analysis of all compounds, but any form of separation will inherently introduce a bias towards the analytes being detected.

Technique	Sensitivity	Throughput	Comprehensiveness
Nuclear Magnetic Resonance (NMR)	Low	Low-high	Low-high
Infrared	Low	High	Low
Liquid Chromatography (LC)-NMR	Low	Low	High
LC-Mass Spectrometry (MS)	Medium	High	High
Gas Chromatography-MS	High	High	High
Capillary Electrophoresis (CE)-MS	High	Medium	High
LC-Electrochemistry-MS	High	High	High
LC-Ultraviolet	Medium-high	High	Very low

Sources: Sumner et al.<sup>1</sup> and Weckwerth and Morgenthaler<sup>22</sup>

Although NMR spectroscopy can yield detailed information on the quantities and identities of metabolites present in extracts, the chemical elucidation of NMR-detected compounds can be highly complex as a result of overlapping signals and shielding effects by neighbouring electrons. Moreover, the inherent low sensitivity of NMR, its sensitivity to the chemical environment (pH, ionic strength, temperature, etc.) of the sample and the differential sensitivity of metabolites to the chemical environment, hamper the quality of NMR analyses of complex samples.<sup>30,33</sup> However, the disadvantages of low sensitivity and resolution are addressed by the development of cryogenic probes, higher strength superconducting magnets, miniaturised radio frequency coils and multidimensional (e.g. 2D-J-resolved and heteronuclear single quantum coherence) techniques.<sup>5,30,34</sup> By using 2D-NMR that spreads the spectral content over a two-dimensional plane, the identification of compounds can be facilitated and minor compounds can be better observed, even allowing for structural elucidation in crude extracts.<sup>3,5,30</sup>

Column chromatographic techniques (GC/LC), on the other hand, have medium and high sensitivity, and provide separation of the sample components based on the partitioning of an analyte between stationary and mobile phases, according to its physicochemical properties. A better chromatographic separation of an inherently multidimensional sample can significantly enhance the quality of MS analysis and subsequent

compound identification by reducing the complexity of the mass spectra and the matrix effect. To optimise chromatographic separation of such complex samples, factors including column chemistry, the elution method for LC (gradient or isocratic) and the programmed-temperature method for GC are to be considered. Recent developments in enhancing chromatographic separation of complex samples include the use of multidimensional separation systems such as two-dimensional gas chromatography (GC×GC) and two-dimensional liquid chromatography (LC×LC). Separation involved in these techniques is based on the orthogonality of the two columns (of different chemistries) used. These techniques thus permit an improved chromatographic separation of complex samples by providing an expanded separation space, thereby minimising possible co-elution of an analyte or one-dimensional peak overlap.<sup>35-38</sup>

Coupled to MS detection, these chromatographic techniques are more sensitive and capable of detecting metabolites in low abundance. GC-MS is a versatile, robust, technically reproducible and sensitive technique in the nano- to picomolar range. It is well suited to non-targeted metabolite profiling of volatile and thermally stable non-polar or derivatised polar metabolites or the targeted analysis of derivatised primary metabolites.<sup>39</sup> LC-MS, on the other hand, is technically more demanding, but caters well for metabolites that are non-volatile, polar or thermally labile. In plant metabolomics, LC-MS is frequently used to profile secondary metabolites. As chromatographic separation is based on the chemical nature of the analytes and that of the stationary and mobile phases of the column, more than one type of column chemistry might be needed to cover a wide range of analyte classes, especially for secondary metabolites. In combination with ESI-MS (electron spray ionisation-MS) it offers a powerful and sensitive technique in the pico- to femtomolar range.<sup>1,31</sup>

Here, the detection of the mass-to-charge ratio ( $m/z$ ) and abundance of the various analytes generated during ionisation is a key aspect of the analysis. The three main components in all types of MS instruments are (1) an ionisation source such as electron impact- (EI), electrospray- (ESI) and atmospheric pressure chemical (API) ionisations, (2) a mass analyser such as time-of-flight, quadrupole mass filters and quadrupole ion traps and (3) a detector such as an electron multiplier-based detector or a micro-channel plate linked to a time-to-digital converter. To optimise the transmission of ions to the analyser and detector, all three MS components are maintained under vacuum. The detected ions are recorded as pairs of  $m/z$  and abundance values, processed, and displayed in a mass spectral format.<sup>14,31</sup> To enhance detection efficiency, mass resolving power and mass accuracy of MS, various technologies (in ionisation methods, mass analysers and detectors) have been developed for different applications and for use in multi-MS platforms.<sup>14,40-43</sup>

