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## The Assessment of Plant Metabolite Profiles by NMR-Based Methodologies

### Abstract

NMR-based metabolic profiling techniques can simultaneously track changes in many plant metabolites and have found a number of applications in both systems biology and biosafety. Together with multivariate statistical analyses, NMR spectroscopy has been successfully applied to the characterisation of various herbs and plant products for quality control, authentication, determining geographical origin and for detecting adulteration of products. Additionally, the metabolic consequences of plant extracts have been demonstrated in experimental animals and in

man using NMR-based metabonomics approaches to characterise the response. Here the application of NMR spectroscopy and chemometric tools for analysing plant-based products and their metabolic consequences are considered with particular emphasis on deconvolving biological complexity and minimising confounding biological variability with analytical 'noise'.

### Key words

NMR · metabolic profiling · metabolic fingerprinting · metabo-  
mics · metabonomics · phytomedicines · traditional Chinese  
medicine · genetically modified plants · toxicology · xenobiotics

### Introduction

Plants, plant-derived products and plant-derived chemicals serve mankind as medicines, nutraceuticals, functional foods, poisons, fragrances, and staple foods [1], [2]. Any of the utilised plant materials show characteristic profiles of low molecular weight biomolecules, often summarised as "metabolites". Most of the observed bioactivities can be correlated to certain metabolites or to metabolite groups. Abiotic environmental factors such as UV radiation, temperature, or moisture and biotic factors such as xenobiotics (e.g., toxins, pesticides, pharmaceuticals), nutritional sources (e.g., limited nitrogen or carbon sources, salt stress, starvation), progressing diseases, organism development, or genetic modifications lead to very specific qualitative and

quantitative shifts in metabolite patterns. Detecting and identifying pattern changes from normal to anomalous is the first and often crucial step in understanding underlying biochemical changes (e.g., genetic disorders, silent mutations, intoxication) of the organism. In the case of authentication and quality control of biological materials used as food, herbal medicinal products or nutraceuticals, changes of metabolite profiles can be associated with factors like adulteration or spoilage. Here, reliable discrimination between properly treated materials and altered samples is mandatory. Hence, analytes identified as discriminators by the use of unbiased approaches and multivariate statistical methods might serve as diagnostic markers for further routine analysis [3].

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Most of the known plant metabolites are often referred to as secondary metabolites due to their origin from biogenetical pathways not involved in primary life-sustaining functions [1], [4], [5]. These small organic compounds are of astonishing structural diversity, and structural hybrids combining unrelated biogenetical pathways are often encountered. It has been estimated that hundreds of thousands of structural diverse metabolites exist within the Plant Kingdom, many of them with well assigned bioactivities [6], [7]. Metabolite groups are either confined to certain taxa [5] or can be spread over unrelated species [8]. Within a single species, accumulations of several dozens of chemically highly diverse secondary metabolites are commonly encountered, especially if essential oils and volatiles are involved [9]. Secondary metabolism is highly adaptive and singular, its diverse products often participate in survival related actions, e.g., in the context of active or passive chemical defence or in inter- and intraspecies communication [4], [5]. The qualitative and quantitative analysis of secondary metabolites including their structural characterisation has been a major driving force in the development of both organic and analytical chemistry in the last century [1], [10]. Highlights of this development have been the discovery, structural characterisation, synthesis, and mode of action analysis of most potent natural products like the antibiotic vancomycin [11], [12] or the anticancer agent taxol – a plant-derived metabolite of fungal origin [13], [14]. Another focus has been the development of powerful hyphenated analysis techniques for characterising the complex multicomponent chemical composition of biological materials, such as GC-MS or diverse HPLC hyphenations recently culminating in the LC-SPE-NMR set-up [15], [16].

Primary metabolism, in contrast, is largely uniform and universal. The highly conserved metabolic pathways responsible for the formation of primary metabolites can be found throughout nature, e.g., glycolysis, the Krebs cycle or the pentose phosphate pathway. The spanned metabolic network is indispensable for regular development and growth. Primary metabolites are, in contrast to secondary metabolites, of limited structural variability. Studying their generation and interconversion within the metabolic network by flux analysis has been a major topic in biochemistry over the last decades and has become an integrative part of systems biology. Current methodologies often involve the use of stable isotope enriched substrates like  $^{13}\text{C}$ -glucose or  $^{13}\text{C}$ -carbon dioxide. Mass spectrometry and NMR spectroscopy coupled to different chromatographic techniques are the major analytical techniques utilised for this approach [17], [18], [19], [20], [21].

Proper analytical techniques are needed for the observation of analyte pattern changes. Classical approaches used in plant biology like TLC, FT-IR, NIR, HPLC-UV, HPLC-DAD, or GC-MS are strongly biased by limited separation and/or detection capabilities owing to the physico-chemical properties of the analytes under investigation (e.g., poor volatility, poor ionisability, lack of chromophores etc.). Only limited metabolic profiles are obtained and the analyte signature is dependent upon the analytical equipment used. For most analytical platforms confirmative qualitative measurements are needed for analyte identification due to the low information content of conventional detection methods [22]. A notable exception is GC-MS where retention

time index formation (e.g., the Kovats index) and database comparison support analyte identification (e.g., as employed in the analysis of waxy constituents from *Hypericum perforatum* [23]). Combinations of different analytical platforms like NIR, IR, HPLC-DAD, GC-MS with statistical pattern classification procedures have been used for a broad variety of applications including the analysis of olive oil [24], [25], St. John's wort (*Hypericum perforatum*) [26], *Echinacea purpurea* roots [27], and essential oils [28].

Within the last years comprehensive approaches associated with buzz words like metabonomics, metabolomics, or metabolic profiling originated. All of these technologies attempt to give detailed qualitative and quantitative metabolite overviews in whole organisms, exudates or extracts. Metabonomics is a whole systems approach reaching for the “quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” as defined in 1999 [29]. Metabolomics, introduced shortly thereafter as an additional term [30], has been defined in 2002 as “a comprehensive analysis in which all the metabolites of a biological system are identified and quantified” [31]. Metabolic profiling, in contrast, is defined as a methodology using predefined metabolites which are biogenetically closely related and limited in number [32], [33]. Hence it has to be considered a biased approach. Metabolic fingerprinting, the term metabolomics originally derived from [30], [31], and nowadays often used in the context with NMR-related techniques, is presently understood as “high-throughput, rapid, global analysis of samples to provide sample classification”. Analyte identification and quantification is said not to be used in this context [32], [33].

This somewhat artificial terminology, which depends solely on the quality of conclusions drawn from the obtained classifications and the amount of variance explained by the identified and quantified analytes, has been recently used to classify NMR-based analyses in plant sciences. Krishan et al. stated that “NMR either generates a metabolite profile, in which the NMR signals are assigned to specific metabolites, or a metabolite fingerprint, in which the analysis is based on the distribution of intensity in the NMR spectrum rather than the assignment of the signals” [19]. This and other reviews [19], [20], [21] tend to subsume NMR-based metabonomics in plant sciences as metabolite profiling or metabolic fingerprinting approaches stating, for example, that, in contrast to sensitive mass spectrometry approaches, “NMR can only be expected to detect a subset of the metabolites in the sample and as such is best classified as a metabolite profiling technique” [20].

