

Quality Control of Herbal Material and Phytopharmaceuticals with MS and NMR Based Metabolic Fingerprinting

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Abstract

Metabolic fingerprinting techniques have received a lot of attention in recent years and the annual amount of publications in this field has increased significantly over the past decade. This increase in publications is due to improvements in the analytical performance, most notably in the field of NMR and MS analysis, and the increased awareness of the different applications of this growing field. Metabolomic fingerprinting or profiling is continuously being applied to new

areas of research such as drug discovery from natural resources, quality control of herbal material, and discovering lead compounds. In this review the current state of the art of metabolic fingerprinting, focussing on NMR and MS technologies will be discussed. The application of these two analytical tools in the quality control of herbal material and phytopharmaceuticals forms the major part of this review. Finally we will look at the future developments and perspectives of these two technologies in the quality control of herbal material.

Introduction

Plants have been used throughout history as the primary source of food, timber, fuel, and medicine, among other purposes. Due to their amazing ability to produce a vast structural array of chemical components, of which only relatively few have been identified, plants are one of the most important sources for the development of new drugs. Today plants, plant extracts and plant-derived components are being used in a multitude of herbal remedies and phytopharmaceuticals. Well-known plants which are used to treat specific diseases include *Artemisia annua* (to treat uncomplicated malaria) [1] and the female flowering tops of *Cannabis sativa* (to relieve pain and as an appetite enhancer) [2]. A multitude of examples exists for the use of plant extracts to treat a large array of diseases and the WHO estimates that about 80% of the world population uses medicinal plants as their primary source of medication. Many well-known drugs have also been isolated from plants including artemisinin from *A. annua*, paclitaxel from *Taxus brevifolia*, vinblastine and vincristine from *Catharanthus roseus* and galanthamine from the Amaryllidaceae family. In the literature three terms are used to describe the analysis of metabolites produced by plants.

These terms, notably metabolic profiling, metabolic fingerprinting and metabolomics are sometimes used interchangeably. It is not our intention to give a formal definition of metabolomics. For a formal definition and discussion [3,4] should be consulted. A very simplistic view is that metabolomics can be seen in two different ways. Firstly there is the microscopic view (targeted approach) which looks at a specific set of compounds (e.g., phenolics or terpenoids, etc). This approach is also called metabolic fingerprinting or metabolic profiling. The second view is the macroscopic view (untargeted approach) which aims at identifying and quantifying all the metabolites present in a specific organism [3]. Metabolomics is therefore a “systematic study of the unique chemical fingerprints that specific cellular processes leave behind” – and, more specifically, the study of their small-molecule metabolite profiles [5]. The metabolome represents the collection of all metabolites in a biological organism, which are the end products of its gene expression. Thus, while mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic fingerprinting can give an instantaneous snapshot of the physiology of that cell. One of the challenges of systems biology is to integrate proteomic, transcriptomic, and

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metabolomic information to give a more complete picture of living organisms.

The analytical tools used to study the metabolome include mainly chromatography and spectroscopy. Chromatography is used to separate the metabolites which are then being quantified and identified with the use of various detectors. The two main chromatography systems employed in metabolic fingerprinting are gas chromatography (GC) and high-performance liquid chromatography (HPLC). The detectors used in these chromatography systems depend largely on the type of components to be detected and quantified. For a targeted approach, mass spectroscopy (MS) is usually employed as the detector, while nuclear magnetic resonance (NMR) and MS are used without the chromatography step as an untargeted analytical tool. In the 1980s the first reports on the use of NMR for fingerprinting of plant extracts were reported, e.g., Kubeczka and Formáček published a book [6] with ^{13}C -NMR fingerprints of essential oils and Schripsema and Verpoorte applied ^1H -NMR for characterizing plant cell cultures and quantitative analysis of sugars in the cell culture extracts [7]. But, particularly in the past decade, metabolomics is rapidly developing for studying plants and quality control of botanicals. The use of these two analytical tools (MS and NMR) will be discussed in more detail below as well as the applications in the quality control of herbal material and the perspectives of these methods.

Metabolic Fingerprinting using NMR

Recent advances in analytical chemistry, combined with multivariate data analysis, have brought us closer to the final goal of metabolomics, comprehensive evaluation of all metabolites, both quantitatively and qualitatively in living organisms. Among many different technological platforms, NMR and MS have been successfully used for metabolic fingerprinting analysis. These two techniques have their respective advantages and limitations, and are often discussed as being complementary [8,9]. However, as a tool for metabolomics, NMR has some unique advantages over MS-based methods. It can provide a detailed analysis on the biomolecular composition very quickly with relatively simple sample preparation [10–12]. It is a universal detector for all mole-

cules containing NMR-active nuclei, unlike MS where detection of analytes is influenced by selective ionization or ultraviolet spectrometers where only chromophore-bearing compounds are detected. For all proton-bearing molecules, the intensity of all proton signals is absolutely proportional to the molar concentration of the metabolite. Using a proper internal standard, the real concentration of metabolites can be easily calculated [11]. Because NMR spectroscopy is based on the physical characteristics of compounds, it has a very high reproducibility.

In metabolomics, the detection of metabolites is not the final step, but all data obtained from the analytical methods should be further analyzed by statistical methods in order to extract all possible information from the data. The accuracy and correctness of the data to be further analyzed by the statistical methods are inevitably reliant on the robustness of the raw analytical data set. In this aspect, NMR has a unique advantage, the highest reliability in metabolomics. Unlike the retention time in chromatography-based techniques, with a few exceptions the chemical shift, coupling constant, and integral of each signal in an NMR spectrum does not change as long as it is measured under the same conditions: the field strength applied, solvent, pH, and the temperature. Despite the low intrinsic sensitivity, the robustness of data and ability to cover a broad range of metabolites, has enabled NMR to be the favored overall “macroscopic” metabolomics and fingerprinting tool. In addition to the advantages of data robustness, the power of NMR in structure elucidation of metabolites cannot be matched by any other method. NMR has a long history in the natural products chemistry field as the tool for structural elucidation. With a proper database, it can generate data which can be kept almost permanently [13]. In this part, the general procedure of NMR metabolomic analysis will be discussed together with multivariate data analysis. The identification of metabolites is of high importance and this aspect will be highlighted. At the end of this section specific applications will be discussed using NMR as a metabolic fingerprinting technique.