In plant metabolomic analyses, MS is the key platform for compound identification, an essential step in the workflow of any metabolomic analysis. The mass spectral data provides a pattern that is most often compound specific. However, the degree of certainty in elucidating the structural and chemical identity of an MS-detected analyte relies on the efficiency and accuracy of the three principal processes of the MS (ionisation,  $m/z$  analysis/manipulation and ion detection) and on appropriate algorithms. The procedure in metabolite assignments from MS data consists of: (1) acquisition of sufficient and accurate structural information (such as accurate mass measurement and fragmentation patterns), (2) calculation of chemical combinations that fit the measured accurate mass (elemental composition formula), (3) spectral comparison (mostly for GC-MS instruments) or a database search and verification of the fragmentation pattern (for LC-MS instruments), (4) the use of other MS information available such as MS<sup>n</sup> and MS<sup>E</sup> data (the latter is a form of non-selective MS<sup>n</sup> where E is collision energy) and (5) the use of authentic pure standards (when commercially available) or <sup>13</sup>C materials as internal standards.<sup>14,15,44,45</sup>

The usage of parallel analytical platforms can provide additional information or confirmation for a putatively identified metabolite. For example, EI ionisation is the most commonly used GC-MS ionisation technique that generates informative and characteristic mass spectra resulting from the relative high degree of fragmentation which aids in

compound identification. In contrast, ESI-MS, used in LC-MS, usually generates [M+H]<sup>+</sup> and [M-H]<sup>-</sup> ions as the main signals, and these ions are useful for reducing the candidate structures of detected compounds. GC-EI-MS is thus a good approach for targeted analysis of known primary metabolites, whereas LC-ESI-MS is good for untargeted analysis of secondary metabolites.<sup>14,15,34,44,45</sup>

## Data mining and data processing

High-performance instrumentation as described generates extremely large volumes of data. In order to handle these large data sets and to comprehend the metabolome data, automated software is needed that can identify peaks from raw data, align the peaks among different samples and replicates, and identify and quantify each metabolite. Informatics and statistics are therefore essential tools for processing metabolomic data sets.<sup>33,46,47</sup> Data mining comprises data pre-processing, data pre-treatment and statistical modelling of the primary data (Figure 2). The statistical modelling (which is essential and central in data analysis) is briefly explained below and the reader is referred to more advanced discussions of the topic.<sup>46,47</sup>

Because metabolomic analyses reflect the cellular state under defined conditions, metabolomic experiments are designed in such a way as to measure the biological variation in the metabolome.<sup>5,18</sup> However, the total variation in the metabolomic data is actually the sum of the pre-defined or induced biological variation and all other variations (non-induced biological, technical and analytical variation). Hence, in the data mining step the procedures of data pre-processing and data pre-treatment aid in 'cleaning' the data to focus on the biologically relevant information. Various software packages (which depend on the analytical technique employed for data acquisition) have been developed to aid with data mining in an automated manner.<sup>9,48</sup>

Some of the data pre-treatment methods include centring, scaling and transformation. The centring procedure enables the conversion of all concentrations to fluctuate around the zero value of coordinates, by calculating the average of each variable and subtracting it from each observation. This process adjusts differences in the offset between high and low levels of compounds in samples. It thus simplifies the estimation of regression coefficients. Scaling, on the other hand, involves dividing each variable by a function related to its standard deviation (scaling factor) to adjust for the variation in fold differences between detected metabolites. Lastly, mathematical transformation processes are performed on the raw data because of the possibility of non-linearity in variables from a biological system. These methods transform the data into data matrices suitable for linear modelling techniques by converting multiplicative relations into additive relations, and correcting for heteroscedasticity (random variables with different variability).<sup>47-49</sup> The cleaned data are then subjected to statistical analysis which provides model-based descriptions of the biological variation in the system under study. These statistical models specifically single out representatives of metabolites of interest (annotated peaks), which can further be chemically or structurally identified in a definitive manner.

## Statistical modelling and multivariate data analysis

Metabolomic studies generally generate high-dimensional and complex data sets that are difficult to analyse and interpret by visual inspection or any traditional univariate statistical analyses. Multivariate data analysis (MVDA) methods – mathematical modelling approaches – are therefore used to extract meaningful information from these large empirical data sets.<sup>4,49-51</sup> These chemometric techniques provide models that are well suited to handle confounding and covariance patterns (both within and between variables) – analyses that are normally beyond the capacity of traditional univariate statistical methods.<sup>52-54</sup> Table 3 lists a number of chemometric methods that are used for MVDA. Depending on the research objectives, the most appropriate method should be exploited. Detailed explanations of the mathematical algorithms on which these chemometric models are based, can be found in the cited literature. It may suffice to underline that most of these MVDA models are projection-based methods and apply, in an expanded manner, the

eigenvector/eigenvalues and kernel algebraic notions. Furthermore, these methods are based on the notion of latent variables (assuming that the system under consideration is driven by a definable number of latent variables) that assume a linear (or non-linear) relationship between the descriptor and response variables (see Figure 3: X and Y matrices: observed variables and pre-defined response outputs).<sup>53-55</sup>

