Advances in Techniques for Profiling Crude Extracts and for the Rapid Identification of Natural Products: Dereplication, Quality Control and Metabolomics

Jean-Luc Wolfender*, Guillaume Marti and Emerson Ferreira Queiroz

School of Pharmaceutical Sciences, EPGL, University of Geneva, University of Lausanne, 30, quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland

Abstract: Thanks to the important advances registered over the last decade in analytical techniques, profiling methods for the analysis of crude extracts from plant origin or from other biological sources have evolved into powerful tools for dereplication, quality assessment and metabolomics. Metabolite profiling of crude extracts represents a challenging analytical task since these mixtures are composed of hundreds of natural products. According to the type of study the focus can be put on major bioactive constituents or minor significant biomarkers. In many cases, a rapid on-line or at-line identification of the compound(s) of interest and in some case of all detected constituents (metabolomics) is required. The most common techniques for these types of analyses consist of a spectroscopic method hyphenated to high performance liquid chromatography (HPLC), such as liquid chromatography photodiode array detection (LC-PDA), mass spectrometry (LC-MS) or nuclear magnetic resonance (LC-NMR). With the evolution of multivariate data analysis (MVDA) methods, profiling extracts may also rely on direct NMR or MS analysis without prior HPLC separation, which requires high resolution instruments. In this review most of the current technologies for profiling natural products in various matrices will be presented and discussed. Various applications in the field of dereplication, quality assessment or metabolomics will illustrate the latest analytical strategies employed.

Keywords: High performance liquid chromatography, natural products, crude plant extracts, profiling, fingerprinting, dereplication, metabolomics, mass spectrometry, nuclear magnetic resonance.

1. INTRODUCTION

Natural products (NPs) have provided the inspiration for most of the active ingredients in medicines. The reason for this success in drug discovery can probably be explained by their high chemical diversity, the effects of evolutionary pressure to create biologically active molecules and/or the structural similarity of protein targets across many species [1]. This large chemical diversity is also directly linked to a high variability of their intrinsic physicochemical properties that render their detection and characterisation challenging. It is also well known that crude extracts from plant or fungal sources, marine organisms, microorganisms or of animal origin represent a rich source of bioactive NPs; however, the chemical composition of extracts can be very complex. In this respect, the total number of metabolites in a given organism, also called 'metabolomes', is still unknown but estimated to be in the thousands [2]. For drug discovery purposes, the most interesting of these compounds consist of secondary metabolites, and their numbers differ largely between organisms. While the most abundant metabolites can be present in the range of a few tenths of mg/kg, others can be present in the microgram range.

In the lead discovery programs, profiling methods are playing an increasingly important role in the study of the effects of phytopharmaceuticals and in the quality control of natural remedies. Currently, these methods are based on spectroscopic methods hyphenated to chromatography (mainly to HPLC) such as LC-PDA, LC-MS and LC-NMR. The role of these profiling methods is manifold: they can be dedicated to rapid on-line identification of known NPs to avoid tedious isolation (dereplication), they can be used in the detection of the largest number of metabolites possible for a comprehensive study (metabolomics), or they can be of interest for the standardisation or the quality control of a given complex extract used as a remedy. For all these profiling methods, HPLC has become a key method for the separation of NPs in their original biological matrices [3] because of its versatility and applicability to the analysis of crude mixture without the need for any prior purification procedure. However, none of the available HPLC detectors are able to detect all the NPs in a given extract within a single analysis [4]. This limitation is well known in metabolomics [5], which aims at measuring all of the metabolites in an organism qualitatively and quantitatively. The complex and convoluted nature of the crude extracts analysed also contributes to the difficulty of NP detection. Furthermore, the analytes can be present in large or very small amounts, and, according to the type of study (quantification, standardisation, fingerprinting, screening, trace analysis, etc.), very sensitive and selective methods may be needed for their detection. Metabolite profiling is thus not an easy task to perform. Furthermore, if the nature of the metabolites has to be determined, which is often the case, complementary high quality spectroscopic information have to be recorded on-line. When spectral databases exist for methods such as LC-MS-MS, the dereplication process can be efficiently automated. With the exception of some proprietary databases at the industry level, these procedures are still rarely applied for NP dereplication. Thus, in many cases either a partial structure assignment can be performed by combining UV or MS information with chemotaxonomic data or a *de novo* elucidation has to be made, based on a full set of on-line or at-line spectroscopic data (mainly UV, MS and NMR). In this case, the order of atoms and stereochemical orientations have to be elucidated *de novo* for each compound in a complex manner, and the compounds cannot simply be sequenced as is the case for genes and proteins. Often LC-PDA and LC-MS provide limited on-line structural information. This might be sufficient for the dereplication of

*Address correspondence to this author at the School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest Ansermet, 1211 Geneva 4, Switzerland; Tel: +41 22 379 33 85; Fax: +41 22 379 33 99; E-mail: jean-luc.wolfender@unige.ch

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well-known natural products, but this will only yield a partial structural determination. Access to ^{1}H-NMR data by on-flow LC-NMR or ^{13}C-NMR by indirect experiments with at-line techniques such as SPE-NMR or CapNMR is often mandatory when compounds cannot be easily dereplicated by LC-PDA-MS.