General procedures of NMR-based metabolomics

NMR-based metabolomics, like other methods using MS or chromatography, include sample preparation, extraction, multivariate data analysis, and identification of metabolites. **Fig. 1** shows

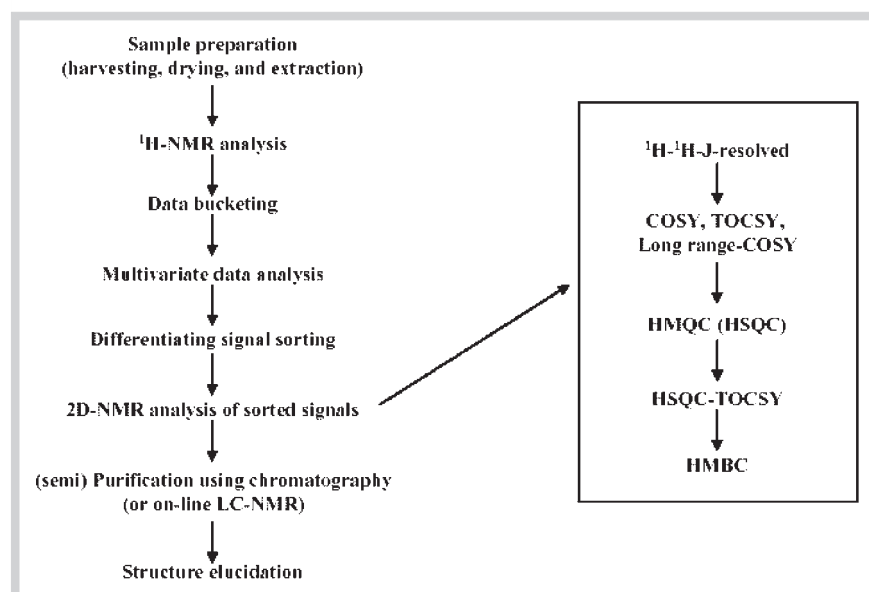


Fig. 1 Schematic flowchart of NMR-based metabolomic analysis.

the generally accepted procedure of NMR-based plant metabolomics. The sample preparation steps need much caution in order to obtain reproducible and reliable results. Because in the metabolomic analysis, the age, type of tissues, developmental stage, environmental conditions (e.g., water drought, sunlight, temperature, and pH of soil), and harvesting time greatly affect the metabolome obtained even from the same genotype. For these aspects sampling of the target materials should be carefully planned to avoid increased biological variation interfering with the final data interpretation [10]. After harvesting, the sample should be frozen immediately to avoid any (bio)chemical change in the material. The next step is grinding and extraction of the material to liberate the metabolites from the cells. During the extraction (bio)chemical reactions may occur in the material, reflected in changes in the metabolome. This can be avoided by drying the material before extraction either by heat or freeze-drying, keeping the material at a low temperature, or grinding at low temperatures and/or in the presence of a solvent that denatures enzymes involved in metabolite alteration. Denaturation can also be achieved by a brief microwave treatment. For the development of an optimum extraction method, Ultra-Turrax®, deep-freezing in liquid nitrogen followed by mechanical grinding, sonication with a probe head or cup horn and bead beating prior to solvent extraction were evaluated for the efficient breakage of the cell using a mycobacteria, *Mycobacterium bovis*. Of the evaluated methods sonication was found to be superior to others [14].

As metabolomics aims at comprehensive fingerprinting of all metabolites the extraction methods used should cover all possible metabolites in an organism. Unfortunately, there is no single extraction method to achieve that goal. The polarity of the solvent limits the range of metabolites that can be extracted. Also the pH is a factor that affects the profile of metabolites extracted, e.g., alkaloids are soluble in non-polar solvents at high pH, and in aqueous solvents at low pH. To overcome these problems in part, a two-phase solvent system consisting of chloroform-methanol-water (2:1:1) has been applied to extract both polar and non-polar compounds in a single extraction [15–18]. Also, this method was applied to metabolic fingerprinting of *Ephedra* with a slight modification, by addition of NH₄OH, for benzylamine alkaloids extraction [19]. However, this elaborate two-phase extraction method was found to be problematic when dealing with a large number of samples because of the long processing time and the possibility of degradation or losses during processing. Recently, a simple direct extraction method with deuterated NMR solvents has been developed for sample preparation, which in general gave better quality NMR spectra [20–24].

Single solvent systems have been used for some studies. Methanol with TFA was found to be a suitable solvent for the extraction of alkaloids [25] while perchloric acid was routinely used for the more polar metabolites, which prevents enzymatic degradation of metabolites [26]. However, a combination of CD₃OD and D₂O was found to be a more preferable solvent, since it can extract more diverse metabolites. The combination of these two solvents was used in different ratios, e.g., 30% to 70% of CD₃OD depending on the study [20, 23, 24]. In most of our work, a mixture of methanol-*d*₄ and KH₂PO₄ buffer in D₂O (pH 6.0) has been used, which extracts a wide range of metabolites including amino acids, carbohydrates, fatty acids, organic acids, phenolics, and terpenoids in a single step [19–22]. The direct extraction method with deuterated NMR solvents can save time and make it possible to deal with a large number of samples. Moreover, the buffer in the sol-

vent can avoid possible fluctuation of chemical shifts of signals in the NMR spectra.

However, when commercial herbal preparations are analyzed a more targeted approach is used focussing on the known major bioactive compounds in the plant material. For instance, in the case of St John's wort (*Hypericum perforatum*), both tablets and capsules were analyzed using a mixture of MeOH-pyridine (6:4, v/v) [27].

¹H-NMR analysis to detect all metabolites extracted from samples

For NMR-based metabolomics, the ¹H-NMR spectrum provides a wealth of chemical information. With the aid of chemical shifts and coupling constants some metabolites (amino acids, phenolics, sugars, and TCA-related organic acids) can be easily identified. The observed chemical shift positions and spin-spin coupling pattern for each proton provide information as to what kinds of protons are found in the molecules and subsequently how the protons are arranged. Furthermore, the concentration of each metabolite in the sample can be easily calculated from the integration of the signals in the spectra. There is no requirement for calibration curves to convert signal intensity into concentration as used in other methods [8, 28–30]. For obtaining reproducible results on concentration, the following parameters are important: relaxation delay, the pulse width, and the acquisition time. The optimum range of these parameters in ¹H-NMR was excellently reviewed by Pauli et al. [9]. One of the problems in measuring ¹H-NMR spectra is the water signal, a huge signal caused by residual water, which overlaps with the anomeric protons of sugars or glycosides ($\delta = 4.8\text{--}5.2$). To suppress this undesired water signal several methods have been applied, e.g., addition of paramagnetic ions like Mg²⁺ followed by Meiboom-Gill modification of the Carr-Purcell (CPMG) spin-echo pulse program [31] and pre-saturation using an additional pulse. When the pre-saturation technique is applied during relaxation delay, the most common method, unwanted reduction of the signal intensity close to the suppressed water might occur. For correct suppression, the temperature and pH of samples should be carefully adjusted since the water signal is greatly affected by those factors. Depending on the molecular size of metabolites, specific pulse sequences have been applied. To filter out the signals from small molecules from those of large ones, spin diffusion differences can be utilized. This was nicely shown in an NMR-based study on toxin-induced changes in lipoprotein profiles [32]. In the case of a matrix containing macromolecules (e.g., proteins or lipid vesicles) the application of a spin-echo sequence like the CPMG pulse sequence allows the attenuation of unwanted resonances from macromolecules [33]. However, in any ¹H-NMR spectra of plant extracts using diverse pulse sequences there are congested signals of metabolites. Multivariate data analysis is a key step for sorting out the discriminating signals from the complex spectra. Prior to the multivariate data analysis all signals in the obtained ¹H-NMR spectra should be digitalized. This could be achieved by the so-called bucketing procedure, which divides the spectrum into small bins and sums all intensities in each bin. Ideally, a smaller bucket size is better to examine subtle perturbations in the metabolome. However, because of unfavorable fluctuations of chemical shifts, even with a fixed temperature and pH of samples, one can only use 8–16 Hz (0.02 to 0.04 ppm in 400 MHz NMR) for the bucket size [34]. It is also possible to use full-resolution NMR data for the multivariate data analysis as shown in the study of Rasmussen et al. [27]. In this work, they