The high-dimensional and complex metabolomic data can be chemometrically analysed in unsupervised and supervised ways (Table 3 and Figure 3). The unsupervised modelling of the data focuses on the intrinsic structure, relations and interconnectedness of the data and is sometimes referred to as descriptive models. Supervised modelling, on the other hand, seeks to transform the multivariate data from metabolite profiles into a representation of biological interest under the guidance of a 'supervisor'. These models are often called predictive models. The basis of supervised modelling is that there are some patterns (such as metabolic fingerprints) in data that have pre-defined responses (such as effects of a treatment or condition), and the goal of supervised methods is to find a model or mapping that will correctly associate the inputs with the responses.<sup>54</sup> The data is thus algebraically represented in two types of matrices – the descriptor

matrix X (observed variables) and the response matrix Y (the pre-defined traits). Geometrically, a multivariate modelling process defines a point in K-dimensional space with the descriptor values as coordinates.<sup>53,54,56</sup> The unsupervised methods are non-parametric analyses and generate models that are independent of the user. The input data, the descriptor matrix X, is presented into the system, which then simplifies and reduces the dimensionality of the data sets, grouping metabolites into different clusters with no loss of information. The supervised models, on the other hand, are mathematical transformations that correctly relate the descriptor matrix X with the response matrix Y.<sup>46,53,54,56</sup>

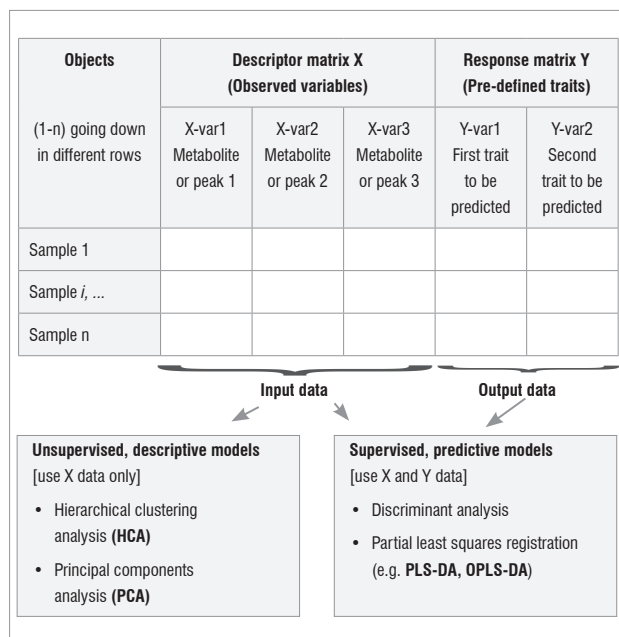
In many metabolomic studies, principal component analysis (PCA) – an unsupervised multivariate linear model – and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) – a supervised model – are often used for data analysis. In all unsupervised chemometric methods, PCA remains the workhorse and gold standard model to deal with high-dimensional and complex data sets.<sup>46,53</sup> PCA is a projection-based method and a mathematically rigorous process that provides a global and qualitative visual representation of similarity or dissimilarity between and within samples (without using class information; e.g. treatment vs. control).<sup>46</sup>

**Table 3:** Some of the chemometric methods used to analyse multivariate data sets. A 'supervised' method is one that requires training with known data sets in which the types of groups expected are pre-defined before being applied to experimental data.

Type of model	Abbreviation	Term	Linearity/non-linearity assumption
Generally 'Unsupervised'	CCA	Canonical Correlation Analysis	Linear
	CD-PCA	Clustering and Disjoint-Principal Component Analysis	Linear
	HCA	Hierarchical Clustering Analysis	Linear
	K-CCA	Kernel-Canonical Correlation Analysis	Non-linear
	K-PCA	Kernel-Principal Component Analysis	Non-linear
	KANN	Kohonen Artificial Neural Networks	Non-linear
	MSCA	Multilevel Simultaneous Component Analysis	Linear
	PCA	Principal Component Analysis	Linear
	SCA	Simultaneous Component Analysis	Linear
	W-PCA	Weighted-Principal Component Analysis	Linear
Generally 'Supervised'	BANN	Back-prop Artificial Neural Networks	Non-linear
	ASCA	ANOVA-Simultaneous Component Analysis	Linear
	DA	Discriminant Analysis	Linear
	K-OPLS-DA	Kernel-Orthogonal Partial Least Squares-DA	Non-linear
	K-PLS-DA	Kernel-Partial Least Squares-DA	Non-linear
	N-PLS-DA	N-way PLS-DA	Linear
	OPLS-DA	Orthogonal Partial Least Squares-DA	Linear
	O2PLS-DA	Bidirectional Orthogonal PLS-DA	Linear
	OSC	Orthogonal Signal Correction	Linear
	PCDA	Principal Component Discriminant Analysis	Linear
	PLS	Partial Least Squares	Linear
	SIMCA	Soft Independent Modelling of Class Analogy	Linear
	PLS-DA	Partial Least Square Discriminant Analysis	Linear