Profiling approaches are methodologically more tightly connected to the concept of quantitative NMR (qNMR) [34], [35], whereas fingerprinting can be understood as a hypothesis-generating tool trying to discover pattern differences with the aid of statistical methods. Both approaches culminate in “omics” technologies, which are not yet fully realisable due to technical limitations. None of the used analytical platforms, either NMR or any of the mass spectrometry-based approaches has yet matured enough to meet the prestigious aim of complete metabolite analysis as requested for an “omics” technique. The assets and drawbacks of the different methodologies have been re-

viewed exhaustively [19], [20], [21], [33], [36], [37]. Assays using different types of mass spectrometers as analyte detectors have been successfully used in metabolic profiling of primary metabolites. Combining analyte derivatisation, chromatographic separations, and state of the art analyte detection by mass spectrometry allows simultaneous identification of dozens of metabolites [38], [39], [40], [41]. In contrast to LC-MS and GC-MS, the use of NMR does not rely on chromatographic separation techniques, which are the most critical source of experimental error in hyphenation set-ups. Compared to flow injection mass spectrometry, which avoids the involvement of chromatography but is suffering from unpredictable ion suppression effects and analyte-specific ionisation efficiencies hampering analyte quantification, NMR signals inherently allow direct quantitative analysis from mixtures if well separated signals are present [35]. This is not always the case, but by using two dimensional recording methods or by switching to magnets with higher field strength, this limitation can be overcome. Lately it has been shown that, by employing sophisticated curve fitting algorithms, analyte quantitation can be achieved from highly overlapping signals [42]. The information content of NMR spectra is exceeding that of mass spectra by far, with each proton or carbon atom in a molecule giving a unique signal. Thus, NMR is currently considered the prime tool for modern structure elucidation in the liquid phase. This is of special relevance for metabolic profiling of matrices with little or no prior information of their composition. In these cases, often encountered when working with plant-derived extracts, mass spectrometry-based approaches are prone to fail due to the inability to achieve unequivocal analyte identification.

Compared to modern mass spectrometry instrumentation, NMR spectroscopy is currently a rather insensitive analytical method [35], [43], [44]. These analytical platforms address different analyte concentration ranges (ppm vs. ppb) with comparable dynamic ranges. About four to five orders of magnitude have been reported for NMR spectroscopy [34], [35], the same holds true for mass spectrometry. One major advantage of NMR spectroscopy over other analytical techniques used for metabolite profiling is its unequalled reproducibility and stability. A recent cross-laboratory comparison performed with NMR magnets of different field strengths showed a coefficient of variation of < 2% [45]. Thus, the typical day to day variation often encountered with MS-based platforms is not a problem for NMR-based analyses.

NMR spectroscopy has a long history in the qualitative and quantitative assessment of secondary plant metabolites. Although NMR-based metabonomics developed only over the past years, both  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR have been already used in combination with chemometrical methods as fingerprinting technologies for more than a decade [28], [46], [47]. The use of NMR spectroscopy as quantitative analysis tool in plant biology has been recently reviewed exhaustively [35]. Examples of successful application include the quantification of ephedrin analogues in *Ephedra* species [48], of kavalactones in *Piper methysticum* (Kava-kava) [49], strychnine and brucine from *Strychnos nux-vomica* [50], and of bilobalide and ginkgolides in *Ginkgo biloba* [44], [51].

In summary, it can be stated that NMR spectroscopy and mass spectrometry platforms are complementary, since different analyte concentration ranges and different analyte classes are ad-

ressed. The somewhat artificial borderlines between fingerprinting, profiling and “omics” approaches as analytical science fields are blurred [52]. It is often hard to assess if the one or the other term has been used according to its original definition. Thus, within this review the terms metabonomics, metabolomics, metabolite profiling and metabolite fingerprinting will be transferred from the cited literature without further commenting on their use.

### NMR Sample Preparation and NMR Analysis

Sample preparation, NMR data generation and statistical data exploration used for the assessment of plants follow NMR-based metabonomics methodologies which strongly evolved over the past years [53], [54] (Fig. 1). In plant biology samples can stem from very different sources (e.g., diverse plant parts or tissues), and a broad variety of matrices will be encountered. Thus, sample preparation protocols have to be adjusted to the sample set under investigation on a case to case basis. As a general rule of thumb it can be assumed that any sample preparation method suitable for HPLC analysis will work for NMR-based metabolomics, if deuterated solvents are used for the preparation of the NMR sample. Unwanted matrix components have to be removed, sample adulteration (e.g., glycoside hydrolysis promoted by pH shifts) has to be excluded, and the analytes of interest have to be well dissolved in the chosen NMR solvent. Furthermore, the physico-chemical characteristics of the samples (e.g., pH, ion strength, solvent composition) are required to be constant over the whole sample set. To meet these requirements, samples have to be in a uniform state prior to extraction (e.g., homogenisation by milling, carefully regulated storage conditions). Weighted aliquots of the samples (typically < 500 mg for proton NMR experiments) are re-dissolved in an appropriate extraction solvent mixture if further purification steps are needed. After extraction and solvent removal (e.g., by the use of a vacuum concentrator) the extracts have to be reconstituted in suitable deuterated NMR solvents. Alternatively, samples can be directly extracted into the NMR solvent of choice [55], [56], [57], [58], [59]. A broad range of solvents suitable for most of the polarities encountered in natural product chemistry is available, e.g.,  $\text{CDCl}_3$ ,  $\text{DMSO}-d_6$ ,  $\text{CD}_3\text{OD}$ ,  $\text{H}_2\text{O}/\text{D}_2\text{O}$  or  $\text{D}_2\text{O}$  buffered with sodium phosphate or mixtures thereof (e.g.,  $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ ) can be used. Depending on NMR tubes and probes available, currently standard sample volumes of 300 to 600  $\mu\text{L}$  are needed. If desired, the use of sample robots combined with a flow NMR set-up allows smaller sample volumes resulting in a remarkable increase in sample throughput. The addition of chemical shift reference compounds (e.g., TSP – trimethylsilyl-1,1,2,2- $d_4$ -propionic acid or TMS – tetramethylsilane) to the NMR solvent is useful for both peak registration and quantitation purposes. In critical cases EDTA can be added to complex interfering ionic contaminations [60], [61]. A final clarification of the NMR samples by centrifugation or filtration through dialytic membranes (e.g., with a cut-off at 10 kDa) is often useful. Using internal standards of known concentration [35] or an electronic calibration signal (ERETIC method) [57] allows quantitative analyses. Currently NMR spectrometers with proton resonance frequencies between 400 MHz and 600 MHz are most often used in NMR-based metabonomics and metabolite profiling, although higher field strengths have been already applied