showed that full-resolution NMR data sets (0.15 Hz per data point resulting in 30 000 variables) gave more precise information about constituents responsible for data clustering, compared to the use of integrated NMR data sets (0.04 ppm bucketing resulting in 200 variables). Some aligning methods were found to improve the separation of NMR data for further statistical analysis [35–37].

Multivariate data analysis of ^1H -NMR spectra to recognize patterns and find discriminating signals

In recent metabolomics studies, many hundreds or sometimes thousands of samples are routinely analyzed and a minimum of several hundreds of signals are detected in each sample. It is crucial to extract the relevant information from such a huge data set. The samples are grouped based on the analysis of the metabolome (pattern recognition) and a discriminating metabolite (biomarker) can be found by multivariate data analysis. The pattern of a class represents the information about the relations between the observations within the class, discerning which are similar, which are different, or which are atypical outliers. Through the pattern recognition some metabolites can be found that are characteristic of a class. Of the multivariate data analysis, principal component analysis (PCA) and partial least squares projections to latent structures (PLS) are on the verge of becoming routine processing steps for raw analytical data. PCA is designed to extract and display the systematic variation in a data matrix and PLS is a regression extension of PCA, which is used to reveal the correlation between two kinds of data sets [38–41]. Both PCA and PLS are projection methods by reducing original data to a few principal components in order to describe maximum variation within the data. All raw data (e.g., the integral values of all chemical shifts in a ^1H -NMR spectra) of samples are plotted in a K -dimensional space (K = number of variables, e.g., chemical shifts in ^1H -NMR spectra) and the plotted samples are projected onto the line generated by least square sense (principal component line). The score of each sample is obtained along the PC line. The next principal components can also be calculated by projection onto the line which is orthogonal to the previous PC line or space. In general, the first three PCs are used for the analysis. For the data analysis, score and loading plots are used. The score plot is useful for observation of any groupings in the data set and in addition will highlight outliers that may be due to errors in sample preparation, experimental conditions or instrumentation parameters. Coefficients by which the original variables should be multiplied to obtain the PC are called loadings. The numerical value of a loading of each variable on a PC shows how much the variable has in common with that component. Prior to the PCA and PLS analysis raw data are scaled in different ways. There are several ways to scale the data. For unit-variance scaling, the standard deviation and the scaling weight as the inverse standard deviation are calculated. Subsequently, each variable is multiplied by the inverse standard deviation. Each scaled variable then has equal variance. This method is useful when the classification relies more on differences between minor compounds, since it increases the influence of weaker signals. However, applying the unit-variance scaling to an NMR data set, it is difficult to interpret the loading plot obtained from the unit-variance scaling method because all signals have the same variance and consequently are different from the original NMR spectra. With mean-centering, the average value of each variable is calculated and then subtracted from the data. This improves the interpretability of the loading plot since it resembles the original NMR spectra. Also,

the spectral noise is removed for the analysis as long as intensities of noise are lower than real signals. However, the influence of minor signals might be excluded since the mean-centering method always retains the original value of signals. In particular, the signals of plant secondary metabolites can be underestimated compared to high levels of primary metabolites. As an alternative technique the so called Pareto scaling has become more common. Pareto scaling gives each variable a variance numerically equal to its initial standard deviation instead of unit-variance. Hence, Pareto scaling is intermediate between the extremes of mean-centered and unit-variance scaling [42], which gives some weight to minor signals and at the same time it provides interpretable loadings. In recent years variable stability (VAST) scaling which weights each variable according to a metric of its stability improved the class distinction and predictive power of PLS-DA models for ^1H -NMR spectra of urines [43].

Two-dimensional NMR spectroscopy to identify differentiating metabolites in the NMR spectra

The signals to discriminate sample groups are sorted by multivariate data analysis. Some metabolites like carbohydrates, amino acids, or organic acids related to the TCA cycle can be elucidated merely based on their chemical shifts in ^1H -NMR spectra. However, for most cases, the spectral complexity and signal overlap in the ^1H -NMR spectra are too high for identification. Chemical structures of metabolites, especially species-specific plant secondary metabolites, should be confirmed by additional methods. Diverse two-dimensional (2D) NMR spectroscopy can give information on chemical structures. Indeed one of the advantages of NMR spectroscopy in plant metabolomics is the power of structure elucidation of molecules in a complex mixture. The 2D-NMR spectroscopy available for metabolomics can be divided into two types, resolved and correlated spectroscopy according to the type of dispersion in the second frequency dimension [8]. A typical resolved technique is the 2D- ^1H - ^1H - J -resolved spectra. It provides additional information on spin-spin coupling constants, together with chemical shifts. One of the difficulties for interpreting a ^1H -NMR spectrum is the interpretation of the splitting pattern of each signal. If a target signal is detected in a crowded region overlapping with other signals, the first step is to identify its splitting pattern. In addition to this use of J -resolved spectra in structure elucidation, projected spectra on to the axis of chemical shift have been applied in several studies [20–22, 44]. The projected spectrum produces a ^1H -NMR spectrum consisting of singlets for each ^1H chemical shift, i.e., a fully proton-decoupled proton NMR spectrum. In the projected spectrum all signals from macromolecules which have short T_2 relaxation times are readily suppressed because the pulse sequence of the J -resolved technique is based on a spin echo sequence [8]. Thus, baseline shifting caused by proteins, oligo- or polysaccharides, which might overlap minor signals or result in overestimation of target signals, are clearly reduced in the projected spectra and lead to better separation in multivariate data analysis. Another promising resolved technique is diffusion-ordered spectroscopy (DOSY) where each NMR signal is resolved based on the molecular diffusion coefficient [45]. DOSY can be used for molecular weight evaluation of unknown, purified constituents by preparing calibration curves with known compounds [46]. Although DOSY has a potential to differentiate metabolites having the same chemical moieties and different molecular weights in plant extract, e.g., glycosides from their aglycones, the application in metabolomics has been limited so far to the analysis of

polymers like polysaccharides from insects or mushrooms [47, 48], interaction between small molecules and ligands [49, 50], and hydrocarbon mixtures [51].