Sources: Vichi and Saporta<sup>51</sup>, Bylesjö et al.<sup>52</sup>, Trygg et al.<sup>53</sup>, Fonville et al.<sup>55</sup>

Note: For a basic plant metabolomic analysis, PCA (score and loading plots) and OPLS-DA (S-plots) are most often used (see Figure 4).

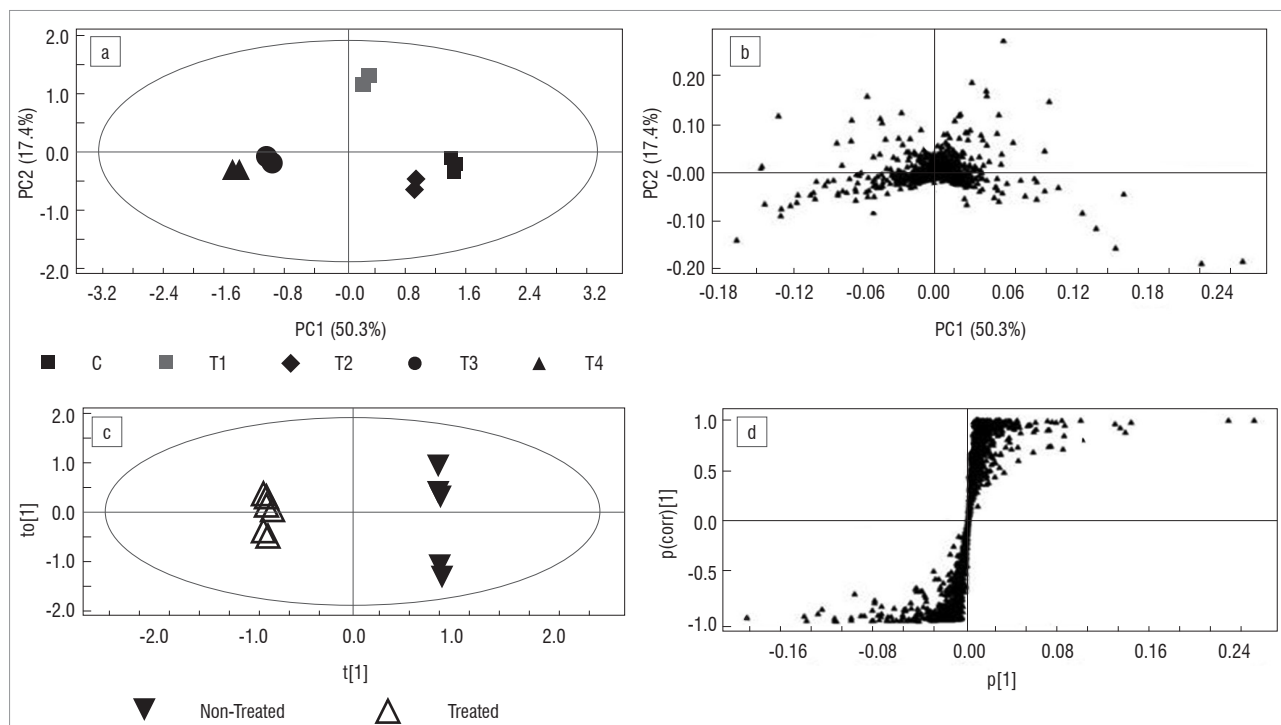


**Figure 3:** Chemometric analysis of metabolomics data. Multivariate data analysis models are classified into two groups: 'unsupervised' and 'supervised' methods. The unsupervised methods use only the descriptor matrix X ( $N \times K$ ), wherein  $N$  is the number of samples and  $K$  is the number of variables (spectral measurements, peaks) in X, and cluster metabolites into groups, independently of the user (with no pre-defined parameters). The supervised models use both descriptor matrix X and the response matrix Y ( $N \times M$ ), wherein  $M$  denotes the number of variables (pre-defined traits) in Y.<sup>52,54</sup>