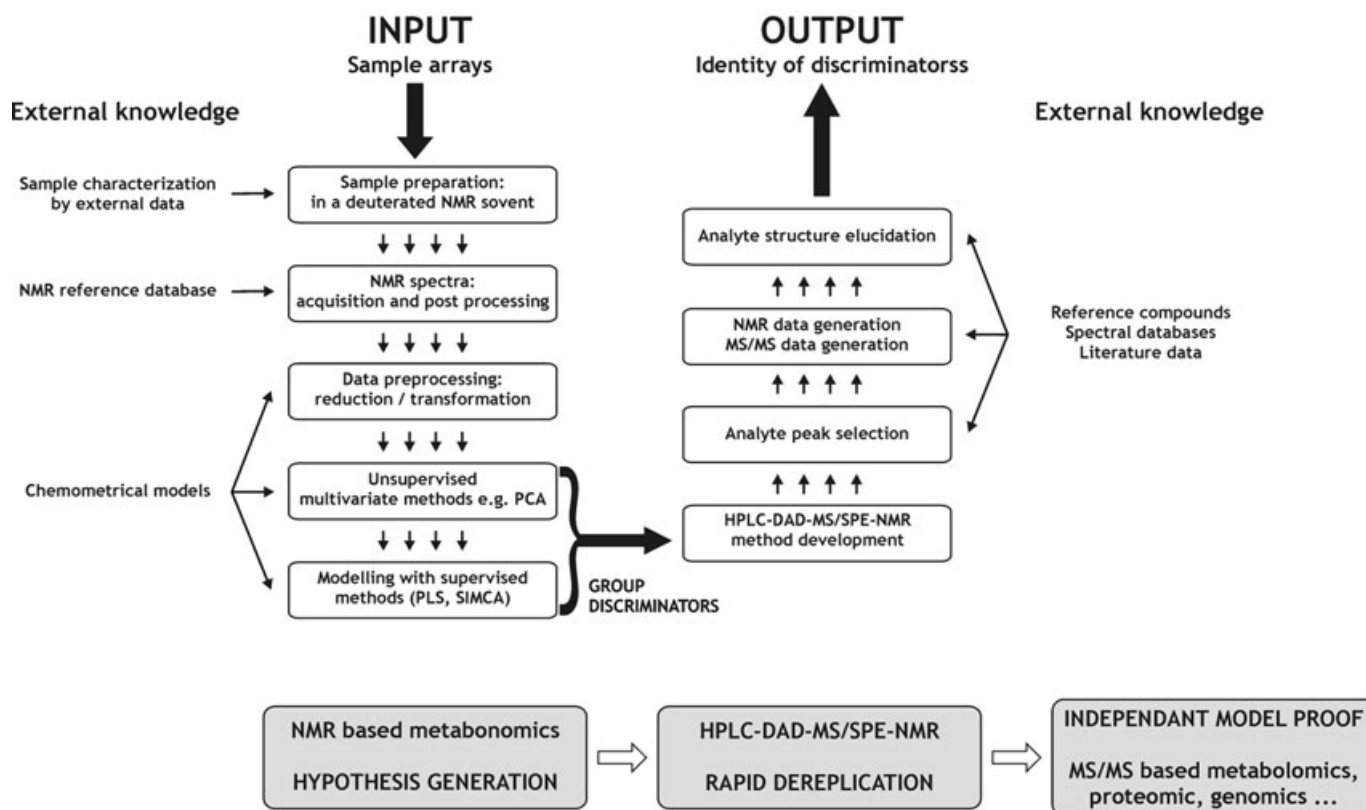


Fig. 1 Schematic overview summarising the process of pattern recognition in complex biogenic matrices by combining NMR-based metabonomics as discovery tool and modern hyphenated assays as analyte identification tools.

to measure biofluids [62], [63]. Typically, recorded free induction decays (FIDs) are stored in 32k data points and zero-filled to 64k data points. The number of repetitions differs greatly and depends on the concentration of the samples. Suppression of undesired solvent signals (e.g., the residual water signal) is usually achieved by pre-saturation during the relaxation delay. In the case of matrices containing macromolecules (e.g., proteins or lipid vesicles) the application of a spin-echo sequence as CPMG (Carr-Purcell-Meiboom-Gill) allows the attenuation of unwanted resonance envelopes [64], [65]. Occasionally a technique known as high-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy, adapted from solid state NMR, is used to generate metabolite profiles for intact plant tissues [66], [67]. However, this technology is more often used to assess states of hydration or to monitor localisation or distribution of metabolites rather than in metabolite profiling since it requires rather elaborate sample preparation techniques.

To allow further characterisation or even identification of analytes of interest (the putative biomarkers), the complete set of homonuclear and heteronuclear two-dimensional NMR methods can be applied to the samples [68], [69]. One particular interesting technique often used is the *J*-resolved experiment (*J*-RES) separating chemical shift and spin-spin coupling data onto different axis. Multiplet proton signals collapse to single lines by the application of a skyline projection on the chemical shift axis. Thus a less congested proton spectrum with full shift information is available. Furthermore, investigating the spin-spin coupling data on often superimposed signals allows further insight into the nature of analytes, e.g., in the case of (*E*) configured dou-

ble bonds with typical coupling constants [65], [70]. However, due to signal overlap and limited sample weight, complete signal assignments are not always possible. Therefore, additional options for molecular structure elucidation have to be used. These include hyphenation of the NMR spectrometer to HPLC (HPLC-NMR, HPLC-SPE-NMR) or the utilisation of an HPLC-MS system allowing chromatographic work-up of the analysed mixture prior to desired structural characterisation [22], [71].

### Pattern Recognition Analysis of NMR Spectra

The complexity of  $^1\text{H}$ -NMR profiles of plant extracts can be reduced, and the interpretation of biological influences made easier, by using multivariate computer-based pattern recognition (PR) methods. Spectra can be analysed either in full resolution or more often after a primary data reduction step whereby each spectrum is digitised into integrated regions over fixed spectral intervals, typically 0.01 – 0.04 ppm. Any regions containing suppressed solvent signals are removed from the analysis and the spectra are generally normalised to the sum of the total spectral intensity to compensate for differences in the amount of plant material analysed for each sample.

Multivariate statistical analysis of the samples can then take several forms. The simplest methods such as cluster analysis and principal components analysis are termed unsupervised methods whereby the spectra are grouped according to their similarity without using the knowledge of sample class. Thus these methods generate an unbiased overview of natural clustering

among biochemically similar samples. These methods work well for classification of plant materials or detection of adulteration. However, for more complex situations, e.g., if assessing the metabolic effects of phytochemicals on mammalian metabolism, where the high degree of physiological variation can confound the analyses, more sophisticated statistical methods are necessary. These methods have been comprehensively documented in the literature and are covered only briefly in the current review [72], [73], [74].

Principal components analysis (PCA) performs a linear transformation of the initial variables (or NMR descriptors) associated with the samples into a smaller set of variables termed principal components (PCs), which are orthogonal to each other and explain progressively less variance in the data set. This has the effect of displaying the variance in a data set without including vast amounts of irrelevant 'noise'. These new variables can be displayed in two- or three-dimensional "scores plots", allowing the analysis of the distribution and grouping of the samples in the new variable space. Since each component is a combination of the original input variables, each variable (or NMR signal) is given a weight (loading) which indicates the strength of influence that variable has on the overall profile for a set of samples. These weights can also be visually inspected in usually one- or two-dimensional "loading plots" allowing the identification of influencing variables (regions of the NMR spectrum) visually.

Supervised methods such as projection to latent structure discriminant analysis (PLS-DA) [72] make use of the class of sample (e.g., treated or untreated) to maximise the difference between two or more classes. This provides a means for identifying candidate biomarkers. However, if enough components are used it will always be possible to separate two classes perfectly. Therefore, in supervised analysis, the data are generally divided into training and validation set where a model is built on samples in the training set and then used to predict the class of independent samples in the validation set. Newer methods based on PLS have been developed recently to accommodate the large amount of extraneous variation unrelated to sample class that is often an inherent part of biological data. An inbuilt orthogonal filter incorporated into the PLS algorithm allows the calculation, examination and removal (if warranted) of orthogonal components which contain information on systematic variation deriving from factors such as age or origin of plant metabolism that may be unrelated to the sample class [75]. Further developments based on the calculation of correlation matrices for selected signals have provided a tool for metabolic structural elucidation whereby correlations between signals from the same or closely related molecules are highlighted [76].

### The Influence of Sample Preparation and Processing on Metabolite Profiles

NMR-based metabolite profiles depend both upon chosen sample processing techniques and NMR acquisition methods. Factors such as the extraction solvent used, the intrinsic pH of the extract, NMR probe temperature stability, and quality of residual solvent suppression will all affect the robustness of the multivariate models produced [74], [77]. Most of these effects are not

peculiar to NMR but present a challenge to many spectrometric and analytical data types. Defernez and Colquhoun have summarised some possible pitfalls and solutions in a recent review, including signal alignment methods that can be applied post-acquisition to sub-optimal data sets [74]. Notwithstanding NMR-based metabolite profiling is a robust analytical technique with much to offer in several key application areas.