Following the resolved spectra, information on correlation between protons or carbon-proton can be obtained by homonuclear and heteronuclear experiments. A typical proton homonuclear technique is correlation spectroscopy (COSY) that shows which signals in a proton spectrum have mutual spin-spin couplings. The resulting spectrum has the conventional 1D NMR spectrum along the diagonal cross-peaks at chemical shifts corresponding to pairs of coupled nuclei. In general protons within three bonds are well correlated in the COSY spectrum but in sp^2 spin systems such as olefinic and phenolic compounds, even the correlation beyond three bonds is readily detected. A modified pulse sequence of COSY, long range COSY, can selectively detect correlation between protons far from each other (more than three bonds). It can show structure confirmation of connectivity between two molecules which is not possible to detect in a normal COSY spectrum.

It is one of the most time-consuming tasks to define NMR signals belonging to the same molecule in the complex ^1H -NMR spectra of a mixture. In particular, most ^1H signals of carbohydrates are detected in an extremely crowded region ($\delta = 3.0\text{--}4.5$) overlapping with each other or with the signals of amino acid protons close to the amino group. A powerful 2D-NMR technique, total correlation spectroscopy (TOCSY) or the Hartmann-Hahn experiment (HAHAHA) provides information on unbroken chains of coupled protons in the same molecule. For example, if anomeric protons of carbohydrates are defined, the rest of the signals in the crowded region can be easily assigned using the correlation in the TOCSY spectrum.

All organic molecules have carbons as their chemical backbone. The information on the carbons is thus crucial for structure elucidation. In fact measurement of a ^{13}C -NMR spectrum is an important, if not indispensable, step to elucidate the chemical structure of a pure compound. However, the magnetically active ^{13}C isotope has too low a sensitivity for the application to the analysis of the metabolome since the natural abundance of ^{13}C is only 1.1% of ^{12}C and its magnetogyric ratio one fourth of ^1H . It results in 1/57 000 of the ^1H sensitivity. Also, the long relaxation time of ^{13}C requires a long measuring time in order to obtain quantitative results. To overcome the low sensitivity of ^{13}C heteronuclear correlation techniques are used from which the information on ^{13}C chemical shifts is indirectly obtained.

Direct correlation between carbons and protons can be detected by heteronuclear multiple quantum coherence (HMQC) or heteronuclear single quantum coherence (HSQC) which gives better sensitivity than HMQC but needs phase correction. In both HMQC and HSQC spectra any carbon attached directly to a proton is detected, consequently the chemical shift of a ^{13}C connected to a specific proton can be obtained. The assignment of C-6 and C-8 of 5,7-dihydroxyflavonoids can easily be learned from the HMQC spectra of the extract of aerial parts of *Genista tenera*. In the HMQC spectra the unusual upfield-shift of those aromatic carbons compared to other aromatic carbons, caused by the adjacent oxygen, confirmed the presence of 5,7-dihydroxyflavonoids [52]. Long-range correlations (two and three bonds) between carbons and protons can be observed by heteronuclear multiple bond correlation (HMBC) spectra. It is an extremely powerful tool for structure elucidation since carbon-carbon correlations can be indirectly obtained with the aid of HMQC or HSQC. In addition, correlations between quaternary carbons like carboxylic acid and

nearby protons can be seen in the spectra. The long-range couplings in HMBC spectra can give information on the connectivity between two moieties which are connected only by quaternary carbons or hetero atoms (e.g., *O*-glycosides). One of the advantages of NMR-based metabolomics is that there are numerous combinations of 2D-NMR experiments that provide information on structures by diverse correlations. Aiming at specific correlations, enhancing resolution, or simplifying spectra HMBC-*J*-resolved [39], DOSY-selective TOCSY [53], 2D-*J*-DOSY [54] and DOSY-HMQC [55] have been applied to the analysis of complex mixtures. The combined HMQC (or HSQC)-TOCSY sequence experiment leads to a ^{13}C -edited TOCSY spectrum, and information on all connectivities of carbons in each molecule in a mixture.

Application to Plant Products



Classification

NMR-based metabolic fingerprinting has been applied to diverse fields of plant research. Classification and identification of adulteration of plant products are among the major interests. Using NMR-based metabolic analysis, it was possible to discriminate different species of plants. An example is the study of *Ilex* [17]. Eleven species of *Ilex* were analyzed and based on their metabolites, each species could be discriminated, especially Yerba mate (*I. paraguariensis*) from its adulterants. The contributing metabolites were arbutin, phenylpropanoids, caffeine and theobromine. Caffeine and theobromine were only found in the *I. paraguariensis*, whereas arbutin was found only in other species. The study of flower heads of chamomile (*Matricaria recutita*) showed that this technique can be applied to assess the relative amount of stalk materials in the different batches of flowers [56]. Another example of NMR analysis combined with PCA is the application to characterize 3 different *Strychnos* species using different parts of the plants (seeds, roots, leaves and barks). All samples were clearly classified based on their metabolites such as brucine, loganin, *Strychnos* icaja alkaloids (icajine, sungucine) as well as fatty acids [57].

Twelve *Cannabis sativa* cultivars were discriminated by analyzing the organic fractions and aqueous fractions. THCA and CBDA were the most important metabolites in organic fractions, while primary metabolites found in the water fraction such as glucose, asparagine and glutamic acid served as discriminating metabolites [15].

Quality control in traditional Chinese medicines

Traditional Chinese medicines (TCM) are often used as a mixture of several plants, with a hardly defined composition. Even though there are regulations for quality control, this is often restricted only to a certain compound or group of compounds. NMR-based metabolic fingerprinting combined with pattern recognition may be a very good tool for the quality control of TCM. Chemotaxonomic analysis including quality control issues was applied in the study of *Ephedra* [19]. *Ephedra* is one of the oldest medicinal plants known to mankind. However, 3 different species of *Ephedra*, *E. sinica*, *E. intermedia*, and *E. equisetina* are commonly and widely used without strict distinction as long as there is a certain amount of ephedrine alkaloids (0.8% of dry weight). Using NMR-based metabolic fingerprinting, it was possible to discriminate *E. sinica*, *E. intermedia* and *E. distachya*. Not only the ephedrine type of alkaloids, but also another secondary metabolite (benzoic acid analogue) was found to be an important discriminating metabo-