In PCA modelling, the variance in a data set is algebraically described in terms of underlying orthogonal variables, also called principal components (PCs). The original variables are thus expressed as linear combinations of these PCs (latent variables), each consisting of two parts – a score ( $t_i$ ) and loading ( $p_i$ ). All PCs are mutually linearly orthogonal to each other and each PC counts for a portion of the total variance in the data set, the first two or three PCs accounting for the largest part of the total variance. The descriptor matrix X is thus mathematically projected into a low-dimensional space, providing interpretable visualisation of the original complex data set thereby highlighting similarities or differences. The score plot gives information about relationships between objects (e.g. trends, groupings and outliers). The Y- and X-axes (e.g. PC1 vs. PC2) of a score plot illustrate the variation within and between groups, respectively. The loading plot illustrates the putative discriminating variables responsible for sample clustering and also explains the variation in scores.<sup>46,53,54</sup> Figure 4a and 4b illustrate typical PCA-derived score and loading plots, respectively.

OPLS-DA is a linear regression method, which has been successfully used for prediction modelling in metabolomics and biochemical applications.<sup>52,53,55</sup> It is a supervised classification model that differs from PCA by the addition of grouping variables that indicate in which class the samples belong. Where PCA modelling is a descriptive method, OPLS-DA method is an explicative or predictive analysis. The latter facilitates the identification of the metabolite ions responsible for the discrimination between groups.<sup>57,58</sup>

OPLS-DA is a modification of the PLS-DA (projection to latent structures-discriminant analysis) method, with an integral orthogonal signal correction filter. The OPLS-DA modelling aims at finding predictive components that simultaneously maximise the covariance and correlation between X and Y matrices.<sup>57</sup> Algebraically, the model uses information in the response matrix Y to decompose the descriptor matrix X into correlated, orthogonal and residual structures of information, respectively. The power of this regression model lies in its ability to separate modelling of Y-predictive (response-related)



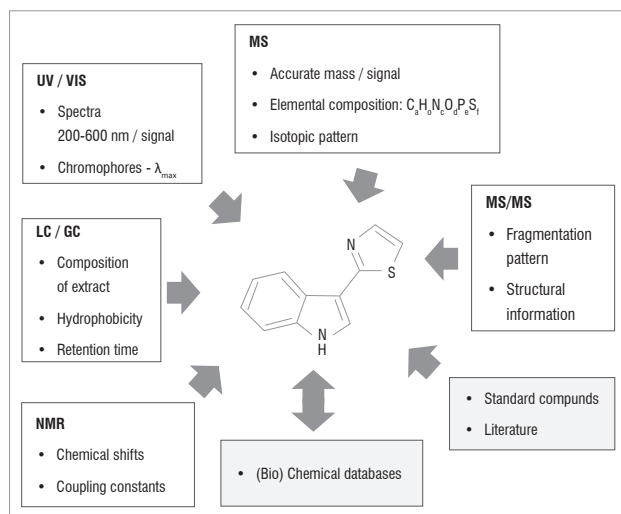
**Figure 4:** Graphical representations of multivariate data analysis. A principal components analysis (PCA) model reduces the dimensionality of a data table forming a low-dimensional model plane. (a) A PCA score plot permits the visualisation of the relation among the observations or samples, showing groupings, trends, or outliers. It shows differences between groups along the X-axis (PC1) and differences within groups along the Y-axis (PC2). (b) The PCA loading plot defines the influence of the variables in the model plane, and the relationship among them. (c) The orthogonal projection to latent structures-discriminant analysis (OPLS-DA) score plot, similar to the PCA plot, indicates differences in the molecular composition of samples while (d) the OPLS-DA S-plot identifies putative biomarkers (bottom left and upper right) responsible for the group separation.<sup>53</sup>

and systematic Y-orthogonal (response-orthogonal) variations in data, while simultaneously maximising the covariance between X and Y. The Y-orthogonal variation can be described as systematic effects needed to characterise the system but are unrelated to the model predictions.<sup>52,55,57</sup>

OPLS-DA methods therefore model data according to a *a priori* class information (such as treated vs. non-treated) assigned to samples before the analysis. This separation of Y-predictive (discriminating) variation and Y-orthogonal variation (that which does not contribute to the class separation) greatly facilitates the data interpretation. As such, the OPLS-DA model is a suitable tool to extract information on changes or differences in the molecular composition of samples (Figure 4c). While the OPLS-DA loading S-plot (Figure 4d) enables the extraction of statistically and potentially biochemically significant metabolites or biomarkers in the samples, the more advanced shared-and-unique-structures plot (not shown) enables the identification of metabolites that are shared between groups or that are unique to a group.<sup>53,57,59</sup>