### Characterisation, Classification and Identification of Adulteration of Plant Products

Using NMR- and MS-based metabolite profiling strategies, several studies have shown that it is possible to characterise and distinguish not only plant species and strains but also to discriminate between different plant tissues within the same plant. The biochemical composition of plant organs and tissues are known to differ. For example, a combination of GC-MS and  $^{13}\text{C}$ -NMR unveiled that *Isolona cooperi* root bark was dominated by 5-isopentenylindole and (*E*)- $\beta$ -caryophyllene, whereas leaf and stem bark oils were monoterpene-rich [78]. Other studies have combined NMR spectral analysis with pattern recognition techniques to characterise seeds, roots, leaves and barks of various *Strychnos* species [50], to assess the relative amount of stalk material in batches of chamomile flowers [77] (Fig. 2), and to differentiate

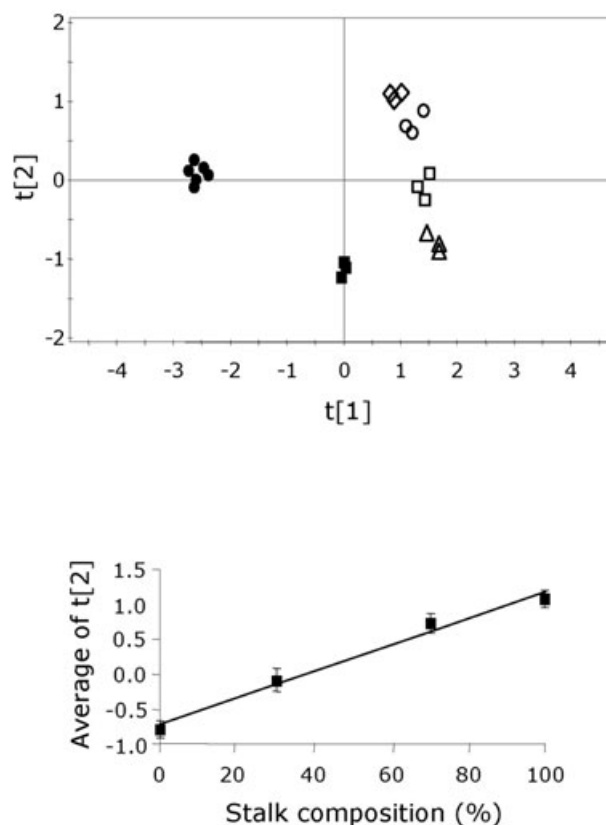


Fig. 2 Top: Scores plot of a principal component analysis (PCA) of  $^1\text{H}$ -NMR spectra from different chamomile extracts obtained from Slovakia ( $\Delta$ ), Egypt ( $\bullet$ ), and Hungary ( $\blacksquare$ ). Samples from Slovakia ( $\Delta$ ) were mixed to different percentages of stalk material [100% ( $\diamond$ ), 70% ( $\circ$ ), 30% ( $\square$ ), 0% ( $\triangle$ )]. Bottom: The average t[2] score for the Slovakian samples with various amounts of stalk material as a function of the percentages of stalks in the final mixture. Modified from [77].

*Arabidopsis thaliana* ecotypes collected throughout the northern hemisphere [79].

NMR-based metabolic profiling has been successfully employed in the analysis of food and food products, such as fruit juices (tomato [80], mango [81], apple [46], [82], orange [83], [84]), vegetables [85], wine [59], [86], [87], olive oil [88], beer [89], [90], [91], port wine [92], tea [93], and coffee [94]. One of the earliest NMR studies to characterise the metabolite profile of fruit juices and milk was by Eades and Bryant in 1986 [95]. Early applications of NMR fingerprinting in plant analysis, focused on fruit juices, were aimed to validate the ability of these techniques to discriminate between samples derived from different varieties of the same fruit. In one of the first investigations it was shown that combining  $^1\text{H}$ -NMR spectroscopy and multivariate data analyses could be used to distinguish between three types of apple juices with a success rate of up to 100% [82]. Principal component loadings identified sucrose and malate as the major components responsible for classification. NMR fingerprinting and multivariate data analysis as illustrated above can be extended to the detection of intended sample changes. A study by Le Gall and co-authors explored the adulteration of orange juices with pulp wash and showed that mixed juices could be clearly differentiated from pure orange juice on the basis of dimethylproline amongst several other components [84].

A technique referred to as SNIF NMR can be applied to the detection of the geographical origin or adulteration of wines by measuring the site-specific deuterium content of ethanol [96]. In addition, both  $^{13}\text{C}$ - and  $^1\text{H}$ -NMR data have been used in conjunction with multivariate data analysis to characterise and distinguish wines from three Slovenian wine-growing regions and from Apulia in southern Italy. Samples were analysed by the combination of NMR and chemometric classification. Excellent separation was achieved according to their geographical origin and year of production. Amino acids, glycerol, butylene glycol and succinate were identified as the major components contributing to the differentiation of samples [97], [98]. An elegant example of the combination of two-dimensional  $^1\text{H}$ -/ $^{13}\text{C}$ -NMR data and a multivariate statistical method was carried out by Forvefle et al. studying polyphenol extracts from grapevine clones harvested in the Bordeaux region. The obtained results showed that clones could be divided into three groups according to the cultivar. This method could also be applied to polyphenol extracts from grape seeds and leaves. It has been proven useful to identify grapevine species, cultivars or clones as well as any other plants which produce polyphenols [59], [99].

Beers have been another interesting target for the application of NMR-based metabolic profiling. A recent PCA analysis of  $^1\text{H}$ -NMR spectra of 50 beers of different label and type (ale, lager, alcohol-free) showed the separation of four groups distinguished by the predominance of different carbohydrates such as dextrans, maltose, and glucose. It also took advantage of analysing the aliphatic and aromatic regions separately; therefore the contribution of minor components could be retrieved more easily. For example, most ales, lagers, and alcohol-free samples could be distinguished based on their aromatic composition which indicated differences between the fermentation processes for the production of different beers [89], [90]. Furthermore, this methodology

allowed differentiation of breweries on the basis of a PCA classification of the  $^1\text{H}$ -NMR data. Lately it was shown by Manfred Spraul and co-workers, that quality control of beer can be performed on a routine basis by combining flow injection NMR utilizing a sample robot with multivariate statistical analyses. From eighty beer samples submitted by local authorities, principal component analysis allowed one to distinguish barley malt from wheat malt beers as well as the detection of beer spoilage by lactic acid, stemming from a bacterial contamination. Partial least square analyses did prove that the quality control parameters "original gravity" (concentration of solids in the unfermented wort) was predictable from NMR spectra with a correlation coefficient of 0.998 and a slope of 0.993. In addition, both ethanol and the spoilage parameter lactic acid were predictable with correlation coefficients exceeding 0.95 [91].

The consumption of unfermented green tea has been associated with health benefits including protection against cardiovascular diseases and cancer. The quality of green tea is judged on a combination of its appearance, flavour and aroma with amino acids, catechins and caffeine contributing strongly to the overall quality. Chromatographic (HPLC, GC-MS) and near infra-red (NIR) spectroscopic techniques have been used to profile and quantify selected compound classes such as the catechins but few studies have scoped the wide range of compounds present in green tea. In a recent study by Le Gall et al., NMR spectroscopy was used to characterise 191 commercially available green tea batches predominantly from China but also including samples from Japan, Indonesia, India, Bangladesh, and Vietnam [93]. By using PCA it was possible to separate Chinese teas from the non-Chinese teas in the first component. Moreover, "high quality" Chinese teas were also well clustered. By analysis of the PCA loading plots higher levels of theanine, theogallin, epicatechin gallate, gallic acid, caffeine, and theobromine were assigned to these samples, whereas the relative concentrations of fatty acids, quinic acid, sucrose, and epigallocatechin were higher in lower quality teas. Here NMR analysis was able to generate a more comprehensive overview and better classification of teas than chromatography-based methods.