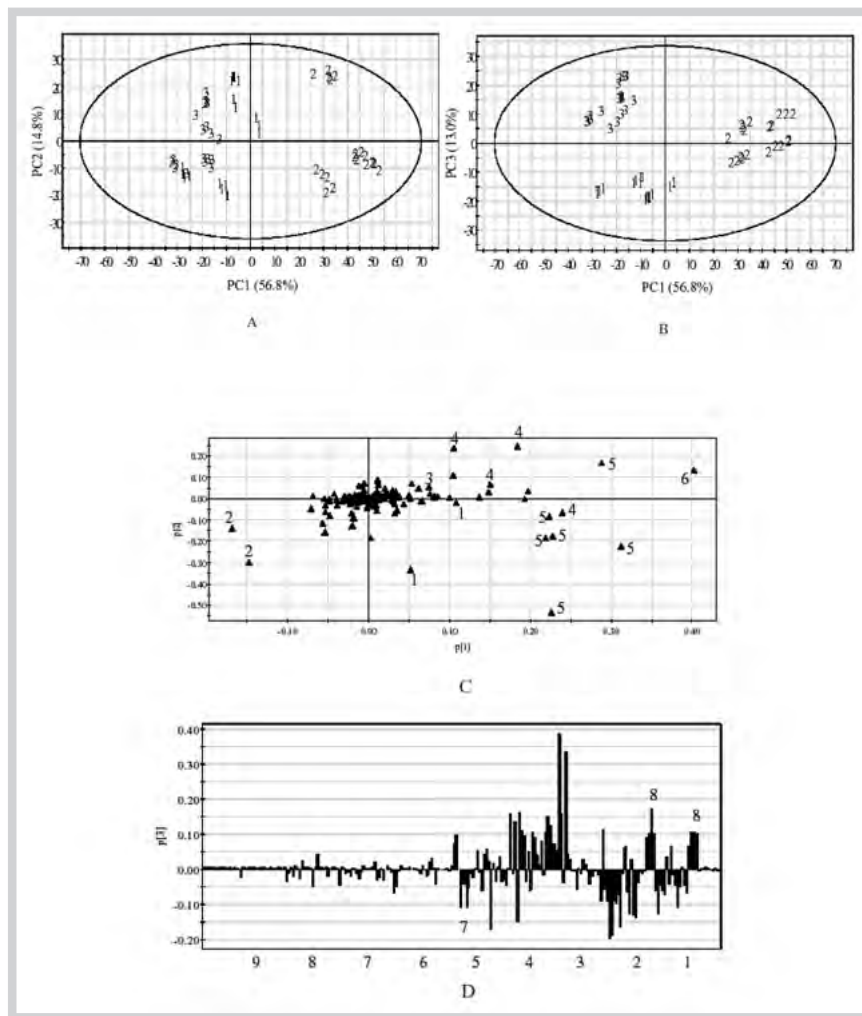


Fig. 2 Score plot of principal component analysis of ginseng preparations using PC1 vs. PC2 (A) and PC1 vs. PC3 (B). 1–3; commercial ginseng preparations. And loading plot of principal component analysis of ginseng preparations. PC1 and PC2 (C) and PC3 (D). 1, alanine; 2, arginine; 3, fumaric acid; 4, inositol; 5, sucrose; 6, acetate; 7, anomeric protons of sugars; 8, methyl protons of saponins (adapted from [58] reproduced with permission of Georg Thieme Verlag KG Stuttgart, New York).

lite. Among nine tested commercial *Ephedra* materials, one was shown to be the mixture of two species. There are several reports on the discrimination of ginseng (*Panax ginseng*) preparations, which is one of the most popular herbal medicines. The first report [58] using NMR-based metabolomics approach showed the differences in ginseng products, different ages (4, 5 and 6 years old) and differently processed (white and red) (● Fig. 2). PCA of *J*-resolved ^1H -NMR spectra of three commercial ginseng preparations showed striking differences among the preparations (● Fig. 2A and B). Loading plots explained that alanine, arginine, fumaric acid, inositol and ginsenosides are important metabolites to differentiate preparations from each other (● Fig. 2C and D). Other studies also showed that this approach can be used for the quality control of fresh ginseng roots of different ages [59] and different origins [60]. Recently, another study for the quality evaluation of TCM was reported by Tarachiwin and coworkers [61]. A combination of an NMR technique and multivariate analysis showed the differences in two *Angelica acutiloba* roots, *A. acutiloba* Kitagawa (yamato-toki) and *A. acutiloba* Kitagawa var. *sukiyamae* Hikino (hokkai-toki), with regard to their geographical and variety origin.

Application of NMR to commercial preparations of *Artemisia* has been reported recently by Van der Kooy et al. [62]. NMR analysis combined with PCA showed clear differences between *A. annua* and *A. afra*. A more targeted analysis combined with LC-MS showed that the content of artemisinin in the commercial prod-

ucts is different from the commercial claim. Other studies of NMR-based methods were applied to the characterization of crude extracts and commercial preparations, including feverfew [63], Kava-kava [64], *Artemisia* [65] and St. John's wort [66]. The details were reviewed in a recent review by Holmes [67].

MS-Based Metabolomics

Hyphenated chromatography-MS systems employed for metabolomic fingerprinting consist of three parts. The first part is the chromatographic separation section, used to separate complex mixtures obtained from the plant material. The most commonly used chromatographic systems are HPLC, GC or CE. The second part consists of the ionization chamber where the molecules are being ionized. Different ionization sources exist such as electron ionisation (EI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). Another ionization source being used, but not in combination with flow-based systems, is matrix-assisted laser desorption/ionization (MALDI). The third part consists of the MS where the ionized molecules are being detected as their mass-to-charge ratio. The most commonly used MS systems include single quadrupole, triple quadrupole, ion trap and time-of-flight spectrometers. More advanced high resolution spectrometers include the Orbitrap or the Fourier transform-cyclotron. For an in depth

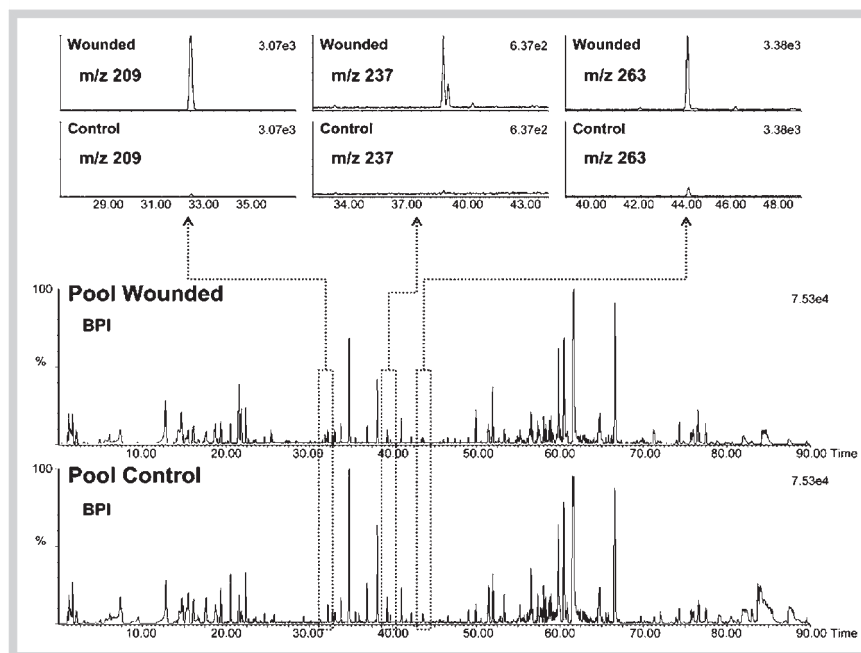


Fig. 3 Metabolite profiling chromatograms obtained by UPLC-TOF-MS of wounded and unwounded plants. In the inset, the comparison of the $m/z = 209, 237$ and 263 is shown ([71] reproduced with permission of Wiley-VCH Verlag GmbH & Co. KGaA).