## Compound identification

Compound identification, the last step in metabolomic analyses, is of great importance because biochemical interpretation of metabolomic data relies heavily on the availability of well-structured databases for the identification of metabolites. In putative identification some molecular properties (such as experimentally determined accurate mass) and mass spectral patterns are used to define molecular and empirical formulae from which metabolites can be derived or identified by comparative searches of available spectral, compound and metabolic pathway databases.<sup>15,34</sup> In such identification procedures, chemical standards are normally not used and the putatively identified metabolites are usually reported with a defined degree of certainty.<sup>14,15,57</sup> Definitive identification, on the other hand, involves the use of more than two molecular properties (retention time, retention index, mass spectral fragmentation, NMR-spectral shifts), comparative searches of libraries (mass spectral, NMR-spectral, retention index), confirmation with authentic chemical standards and the use of *in vivo* labelling methods.<sup>15,18,60</sup> In some instances, analytically detected entities of biological significance are reported as unknown with no structural identification.<sup>10,15,18,31</sup> Figure 5 schematically illustrates typical information generated from different analytical methods, aiding the identification of compounds in a metabolomic analysis.



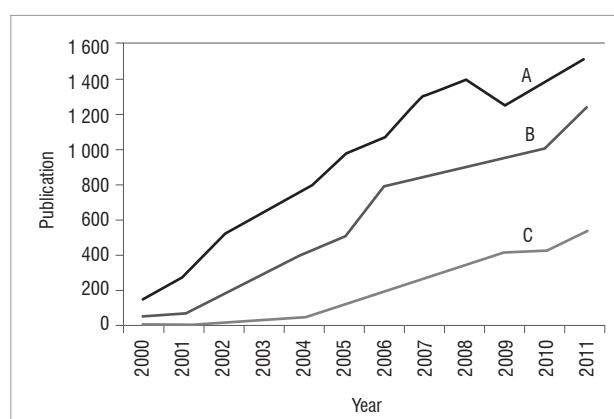
GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy, UV/VIS, ultraviolet/visible range spectroscopy obtained by the photo diode array detector.

**Figure 5:** The identification of a metabolite. Typical information generated by analytical technologies and knowledge resources that are used in the identification of a metabolite. Experimental validation is by means of standard compounds and information present in literature and databases. Resources such as species databases, literature, spectral databases and chemical databases help in narrowing the number of ambiguities for candidate metabolites.<sup>14</sup>

## Applications of metabolomics in plant research

Despite present limitations, metabolomics has proved to be an indispensable tool for characterisation of post-genomic processes in plants with a broader perspective. The uniqueness of metabolomics, firstly, is that it is a data-driven approach with mathematically rigorous data analysis methods and, secondly, is that it has the ability to provide a (relatively) holistic analysis of the actual cellular dynamics of a biological system under consideration. Metabolomic analyses offer ways of elucidating relationships that occur primarily through regulation at the metabolic level and reveal a direct link between a gene sequence and the function of the metabolic network.<sup>1,6,8,54,61</sup>

Plant metabolomics is still a relatively young field. Figure 6 shows the increase in publications in plant metabolomics relative to those related to genomics and proteomics. Metabolomic strategies have much to offer and are increasingly being applied in various areas of the plant sciences.<sup>62</sup> This increase in publications is the result of a divergence in the use of the new technology where different metabolomic approaches (Table 1) is combined with one or more analytical platforms (Table 2).



Source: Data obtained from SciVerse Scopus

**Figure 6:** Growth of plant metabolomics. The graph illustrates the increasing publication trends (and research activity) from the three plant '-omics' approaches: (A) genomics, (B) proteomics and (C) metabolomics, expressed as the number of publications per year from 2000 to 2011. Plant metabolomics is increasingly providing functional information of novel content and value that complements the other plant '-omics' approaches.

Broad research areas where metabolomics are applied include the interpretation of metabolic pathways and networks, biomarker discovery that can assist in the identification of novel molecular targets and bioactive metabolites,<sup>59,63–65</sup> genotyping,<sup>1,8,16</sup> gene function elucidation,<sup>66–68</sup> plant breeding and crop quality assessment,<sup>18,20,69–71</sup> the discovery of metabolites involved in environmental adaptations, abiotic and biotic stress responses, host–pathogen interactions,<sup>72–79</sup> molecular biotechnology, and recombinant DNA technology, including risk assessment of genetically modified crops.<sup>34</sup>