A similar approach was taken in a comparison of the geographical origin of olive oils. Proton NMR was used to analyse 216 extra virgin olive oils collected over a three-year period from different Italian regions. Tree clustering analysis of NMR data was performed without any *a priori* hypothesis and the results showed the method was able to group the oils according to their location of production. In addition, a satisfactory differentiation between different years from the same location was achieved indicating the sensitivity of the approach [100]. Within the last years, several studies have proven the broad applicability of this analysis technique for olive oil classifications [101], [102], [103], [104], [105]. Investigations on olive oil adulteration were also presented. In one publication, a mathematical model was constructed and validated using an artificial neural network based on  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR data with hazelnut oils, pure olive oils, and olive oils blended with 2–20% hazelnut oils. The derived detection limit of adulteration was found to be around 8% [106]. An analogue study, published shortly thereafter, did deal with the adulteration of high quality oil ("extra virgine") by low quality batches. Here the limit of detection for the adulteration was found to be 5% [107].

## Natural Medicines and Traditional Chinese Medicines

Unlike laboratory-synthesised pharmaceuticals, phytopharmaceuticals and especially traditional Chinese medicines (TCMs) are often mixtures from several plant sources with hardly defined or undefined molecular composition. Even the secondary metabolite profiles of many herbal medicines produced from single plant species are far from fully characterised. Given that abiotic factors (e.g., soil, climate) as well as biotic factors (e.g., cultivar origin, plant development state at harvest time) highly influence the metabolite pattern of a plant, raw materials utilised for herbal remedies are often inconsistent in composition. In addition, each of the herbs used for a particular medicinal product often requires specific extraction and treatment. Thus, the quality of both raw and final formulated product is hard to control [108], [109]. Hence, a clear need for further research in terms of quality control, clinical efficacy, and understanding of the molecular mechanism of phytomedicines is present. There have been many attempts to address these issues, most following the routes of conventional drug discovery processes orientated towards well defined single chemical entities [2], [109]. This process includes time- and labour-intensive analyte isolation and identification as well as bioactivity discovery from the obtained single metabolite species. Such approaches have fairly limited throughput and are inefficient when compared with the vast number of traditional medicines to be investigated. Furthermore, this approach does not take into account potentially synergistic effects often encountered when working with plant extracts. Thus, utilising analytical methods capable of measuring multiple components simultaneously is advantageous. In this respect, “omics” disciplines are strikingly aligned with the requirements for the characterisation and evaluation of natural products and TCMs. Up to now NMR-based methods have been applied to the characterisation of crude drug material, extracts and phytopharmaceutical preparations of *Tanacetum parthenium* (feverfew) [55], *Piper methysticum* (Kava-kava) [49], *Arnica montana* [110], *Artemisia annua* [111], *Matricaria recutita* (chamomile) [77], *Ephedra* sp. [48], [112], *Cannabis sativa* [113], *Ilex* sp. [114], and *Hypericum perforatum* (St John's wort) [115] to name but a few.

A combination of high resolution NMR spectroscopy with pattern recognition has been employed to investigate commercially available *Tanacetum parthenium* samples originating from several different manufacturers. The results showed that replicates were tightly grouped indicating the high degree of reproducibility of the method. Two out of fourteen batches were found to be markedly different in chemical composition from the others. The remaining batches classified into discrete groups on the basis of minor differences in overall chemical compositions. Even in products originating from the same manufacturer, batch to batch variation was evident, emphasising the need for better standardisation of such products. Comparing NMR analysis with a more traditional method using HPLC, the NMR-based data were found to give improved discrimination between batches and demonstrated superior reproducibility [55].

*Ilex paraguariensis* is used to produce a herbal tea (mate) drunk predominantly in South America. There are over 500 species of *Ilex*, some of which are used as substitutes or adulterants of *I. paraguariensis* teas. Moreover, the metabolite profile of this

product is known to vary depending on preparation techniques, age of plant materials at the time of harvest, and manufacturer. Usually employed HPLC assays allow the quantification of polyphenols enriched in *I. paraguariensis* compared to other species. However, this method offers only little information about the presence of multiple *Ilex* species in crude drug mixtures. Verpoorte and co-workers were able to characterise eleven different *Ilex* species from both aqueous and organic extracts using an NMR-based method. Of these, only *I. paraguariensis* was found to contain caffeine and theobromine whereas other species contained high levels of arbutin, absent from *I. paraguariensis* [114].

NMR-based metabolomics methods have been employed to profile the metabolite composition of the flower heads of *Matricaria recutita*, the chamomile. Clear differences between samples obtained from Northern Africa (Egypt) and Eastern Europe (Hungary and Slovakia) have been identified. It was also possible to distinguish samples from Hungary and Slovakia based on their metabolomic compositions although they were from closer geographic locations. Furthermore, the applied method allowed the assessment of the percentage of stalk material in the samples. Thus, the combination of unassigned <sup>1</sup>H-NMR spectra and multivariate data analysis can be used for quality control and authentication of this widely used herbal medicine [77] (Fig. 2).

For chemotaxonomic analysis and species authentication including quality control issues, NMR-based metabolomic analysis was applied to three *Ephedra* species and commercial *Ephedra*-based products. In addition to the successful identification of a broad range of metabolites from crude *Ephedra* extracts achieved without any chromatographic separation, this method revealed that the successful differentiation of *E. sinica*, *E. intermedia* and *E. distachya* var. was due to some undisclosed benzoic acid analogues in the aqueous fraction and ephedrine-type alkaloids in the organic fraction. Based on these findings, one of nine evaluated commercial *Ephedra* materials was shown to be a mixture of *Ephedra* species [112].

Application of similar techniques to analyse twelve *Cannabis sativa* cultivars allowed the discrimination of the investigated samples. Both,  $\Delta^9$ -tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) exerted a strong influence on the segregation of the sample groups in the PCA scores plot. Moreover, cultivars were also distinguishable by analysing a water extract. Here sucrose, glucose, asparagine, and glutamic acid were the major discriminating metabolites [113].

Metabonomic studies have been carried out on the extracts of *Artemisia annua* to discriminate samples from different sources and classify them according to their anti-plasmodial activity and their toxicity to cell cultures without prior knowledge of this activity. The use of PLS-DA also allowed the prediction of actual values of such activities for some independent samples not used in model construction [111].

Proton NMR spectra of various extracts of St. John's wort (*Hypericum perforatum*) samples derived from four different accessions obtained by using six different extraction solvent mixtures were chemometrically evaluated and calibrated against non-selective binding to opioid receptors using the PLS algo-

rithm. Subsequently, multivariate data analysis was used to predict the pharmacological efficacy of further St. John's wort extracts on the basis of their  $^1\text{H-NMR}$  spectra. This contribution confirmed the useful application of the presented approach to assess the quality of medicinal herbs and extracts by spectroscopic analysis derived from bioactivity-related quality parameters. However, no details about the spectral regions responsible for the correlation with the pharmacological effects have been disclosed. Thus, the identification of putative molecular carriers of the bioactivity has not been achieved [115].

The varying content of desired beneficial secondary metabolite groups make the quality control of crude plant materials used for the production of herbal medicines indispensable. A recent study proved that NMR-based metabolic fingerprinting allows the discrimination of crude drug materials of St. John's wort. Methanolic extracts obtained from seven randomly chosen lots provided by commercial suppliers were successfully discriminated by a PCA analysis based on  $^1\text{H-NMR}$  spectra. The most influential discriminators were found to be the phloroglucinol-type secondary metabolites hyperforin and adhyperforin, flavonoids, and primary metabolites as sugars and lipids [116]. Tight grouping of the replicates demonstrated the reproducibility of the extraction protocol (Fig. 3). Comparison with a PCA analysis based on HPLC-DAD-derived datasets showed that both grouping within and separation between the lots was comparable. Thus it was concluded that NMR-based fingerprinting has at least the same discriminatory power as the routinely used HPLC-DAD assays. This confirmed previous observations reported from at least two other applications [61], [77], [117].