review of ionization sources and mass spectrometers reference [68] should be consulted. There is little difference in the sample preparation and data handling between MS-based and NMR-based technologies, although the solvents being used differ between the two analytical systems. NMR makes use of deuterated solvents while MS uses non-deuterated solvents.

Sample preparation and extraction

Various authors have made use of targeted extraction methods in order to extract and analyze a specific group of components. Garcia-Villalba et al. [69] used various solvents to extract a major number of compounds from soybean seeds in a comparative study between transgenic and conventional soybeans. The solvents tested for their extraction efficiency included: aqueous methanol (60, 80, 90 and 100%, v/v), aqueous ethanol (60, 80, 90 and 100%, v/v), ethyl acetate and aqueous acetonitrile (70 and 100%, v/v). An alternative extraction procedure was also tested by removing the fat (mainly phospholipids) with hexane prior to the extraction. They concluded that 80% methanol resulted in the highest number of metabolites extracted. In a study to compare the phenolic content between the leaves, stems, roots and fruits of *Vaccinium angustifolium*, 95% ethanol was used as extraction solvent [70]. Grata et al. [71] used 50 mg of lyophilized leaf tissues and extraction with isopropyl alcohol using a ball mill in a case study of the wound response in *Arabidopsis thaliana*.

● **Fig. 3** illustrates the UPLC-TOF-MS chromatograms obtained from the wounded plants in comparison to the control plants. Vorst et al. [72] studied a non-directed unbiased total extraction of metabolites from tubers (*Solanum tuberosum*). The material was ground to a fine powder in liquid nitrogen. Extracts of 0.5 g frozen powder with 2 mL 62.5% methanol, 0.125% formic acid in water at 0 °C were prepared in duplicate. After immediate mixing, the extracts were sonicated for 10 min, spun down (10 min at 1000 g) and filtered through a 0.2 μm inorganic filter. Recently another extraction protocol for a untargeted metabolomic approach for plant metabolomics was published [73], based on reversed-phase liquid chromatography coupled to high-resolution mass spectrometry (LC-QTOF MS) of aqueous methanol extracts.

It can be concluded that no single extraction solvent will be able to extract all metabolites present. This limitation holds unfortunately true for all analytical procedures including NMR-based metabolite fingerprinting.

Sample analysis

The most important limitations of MS-based methods are the inherent lack of ionization of certain components. Ionized components can be identified and quantified at very low quantities making the MS a very sensitive analytical tool for metabolic fingerprinting. The lack of ionization and therefore the inability to quantify or identify components that do not ionize limits the use of MS to the more targeted metabolic profiling techniques. Various probes are being employed to overcome this inherent ionization problem in order to increase the dynamic range of components that can be detected with the use of MS. Other limitations of MS-based technologies are that the equipment needs to be calibrated regularly and fragmentation of molecules occurs which complicates the mass spectra. Fragmentation does however lead to structural information which in turn leads to identification of unknown components with the use of MS-MS-based technologies.

Samples prepared for MS analysis are usually separated chromatographically before the separated compounds are analyzed with the MS. Separation technologies employed largely make use of GC [74, 75], HPLC [70, 76], and capillary electrophoresis (CE) [69, 77, 78]. These technologies are either employed to obtain a targeted chemical fingerprint of a specific plant extract or as an untargeted technique in order to quantify and identify as many metabolites as possible in a plant tissue.

APCI and ESI are the most frequently used with HPLC chromatography as the separation step. These probes are known as “soft ionization” probes, while the detector used with these probes is usually the ion trap or the single quadrupole detector [70, 79–81]. EI ionization, also known as “hard ionization”, is more frequently employed with GC as the chromatography step. More advanced MS technologies and also the more expensive technolo-

gies employed to conduct metabolic fingerprinting include the FT Cyclotron MS [82, 83] and time of flight MS [76, 78].

Data handling

Due to the inherent variation and reproducibility problems associated with any chromatography technique, the generated data should be aligned before any data reduction techniques such as PCA or PLS can be employed. Alignments are used to reduce the variation in retention times between samples. Dedicated software, such as MetAlign, is used for the alignment of chromatographic peaks, automated baseline correction and alignment of all mass peaks across all samples. Subsequent statistics and bioinformatic tools can be used to provide a detailed view on the differences and similarities between samples or to link metabolomics data to other systems biological information [73]. During another study and with the use of the software "Similarity Evaluation System for Chromatographic Fingerprint of TCM", nine common peaks were identified which were used to evaluate 13 batches of Baishi samples from different provinces in China [84]. Various other processing software systems are currently available which aim at reducing variation between sample sets and to extract as much information as possible from the raw data sets. GC-MS data were processed by [85] with MATLAB software 7.1 (Mathworks) while [86] used MET-IDEA to process data generated by GC, LC or CE chromatography in combination with MS systems.

Applications of Chromatography-MS for Quality Control of TCM

Chromatography-MS technologies are mainly employed in a targeted approach when the quality control of TCM products is involved. Kang et al. [84], used an HPLC-PDA-ESI-MS for the chromatographic fingerprinting of furocoumarins in the roots of *Angelica dahurica*. A novel and sensitive HPLC-DAD-ESI/MS method has been developed by Su et al. [87], for the simultaneous determination of five major flavonoids in *Hypericum japonicum* hydroalcoholic extracts. Fingerprinting of TCM is also used to identify substances illegally mixed into TCM preparations. Liu et al. [88] identified aminophylline and prednisone acetate mixed illegally in TCM preparations. Li et al. [89] developed an approach with the use of HPLC/DAD and GC/MS in order to fingerprint aporphinoid and quinolizidine alkaloids of the total alkaloids from *Caulophyllum robustum*. The results were used to construct binary chromatographic fingerprints of the total alkaloids. *Artemisia annua* is a potent antimalarial herbal drug. This activity is ascribed to artemisinin, a sesquiterpene lactone that is very effective against drug-resistant *Plasmodium* species with a low toxicity. As a consequence, in view of a possible use of the phytocomplex rather than pure artemisinin, an HPLC/DAD/MS method was proposed for the simultaneous detection and quantification of both flavonoids and artemisinin. Jensen et al. [90] developed a LC-MS-APCI method in order to evaluate the composition of active constituents in phytopharmaceutical preparations. Several commercial ginkgo preparations on the Swiss market were analyzed, and the ginkgolide and bilobalide contents were evaluated. In another study a comparative analysis of the flavonoid components of the leaves of two medicinal plants known in Brazil as "espinheira santa", namely, *Maytenus ilicifolia* and *M. aquifolium* and a hybrid plant, *M. aquifolium* × *M. ilicifolia*, has been carried out using HPLC coupled with PDA-MS. By combining efficient LC