Metabolomics can be an effective approach for the comprehensive evaluation of the qualities of medicinal plants.<sup>80</sup> The combination of NMR spectroscopy and MVDA was used in a chemotaxonomic study of *Ilex paraguariensis* (a tonic and medicinal plant) and other *Ilex* species.<sup>81</sup> Distinct discrimination of species was observed, based on a large number of metabolites present in organic and aqueous fractions. The major metabolites that contributed to the discrimination were identified as arbutin, caffeine, phenylpropanoids and theobromine. Among those metabolites, arbutin, which had not been reported as a constituent of *Ilex* species, was found to be a biomarker in 8 of the 11 species investigated. With regards to the mining of medicinal plants for the discovery of bioactive metabolites, metabolomics has so far been a valuable tool for high-throughput screening of bioactive substances in order to discover

new phytotherapeutic agents with high selectivity, unique modes of action and acceptable toxicological profiles.<sup>80</sup>

In the development of novel herbicides and pesticides, metabolomics is an invaluable tool because of its non-targeted nature. Modes of action of herbicides determine how plants respond to these chemicals and can be used to predict the suitability of new lead compounds. Here, applications of metabolomics in agroecosystems also include the investigation of ecotoxicological risk assessment of these bioactive compounds.<sup>82,83</sup>

Ecometabolomic studies were the subject of a recent review<sup>83</sup> concerning investigation into the responses of some metabolic pathways in plants to changes in abiotic factors (such as temperature, water, nutrient availability and pollution) and the biotic interactions between two or more species, which provided new biochemical insights that can be useful for systems biology and metabolic or genetic engineering.

NMR-based and MS-based metabolic fingerprints allowed the investigation of a range of chemistries, adding insight into the metabolic changes associated with establishment of disease in *Arabidopsis thaliana* leaves infected with *Pseudomonas syringae*.<sup>76</sup> Significant alterations in the levels of amino acids and other nitrogenous compounds, as well as specific classes of glucosinolates, disaccharides and molecules that influence the prevalence of reactive oxygen species involved in defence signalling were identified. The findings suggest that, superimposed on defence suppression, pathogens reconfigure host metabolism to provide the sustenance required to support exponentially growing populations of apoplastically localised bacteria.

To obtain further insight into the interaction between plants and herbivores, the interaction between cabbage (*Brassica oleracea*) and small cabbage white caterpillars (*Pieris rapae*) was analysed by LC-MS.<sup>69</sup> This study revealed a high correlation in levels of three structurally related coumaroylquinic acids in both plants and caterpillars, which suggests that these compounds represent a 'metabolic interface' in the interaction between the plant and the caterpillars.

Another NMR-based metabolomic analysis of the metabolome of tobacco plants treated with salt contributed to the understanding of the dosage and duration dependence of salinity effects on plant metabolism.<sup>73</sup> The results showed that salinity causes alterations in widespread metabolic networks involving, *inter alia*, transamination, the tricarboxylic acid cycle, glutamate-mediated proline biosynthesis and shikimate-mediated secondary metabolism. These results evidenced the valuable insights provided by metabolomic approaches in understanding the osmotic effects on plant biochemistry.

The composition of secondary metabolites greatly influences the quality and health potential of food and food products, in particular, flavonoids as a result of their antioxidant properties. Bovy and co-workers<sup>84</sup> highlighted the potential of GC-MS and LC-MS based metabolomics in profiling the metabolic changes in the flavonoid biosynthetic pathway of genetically engineered tomatoes and in monitoring the flux into newly introduced branches of the flavonoid pathway, such as stilbenes, auronas, chalcones, anthocyanins and flavones.

Further selected examples (Table 4) illustrate the wide and divergent range of applications of metabolomics and metabolomic approaches in modern plant sciences.

## Current limitations of metabolomics

One of the main challenges of plant metabolomic studies is the enormous complexity and diversity of the plant metabolome and the incomplete knowledge of plant metabolic pathways. Plant primary and secondary metabolites constitute a more heterogeneous group of molecules than the biomacromolecules in terms of physical and chemical properties.<sup>1,3,5,9,10,19</sup> An analysis of the metabolome, with its divergent physicochemical properties and wide variation in concentration ranges, would thus require a wide spectrum of chemistries and instrumentation with wide dynamic ranges. Hence, it is currently technologically impossible to extract and analyse all metabolites in a cell or organism in a single analysis, and the currently characterised plant metabolites represent a very small fraction of the