### Genetically Manipulated Plants

With increasing levels of environmental contaminants, including negative influences of certain agricultural practices and other human activities on soil fertility and crop productivity, there is a need to develop plant species with increased tolerance to suboptimal growth conditions. Modifying plants genetically has been a continuous process for a long time in horticulture; at least since the rediscovery of Mendel's rules of inheritance. Applications include the improvement of environmental adaptability, disease resistance or tolerance to pesticides. Central ambitions are improving crop yields, obtaining higher quality crops or using plants to produce desired chemicals. For example, the development of hybridised rice strains to increase yield and quality has made tremendous progress within the last decades [118]. Recently, genetic engineering has been employed as a new tool in this field by either modifying some endogenous genes or transferring some exogenous genes. Genetic manipulation of plants offers a solution to improve the quality of crops grown for human and animal consumption but also carries a strong element of public concern regarding the safety of genetically modified organisms. Changing a genome can lead to both witting and unwitting consequences of the modification. Therefore, the manipulated organism must be tested to ensure nutritional equivalence/improvement to the parent cultivar and absence of toxin or anti-nutrients accumulations [39], [43], [119]. Tested metabolites are usually chosen from a recommended list based on prior knowledge. Therefore, this biased strategy is open to miss unin-

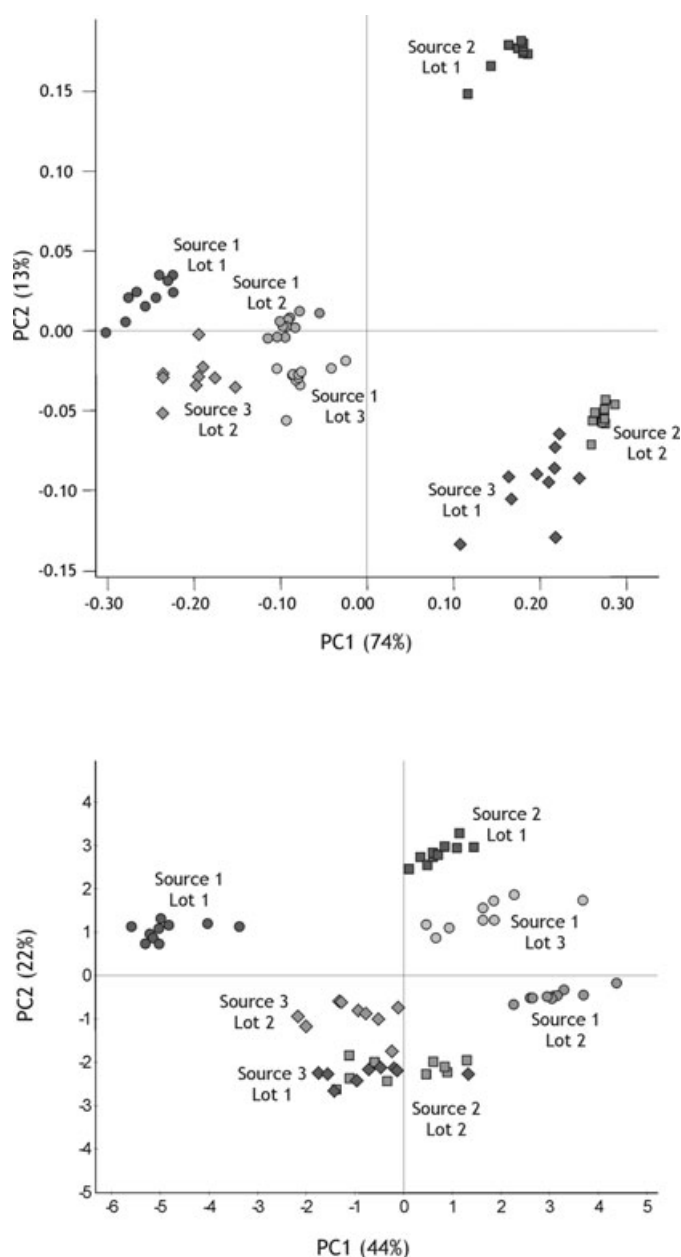


Fig. 3 Comparison of  $^1\text{H-NMR}$ - and HPLC-UV-derived principal component analyses (PCAs) performed on different St. John's wort lots (different grey shades) provided from three suppliers ( $\circ/\square/\diamond$ ). *Top*: Scores plot of a PCA of the aromatic region (8.5–5.8 ppm) of  $^1\text{H-NMR}$  spectra. Group discrimination is related to qualitative and quantitative differences in the flavonoid pattern. *Bottom*: Scores plot of a principal component analysis based on HPLC-UV peaks detected at 280 nm. Group discrimination is related to phloroglucinols and variations in the phenolic constituent pattern. Modified from [116].

tended effects introduced by the genetic manipulation. Unbiased profiling methods, in contrast, allow a comprehensive analysis of qualitative and quantitative changes in metabolite composition, which may mirror alterations in gene functions [30], [39], [43]. Within the last years, NMR-based platforms have been successfully applied to investigate the metabolic consequences of genetic modification or strain differences in plants [61], [79], [119], [120], [121], animals [122], [123], [124], [125], and yeasts [126].

In a study by Le Gall and colleagues, a comparison between wild-type tomatoes (*Lycopersicon esculentum*) and cultivars overex-



pressing regulatory genes from maize was carried out [61]. Using NMR analysis of the extracted whole fruits (Fig. 4) in combination with uni- and multivariate data analyses, the metabolite composition of genetically modified tomatoes was compared with that of controls. Differences were observed in chemicals involved in a number of metabolic pathways. Flavonoids such as kaempferol and naringenin glycosides were upregulated > 10-fold in transgenic fruits. Other smaller (< 2- to 3-fold) but nevertheless statistically significant differences between wild-type and modified fruits included primary and secondary metabolites. Trigonelline, glutamine, and asparagine, were present in relatively higher concentrations in the transgenic tomatoes whilst

citrate, phenylalanine, sucrose, malate, branched-chain amino acids, and cinnamic acids were amongst those metabolites that showed higher concentrations in the wild-type fruits. Although these metabolic differences were present at all levels of maturity of the fruit, the differences between wild-type and transgenic tomatoes increased with ripeness (Fig. 5). The <sup>1</sup>H-NMR spectra showed that the levels of glutamic acid, fructose, and some nucleosides and nucleotides gradually increase from the immature to the ripe stage, whereas the levels of valine and  $\gamma$ -aminobutyric acid decreased during the same process.