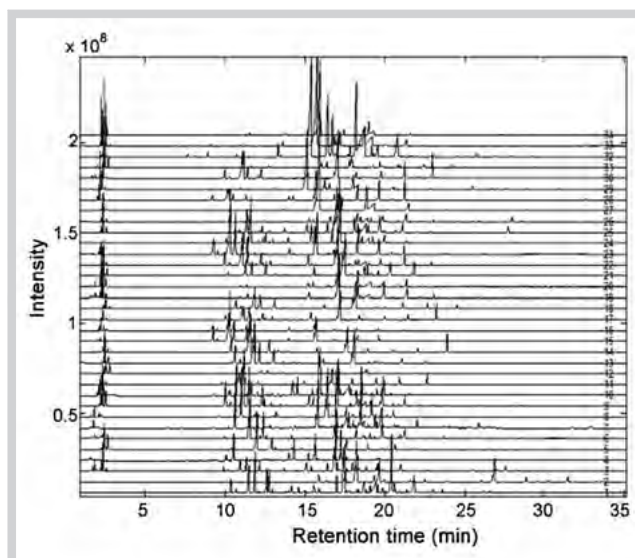


Fig. 4 The total ion current (TIC) chromatograms of 34 GC-MS data of fresh medicinal materials of *Houttuynia cordata* collected from four representative planting areas in China ([74] reproduced with permission of Elsevier B.V.).

separation and ESI-multiple stage mass spectrometry (ESI-MS_n) with UV detection, performed both with and without the post-column addition of shift reagents, the on-line identification of minor flavonoids in *Maytenus* extracts could be readily achieved. Fingerprint analysis of the flavonoid extracts revealed significant differences in the profiles of the two *Maytenus* species, while the hybrid plant contained flavonoids found in both parent species [91]. Bruni et al. [92] examined the qualitative and quantitative phytochemical variations in the dried flowering tops of cultivated *Hypericum perforatum* infected by phytoplasmas of the "ash yellows" class. Methanolic extracts of healthy and infected plants were separated by RP-HPLC to quantify naphthodianthrones and flavonoids, while essential oils were analysed by means of GC/MS. The affected plants exhibited decreased amounts of rutin, hyperoside, isoquercitrin, amentoflavone and pseudohypericin whereas the chlorogenic acid content was doubled. The essential oil yield was drastically reduced in infected material and revealed an increased abundance of sesquiterpenes and a matching decrease in monoterpene hydrocarbons and aliphatics. The consequences of the phytopathological condition of cultivated *H. perforatum* plants on the commercial quality were further outlined. Zeng et al. [74] employed GC-MS to develop a quality control method on thirty-four samples of medicinal materials of *Houttuynia cordata* collected from four different areas. **Fig. 4** illustrates a comparison of the total ion chromatograms of the 34 samples analyzed by GC-MS.

Trends

The greatest challenge that metabolomics studies face is metabolite differentiation and identification. Technical advances of current analytical tools are promising in this perspective. NMR is the tool traditionally utilized by natural products chemists for structure elucidation. The major limitation of NMR is its rather low sensitivity when compared to other analytical methods. Recently

developed probes for high-resolution NMR, such as cryoprobes and capillary NMR, help to increase the sensitivity threshold of the technique. CryoNMR aims to limit the receiver coil noise and to improve the coil quality factor by cooling the receiver coil and the preamplifiers to 25 K or below. With the use of cryogenically cooled probes the detection sensitivity of NMR is at least 3- to 4-fold enhanced in comparison with conventional probes operating at the same magnetic field [93]. Capillary NMR takes advantage from the use of small diameter radiofrequency (RF) coils for mass-limited samples (usually in the order of nanomole quantities). By decreasing the diameter of the receiver coil, the signal-to-noise (S/N) ratio improves [94]. Both cryoNMR and capNMR are very promising analytical approaches for metabolic fingerprinting studies.

The importance of “chemically characterizing” the metabolic compounds has been pointed out by Kopka as one of the main goals for any further development of the metabolomics approach [95]. As no single analytical technique is suitable for the detection and identification of all the metabolites in a biological sample, the most common strategy to clean up or isolate and structurally identify metabolites is to combine chromatographic techniques to MS or/and NMR spectroscopy. The resulting hyphenated methods, such as various HPLC-NMR techniques, are successfully applied to natural products research. An off-line solid phase extraction may sometimes be required to partially purify or concentrate the analytes [96,97]. Pioneering in the field of medicinal plants were Hostettmann, Wolfender and coworkers, who since the late 1990s have applied continuous and stopped-flow HPLC-NMR for the investigation of many natural products [98–102], and many other publications as reviewed by Jaroszewski [103]. More recently Wolfender and coworkers developed hyphenated methods for non-targeted metabolome profiling of minor metabolites from plant extracts [104,105]. The strategy is based on ultra-performance liquid chromatography-time-of-flight mass spectrometry (UPLC-TOF-MS). UPLC is a newly developed analytical technique that seems to be very promising in metabolic fingerprinting and metabolomics studies. The method is based on the use of very small particles (1.7 μm) at elevated pressure, thus achieving superior theoretical plate numbers and resolution in a short time of analysis and with less solvent consumption [106,107]. When applied to natural products analysis, UPLC revealed great advantages in comparison to standard HPLC [108–110]. The combination of the technique with mass spectrometry has been applied for the analysis of biological fluids, achieving very high resolution and sensitivity with the concurrent advantage of reduced separation speed [111–114]. A first attempt at using UPLC-MS for the differentiation of complex herbal mixtures was done by Jacob et al. [115]. The author applied UPLC-MS in combination with chemometrics tools for a chromatographic fingerprinting of 24 different Chinese medicinal herbs. The aim was to detect toxic compounds (aristolochic acids) in the multiherbal preparations of the traditional Chinese medicine. MS is often chosen as detection method coupled to UPLC, but this requires a sample preparation (e.g., pretreatment with solid phase extraction or liquid-liquid extraction) that is considered the rate-limiting factor of the method [107].