**Table 4:** Some illustrative examples of the applications of metabolomics to the biological study of plants

Study	Metabolomic approach	Main findings	Reference
Salt-stressed tomatoes: two varieties of tomato: Edkway and Simgle F1	Metabolic fingerprinting, using FT-IR spectroscopy	Classification of control and salt-treated fruit Key discriminatory regions were identified as nitrile-containing compounds and amino radicals	72
Wound-induced tuberization in potato ( <i>Solanum tuberosum</i> L.)	GC-MS-based metabolite profiling of both non-polar and polar metabolites	Dynamic picture of wound-induced metabolism Suberin-associated aliphatics in non-polar profiles; and organic acids, sugars, amino acids and phenylpropanoids in polar profiles Correlations between known suberin-associated metabolites and several unidentified metabolites in profiles	74
<i>Nicotiana tabacum</i> defence responses against <i>Phytophthora nicotianae</i>	Metabolic profiling using direct infrared laser desorption ionisation mass spectrometry and LC-ESI-MS	Correlation among three metabolic cascades and the defence response: changes in the metabolome indicating that the jasmonic acid/salicylic acid defence cascades and the abscisic acid turnover cascade are involved in stress/infection response Infection-specific changes in the plant metabolome (of phenolics, alkaloids, oxylipins and carbohydrates)	75
Characterisation of the natural variation in <i>Arabidopsis thaliana</i> metabolome	Metabolic distance based on untargeted metabolite fingerprints (using LC-MS and GC-MS)	<i>A. thaliana</i> accessions chemically diverged (in the PCA plane) As a result of chemical divergence, these accessions differed in their interaction with biotic agents Weak correlation between the genetic and metabolic distance, revealing that genetic diversity is not one-to-one translated into metabolic diversity	78
Investigating the committed enzyme for the first step of sulpholipid biosynthesis in <i>Arabidopsis thaliana</i>	LC-MS-based metabolomic analysis with 'gene-to-metabolite' correlation strategy	Identification of a novel gene, <i>UDP-glucose pyrophosphorylase3 (UGP3)</i> , required for sulpholipid biosynthesis Chloroplastic localisation of <i>UGP3</i>	68
Metabolomic analysis of <i>Medicago truncatula</i> cell cultures treated with yeast and methyl jasmonates	Metabolic analysis of intra- and extracellular secondary metabolites, using HPLC-PDA-ion-trap MS	Three phases of intracellular response to elicitation Novel methylated isoflavones identified Highlights flexibility within the isoflavonoid pathway, suggests new pathways for complex isoflavonoid metabolism and differential mechanisms for medicarpin biosynthesis depending on the nature of elicitation	65
Investigating metabolic differences in the three medicinal Panax herbs: <i>P. ginseng</i> , <i>P. notoginseng</i> and <i>P. japonicus</i>	Metabolite profiling of metabolites in the three medicinal Panax herbs, using UPLC-QTOF-MS and multivariate data analysis models (PCA and PLS-DA)	Clear separation of metabolic compositions among the three medicinal herbs Identification of critical markers (accountable for the separation/variations): chikusetsusaponin Iva, ginsenoside-R0, Rc, Rb1, Rb2 and Rg2	64
Ergosterol-induced sesquiterpenoid synthesis in tobacco cells	LC-ESI-MS-based metabolomics following dispersive liquid-liquid extraction	Differential changes in the metabolome of tobacco cells, leading to variation in the biosynthesis of secondary metabolites	77
Activation of camalexin biosynthesis in <i>A. thaliana</i> in response to lipopolysaccharides	LC-ESI-MS-based targeted gene-to-metabolite metabolomics	Upregulation of the camalexin biosynthetic pathway was accompanied by a time-dependent increase in camalexin concentration	79



whole metabolome. A second challenging task is the identification and/or structural elucidation of molecules from analytical detector signals. The lack of universal metabolite-specific libraries and known reference compounds currently represents a major limitation to the definitive identification of metabolites.<sup>15,18,60</sup> Fortunately, a number of strategies, such as advancement in and complementary use of technology (LC-NMR-MS, GC×GC-TOF-MS, highly improved MS instrumentation, etc.) and metabolomic databases,<sup>12,85</sup> are increasingly being brought forward to assist in metabolite annotations and compound identification.<sup>14,15,36</sup>

## Conclusion and outlook

The combination of the capabilities of different analytical instrumentation for the analyses of multicomplex samples and the integration of metabolomics with other ‘-omics’ approaches in the context of a high-dimensional biological approach, is able to provide new insights into cellular function and regulation of metabolic networks. The ultimate aim of ‘-omics’ technologies is to understand and predict the behaviour of complex systems such as plants, through the use of results obtained from data mining tools for subsequent modelling and simulation. Plant metabolomics has developed to the point where it can be applied alone and/or in combination with other technologies of functional genomics. Even with its current limitations, plant metabolomics is an informative tool that is revolutionising plant biology.

## Acknowledgements

The National Research Foundation and the University of Johannesburg are acknowledged for support.

## Authors' contributions

F.T., L.P. and I.A.D. contributed to and wrote the manuscript. I.A.D. was the project leader.

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