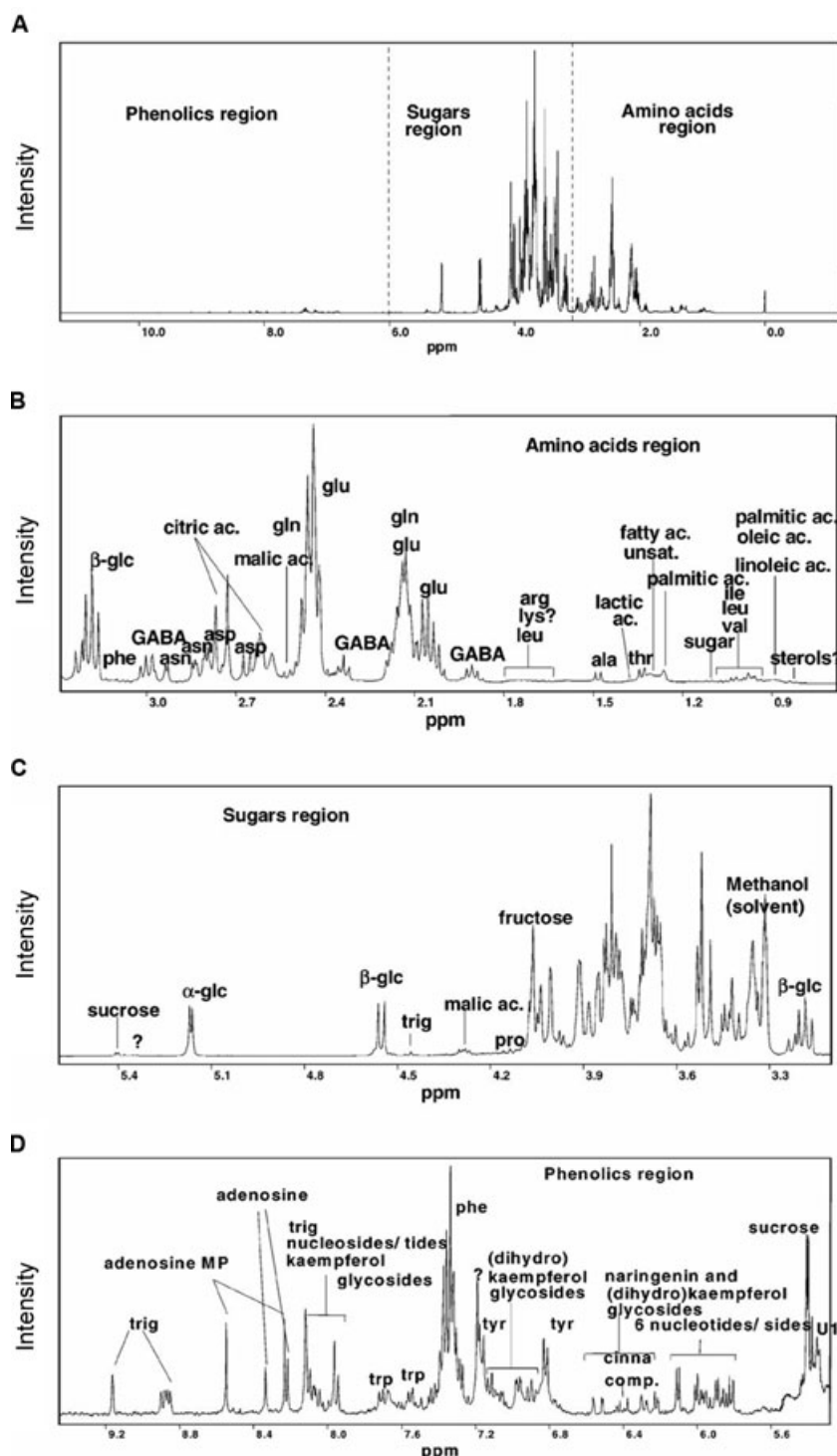


Fig. 4 Details of <sup>1</sup>H-NMR spectrum of a tomato extract demonstrating both signal richness and dynamic range of plant extract NMR spectra. Key: ac., acid; ile, isoleucine; leu, leucine; val, valine; unsat, unsaturated; ala, alanine; arg, arginine; lys, lysine; GABA,  $\gamma$ -aminobutyric acid; glu, glutamic acid; gln, glutamine; asp, aspartic acid; asn, asparagine; phe, phenylalanine;  $\beta$ -glc,  $\beta$ -D-glucose; pro, proline; cinnacomp, cinnamic compounds; tyr, tyrosine; trp, tryptophane; trig, trigonelline, MP, monophosphate. Reproduced from [61] with permission of the American Chemical Society.

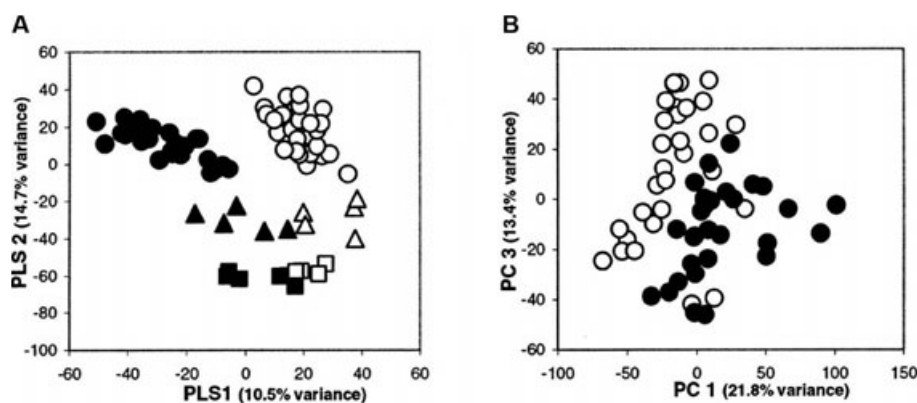


Fig. 5 First two PLS scores for 80 transgenic and control tomatoes at three stages of maturity (A) and PC1 and PC3 for 60 transgenic and control red tomatoes (B): ○, red transgenic; ●, red control; △, turning transgenic; ▲, turning control; □, green transgenic; ■, green control. Reproduced from [61] with permission of the American Chemical Society.

Modification of *Nicotiana tabacum* with genes from *Escherichia coli* and *Pseudomonas fluorescens* resulted in the ability of tobacco plants to synthesise salicylic acid associated with acquired resistance against the tobacco mosaic virus. Previous HPLC-based studies targeting specific classes of metabolites such as alkaloids and flavonoids were not able to differentiate between modified and wild-type plants. Using an NMR-based method with subsequent PCA, Verpoorte and co-workers showed a clear differentiation in the metabolite profiles of leaves and stems [120]. Using an aqueous extract, signals of phenylpropanoids (predominantly chlorogenic acid) and sugars (e. g., glucose, fructose, sucrose) were found to be present in higher concentrations in the wild-type leaves whilst alanine and malic acid were elevated in the genetically manipulated plants. It has been suggested that increased utilisation of sugars and carbohydrates by the manipulated plants may be a result of channelling these substances into resistance to stress, rather than purely for growth. This is consistent with the lower growth rate observed in plants treated with salicylic acid. Furthermore, this study allowed the discrimination of plants inoculated with the tobacco mosaic virus from uninfected plants.

Secondary plant metabolites differ widely between species and are known to play a significant role both in plant survival and in environmental interactions such as defence against herbivores. In a recent study various strains and species of ragwort were investigated. These taxa are known to exhibit very different qualities of toxicity to herbivores, which are mediated by pyrrolizidine alkaloids. *Senecio aquaticus* and *Senecio jacobaea* are closely related sister species forming natural hybrid swarms, which may lead to the biosynthesis of novel pyrrolizidine alkaloid derivatives. NMR-based metabolic profiling was applied to examine hybridisation-induced biochemical changes in F1 hybrids of the two parent species. Numerous changes in the levels of both primary and secondary metabolites between the parents and the hybrids were found. As expected, F1 hybrids were intermediates to the parent species. In particular, jacaranone analogues, chlorogenic acid, flavonoids, glucose, sucrose, and alanine were found in elevated concentrations in one of the parents – *S. aquaticus*. However, due to the limited sensitivity of the NMR approach and due to the high similarity of the alkaloid pattern of the parent species this study failed to address pyrrolizidine alkaloids [58].

Transgenic peas (*Pisum sativum*) were studied for unintended effects beyond those intended by incorporation of a transgene. Multivariate analysis of  $^1\text{H-NMR}$  spectra of  $\text{D}_2\text{O}$  extracts obtained from uniformly grown glasshouse pea plants revealed differences between the transgenic and control group, which exceeded the natural variation of the wild-type plants. A comparison of wild-type plants and a null segregant, which has lost the introduced transgene again, did prove that the encountered differences are most likely stemming from the selection pressure of the transformation event. It was concluded that the population subset being able to undergo transformation has a reduced variation in the metabolite profile, thus differentiating from wild-type plants. Furthermore, it was reported that interplant variations (two plants, six replicated extracts, six replicated NMR measurements) were found to exceed the overall analytical error including the extraction process [127].