Another advanced liquid chromatographic technique that has been applied only a few times in metabolic fingerprinting is the hydrophilic interaction chromatography (HILIC). The method utilizes hydrated silica columns and eluting systems that are moving from high organic solvents miscible with water to high aqueous solvents. Water forms stagnant water layers on the stationary

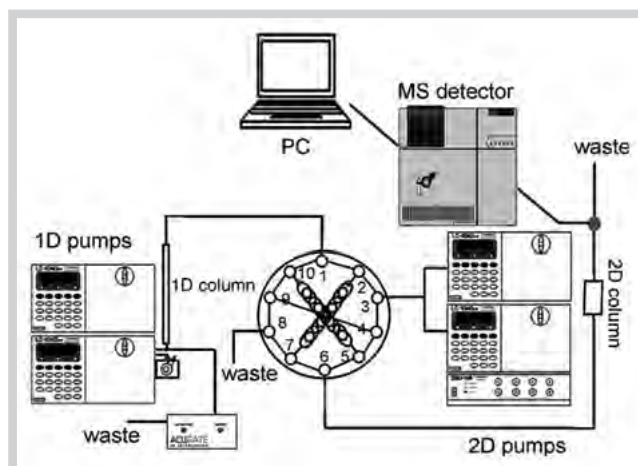


Fig. 5 Conventional LC \times LC set-up using one multi-port switching valve between the two dimensions ([122] reproduced with permission of Elsevier B.V.).

phase surface and the polar compounds are partitioned in this system [116]. The method is applicable to the analysis of hydrophilic compounds that are subjected to high retention by the column. Tolstikov and Fiehn applied HILIC chromatography coupled to mass detectors for the analysis of plant extracts [117] and later for a comprehensive analysis of urine [118]. Oligosaccharides, glycosides, amino sugars, amino acids, and sugar nucleotides were detected in the phloem exudates from *Cucurbita maxima* leaves by the authors. In order to cover a wider metabolome range, Dunn proposed that HILIC chromatography should be connected with reverse-phase liquid chromatography [119]. Although the mentioned enhanced liquid chromatographic techniques have been developed, the peak resolution is still the limiting factor when LC is chosen for comprehensive metabolic profiling. The introduction of a second dimension has proved to be a powerful strategy although, up to now, it has been mainly applied in proteomics [120,121]. When multidimensional chromatography is applied, the sample is subjected to two analytical separations, which are columns with orthogonal properties based on different selectivities. A combination of 2D liquid chromatography (Ag-LC \times RP-LC) and a mass spectrometric detection method (APCI-MS) was applied for a comprehensive analysis of triacylglycerols in commercial natural oil by Dugo and coworkers. **Fig. 5** illustrates a conventional LC \times LC set-up which was used during the analysis [122]. The analytes were clustered in groups in the 2D space thus facilitating their classification, and they were identified on the basis of the APCI-mass spectra. However, the method still suffers of some limitations such as mobile phase immiscibility, precipitation of buffer salts and incompatibility of stationary phase [123].

Analogously to two-dimensional liquid chromatography, also gas chromatography has seen the development of GC \times GC for the analysis of targeted and/or unknown volatile compounds present in a mixture. The separation efficiency is highly increased especially for very complex samples. Advantages are the fast acquisition time of the technique, and with the use of the second dimension the problem of co-elution is in most of the cases reduced. Compared to one-dimensional GC, the sensitivity of the chromatographic technique is much enhanced (trace levels of compounds are detected) and chemically similar compound patterns

are created with a major advantage for the identification of unknown compounds [124]. The combination of the GC × GC system with a mass spectrometer represents a very comprehensive tool for the analysis of complex mixtures. Only a limited number of applications of this platform have been reported so far. The two-dimensional gas chromatography technique coupled with quadrupole mass spectrometric detector (GC × GC-qMS) was employed by Shellie and coworkers for the metabolic differentiation of *Pelargonium graveolens* essential oils [125] and of semi-volatile oils from roots of three different ginseng species [126]. Very high efficiency was obtained, with mass spectra of high quality that facilitated the identification of separated metabolites by matching the results with available libraries. The same authors applied a GC × GC approach coupled to time-of-flight MS (GC × GC-TOF-MS) for a metabolomic study of mammalian tissues [127]. The TOF-MS detector has the advantage of an efficient spectral deconvolution of partially resolved GC × GC elutes that gives more structural information. In the study of Shellie and colleagues, the technique led to the detection of more than 1200 compounds, with a number of peaks characterized by high “analytical purity” that was increased by a factor of seven when compared to the classical one-dimensional GC-TOF method. The authors could structurally characterize only 10% of all the detected compounds. Nevertheless, the method showed to be effective for the determination of some metabolomic biomarkers of obese and lean mice. Hope and coworkers demonstrated the importance of using tandem GC in combination with a TOF mass spectrometer for the analysis of complex samples, such as metabolic extracts from perennial rye grass for an evaluation of the pre- and post-harvest physiology of the samples [128]. This was a very early-stage study of GC × GC-TOF-MS applied to plants for the purpose of metabolomic studies. The results of the analysis were limited only to one single identified compound (malic acid) but the method showed to have advantages of enhanced resolution and increased speed when compared with 1D chromatography. In another metabolomic-based study of plants a combination of GC × GC-TOFMS and principal component analysis (PCA) was applied to chemically differentiate the extracts from basil, peppermint, and stevia. The regions of interest, as resulted from the PCA, were further deconvoluted by the use of the chemometric technique parallel factor analysis (PARAFAC) and the interesting analytes were quantified [129]. The GC × GC technique is a promising tool and it is expected to gain a much wider distribution. However, analytical progresses will not lead to any improvement in the field of metabolomics science if we do not consider also other points of view. Metabolomics “happens” within each cell, and the tissues that are the object of our investigation at the moment are made of several cell types [130]. The next generation of plant metabolome scientists must focus on the cellular and even subcellular compartmentation of metabolism. Researchers have started looking into the metabolomics of single cells with the use of laser microdissection (LMD). In combination with GC-TOF-MS analysis the technique has been applied for the first time to a comprehensive metabolic fingerprinting of vascular bundles of *Arabidopsis* after the choice of cryosectioning the tissue as pretreatment procedure [131]. The study led to the identification of 68 metabolites. For more than half of them the authors could demonstrate whether they were enriched or depleted in vascular bundles. LMD has also been successfully used in combination with cryoprobe NMR for the analysis of specialized cells from the floral and leaves tissues of *Dilatis* spp. coming from different herbarium specimens [132]. The phytochemical pattern of

LMD-derived samples was compared with that of whole leaf extracts and some secondary metabolites were identified and connected with the specific cells. The study revealed the potential of the method in chemotaxonomic applications even of plants that are difficult to raise or to obtain. The same kind of approach, in combination with mass spectrometric measurements (LMD/NMR/MS) was carried out on the stone cells of Norway spruce bark [133].

The next point that must be considered is that metabolism takes place as a network happening within very short time fractions (< msec), and not as individual pathways. It seems thus more realistic to describe the metabolome of a tissue (or a cell) not as a single snapshot, but measuring the actual metabolic flux.

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