Proton NMR analysis of metabolite composition was conducted for strawberry fruit quality, tomato strains over-expressing hexokinase, and *Arabidopsis thaliana* transformants with decreased phosphoenol pyruvate carboxylase activity. The results showed that biochemical phenotyping of strawberry fruits offered a way of detecting quantitative trait loci controlling fruit quality. Comparison of metabolic profiles of the roots of tomato plants revealed that environmental factors, i.e., culture conditions, can significantly modify the metabolic status of plants and thus hide or emphasise the expression of a given genetic background. The decrease in phosphoenol pyruvate carboxylase activity in *A. thaliana* transformants affected the metabolic profiles without compromising plant growth [57].

Metabolite profiling by  $^1\text{H-NMR}$  and HPLC-UV has been applied to address changes of metabolite patterns in potato tubers with genetic modifications in metabolic pathways. Significant differences were observed between two of four plant lines with modified polyamine metabolism and the controls. Proline, trigonelline, and numerous phenolics were identified as discriminators [119].

In another study, NMR-based metabolomics was applied to transgenic maize with the aim of verifying the possibility of identifying and classifying maize seeds obtained from transgenic plants. Based on the inherent variation in metabolite levels, recognition of discriminant metabolites of transgenic samples

was feasible. The established PLS-DA model was used for the classification of non-*a priori* defined samples of maize [128].

High resolution magic angle spinning (HR-MAS) NMR spectroscopy combined with multivariate data analysis including batch processing protocols was applied to the analyses of genetically modified poplar strains. NMR spectra of milled poplar samples collected at the internodes of the tree revealed a growth-related gradient in the plant internodes direction, as well as the discrimination between the genetically modified plants from wild-type populations [129].

### Toxicity and Xenobiotic Metabolism in Plants

Plant viruses represent a threat to agricultural and commercial crops. The effects of phytoplasma infections on *Catharanthus roseus* leaves were assessed by metabolomic profiling using one- and two-dimensional NMR spectroscopy followed by PCA. An increase of metabolites related to phenylpropanoids or terpenoid indole alkaloids (e. g., chlorogenic acid, loganic acid, secologanin, vindoline) was found, suggesting an infection-related stimulation of the involved biochemical pathways. Furthermore, infected leaves showed increased levels of several primary metabolites [121].

Certain plants have adapted to tolerate higher levels of pollution than other species. However, the accumulation and tolerance of potential toxicants in these plants may have detrimental consequences on organisms higher in the food chain. The value of NMR-based metabolomics in the investigation of cadmium tolerance in plants has been demonstrated for *Silene cucubalus*. This species is known to respond to cadmium exposure by chelation of the heavy metal ions to phytochelatin, peptide ligands which consist of repetitions of  $\gamma$ -Glu-Cys sequences. It was shown on *S. cucubalus* cell cultures that  $\text{Cd}^{2+}$  exposure resulted in biochemical changes related to the energy production and the Krebs cycle. In addition, while non-tolerant species show an increase in the levels of the stress biomarker proline after exposure to  $\text{Cd}^{2+}$ , no increase in proline was observed in this study [130].

Over three hundred herbicides currently used in agriculture today affect less than thirty biochemical pathways. Nineteen herbicides with different modes of action such as inhibition of enzymes, oxidative phosphorylation, auxin transport, microtubule growth or mitosis were successfully characterised in corn (*Zea mays*) plants using NMR spectroscopy in combination with artificial neural network analysis [131].

### Characterisation of the Metabolic Effects of Consumption of Plants and Plant-Derived Products

Although spectroscopic methods have been frequently used in combination with pattern recognition techniques to characterise and assess the biological activity of individual plant-derived chemicals, to date only few studies have addressed the effects of plant products on mammalian metabolism. Identifying potential metabolic effects of phytopharmaceuticals and phytochemicals in mammals against the background of environmental variation

presents a significant analytical challenge and is particularly problematical in humans. Thus, one approach in establishing the biochemical consequences of plant products is to use animal models in a well-controlled environment.

The ingestion of both green tea and black tea polyphenols is known to be linked to a number of health benefits including anti-inflammatory action, cardioprotectant activity, and cancer prevention. Metabonomic methods utilising  $^1\text{H-NMR}$  spectroscopy of biofluids and PCA were applied to investigate the bioavailability and metabolic responses of rats to a single dose of epicatechin dissolved in water. NMR analysis of urine samples obtained after analyte consumption showed that this compound was both bioavailable and biochemically active. Metabolites of epicatechin were observed in the urine together with changes in the urine profile of endogenous compounds such as taurine, citrate, dimethylamine and 2-oxoglutarate [132].

Timed samples obtained pre- and post-intervention from the same individuals coupled with strict dietary control can be helpful in elucidating the metabolic effects against the background of genetic and environmental diversity. In one such study a group of pre-menopausal women was sampled at multiple time points pre- and post-consumption of unconjugated (soy) or conjugated (miso) isoflavone-containing food. The observed biochemical changes suggest that soy isoflavone ingestion had significant effects on metabolic pathways associated with osmolyte fluctuation [133]. Clear differences in the plasma lipoprotein, amino acid, and carbohydrate profiles were also observed following soy intervention, suggesting a soy-induced alteration in energy metabolism [134].

Bioavailability and biotransformation of black tea flavonoids has been assessed via screening by  $^1\text{H-NMR}$ . Urine samples were obtained from human volunteers before and after a single dose of black tea and analysed using a combination of NMR and pattern recognition techniques. Hippuric acid was confirmed as the major urinary black tea metabolite. One previously unknown metabolite was also detected and identified as the sulphate conjugate of pyrogallol [135].

In a study on the metabolism of chamomile tea in humans an orthogonal signal correction (OSC) technique in combination with projection to latent structure analysis was employed to remove extraneous variation in the data. This allowed the detection of biomarkers of chamomile tea intake which were obscured by the great diversity in physiological state and diet of the volunteers. A strong increase in hippurate and glycine levels was observed together with a decrease in creatinine. The observed increase in levels of hippurate and glycine remained stable for at least two weeks after ceasing consumption of the tea indicating that the metabolite changes were more likely to result from altered gut microfloral status than being a direct metabolite of the tea *per se* [136]. The development of sophisticated mathematical filters holds promise for characterising the metabolic effects and assessing risks associated with consumption of multicomponent plant products in humans.

## Future Directions of Metabolomics in Plant Science

Metabolomics has not only a potential as a tool for the biochemical characterisation of plant products and phytomedicines but also as a full-scale discovery platform with the potential to advance our understanding of multidimensional plant systems [33]. Despite the fact that metabolomics has not yet reached its full maturity, and retains technological limitations, several authors have recognised the unequalled potential of the technology in phenotyping, particularly in the analysis of large mutant or transgenic libraries of model experimental plants [137] and in exploring the dynamic responses of plant systems [138]. Perhaps the most promising direction of NMR-based metabolomics lies in profiling and understanding the synergy between components of traditional medicines, particularly with respect to characterising the metabolic effects in test organisms or humans [139]. Although independently valuable, as illustrated in this review, the power of metabolic profiling increases vastly when combined with gene and protein expression analysis. In combination with data from the transcript or protein level, metabolic profiling can provide a holistic picture of a system's response to a biological challenge. Moreover, the comprehensive and non-targeted profiling of metabolites in plants presents exciting opportunities as a discovery tool for functional genomics and systems biology in plant sciences [140]. It is obvious, when considering the relative advantages and disadvantages of NMR- versus MS-based technologies, that a combination of both approaches, together with the armoury of increasingly sophisticated chemometric and bioinformatics tools will provide a uniquely powerful tool in a multitude of plant biology applications. Linking derived data sets to other system biology platforms like transcriptomics or proteomics ensures a deeper insight into metabolite fluxes, the biochemical origin of pattern shifts, and plant biochemistry in general. Public availability of metabolite databases, software, and standard protocols will certainly boost both MS- and NMR-based techniques of metabolome analysis within the next years [140], [141], [142], [143], [144].

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