With the latest development recorded in mass spectrometry and NMR spectroscopy, the profiling of complex mixtures can also be performed without hyphenation to HPLC. In this respect, different approaches have recently emerged such as direct MS and direct NMR profiling that involve multivariate data analysis (MVDA) of the convoluted spectroscopic data. These analytical strategies are mainly used for metabolomic or quality control studies. The advances made in new generation HPLC, MS and NMR instruments provide unsurpassed performances in terms of resolution and sensitivity and have strongly influenced the development of profiling methods. With state-of-the-art methods high resolution profiling of very complex mixtures such as multi-herb preparations has become feasible, and it is now possible to determine the complete *de novo* structure determination of minor bioactive NPs based on sets of high quality spectra obtained in mixtures.

This paper will provide a brief overview of the current methodologies for profiling complex extracts, and the latest applications in the field of natural product research will be discussed.

2. HPLC-HYPHENATED METHODS FOR NATURAL PRODUCT CHARACTERISATION

2.1. HPLC Separation of Crude Extracts

HPLC represents the most robust and versatile technique for the separation of natural products because it gives the possibility to analyse metabolites without need for derivatisation directly in the crude extracts. These extracts are usually obtained after maceration with different solvents and can be enriched in a given class of constituents by liquid-liquid extraction (LLE) or solid phase extraction (SPE).

HPLC has significantly developed through the years in terms of convenience, speed, choice of stationary column phases, sensitivity, applicability to a broad variety of sample matrices and ability to hyphenate the chromatographic method to spectroscopic detectors [6]. From a chromatography viewpoint, the development of columns with different phase chemistry (especially reversed phase) enabled the separation of almost any type of NPs. The latest developments in HPLC, including the recent introduction of highly pH-stable phases, sub-2-μm particles [7] and monolith columns, have considerably improved the performances of HPLC systems in terms of resolution, speed and reproducibility. Efficiencies exceeding 100,000 plates and peak capacities over 900 can be attained by coupling columns together [8]. For the separation of crude extracts, either raw mixtures or samples enriched by extraction via simple SPE or LLE are injected. The separations are mostly performed on C_{18} material with MeCN-H_2O or MeOH-H_2O solvent systems in gradient elution mode. To improve the separation efficiency, various modifiers are added to the mobile phase that can strongly influence the sensitivity of detection. In multiple hyphenated systems, the presence of several different on-line detectors (hypernated systems) leads to the need for an eluent composition that is compatible with each of the detectors [9].

For profiling studies, two main types detectors can be defined: simple detectors used for the recording of chromatographic traces for profiling or quantification purposes (e.g., UV, ELSD, and ECD) and detectors for hyphenated systems that generate multidimensional data (chromatographic and spectroscopic) for on–line identification and dereplication purposes (e.g., PDA, MS, and NMR). This paper will focus mainly on this last category. These hyphenated methods are namely LC-PDA [10], LC-MS, LC-MS-MS or LC-MS^n [11] and NMR (either directly hyphenated with LC (LC-NMR) [12], used at-line with preconcentration methods such LC-SPE-NMR [13] or after microfractionation with microflow LC-NMR (CapNMR) [14]).

Ideally these hyphenated methods should provide the same type of spectroscopic information that could be acquired for isolated pure products. However, this is not always true for on-flow methods and restricted structural information are often only available because of instrumental constraints and compromises related to the nature of the HPLC mobile phase (e.g., solvent or modifiers with high UV cut-offs in LC-PDA that will hinder the recording of relevant absorption bands of the analytes, eluent modifiers that can cause ion suppression in LC-MS or the use of a non-deuterated solvent in LC-NMR that may mask the structure-informative region of the spectra). These constraints are even worse when multiple detectors are coupled in LC-NMR-MS systems [15] or in even more extended hypernated systems. When such setups are coupled, there are even more compromises in terms of compatibility of sensitivity dynamic range and eluent composition, but there is the advantage of obtaining all structural information within a single run [9].

2.1.1. UHPLC

One of the most important breakthroughs over the last few years has been the introduction of Ultra High Pressure Liquid Chromatography (UHPLC) for both high resolution profiling and rapid fingerprinting of crude extracts [16].

From a theoretical point of view, the introduction of sub-2-μm particle supports induces a concomitant increase in efficiency and optimal velocity due to improved mass transfer [7]. Therefore, high-throughput separations can be obtained with short column lengths for metabolite fingerprinting (Fig. **1A**) whereas highly efficient separations are achieved with relatively long columns for detailed metabolite profiling (Fig. **1C**) [17]. However, the use of such small particles requires dedicated systems that are able to work at pressures higher than 600 bar. Since this approach provides very narrow LC peaks, MS detectors with very fast response times are generally mandatory, and, in recent years, UHPLC-TOF-MS has been recognised to be very efficient for profiling studies in metabolomics [16, 18].

As an example a comparison of the chromatograms obtained with NI ESI-TOF-MS of the crude isopropanol extract of *Arabidopsis thaliana* using a 5:95 H_2O:MeCN gradient in HPLC and UHPLC is presented in Fig. (**1**). As shown, a considerable improvement in resolution was obtained when transferring the standard HPLC method (Fig. **1D**) (60-min gradient, 250×4.6 mm I.D., 5-μm column at 1 mL/min) to a UHPLC (1×150×2.1 mm I.D., 1.7 μm, 200 μL/min) (Fig. **1B**) and even to two 150 mm UHPLC column coupled in series (300×2.1 mm I.D., 1.7 μm, 200 μL/min) (Fig. **1C**) providing the baseline separation of more than 250 constituents. This, however, generated longer analysis time. The use of high temperatures (HT) (90°C) (data not shown) enabled a twofold reduction in the analysis times, giving this type of HT-UHPLC profiling a much higher peak capacity than that of standard HPLC profiling [16]. A transfer of this gradient to shorter UHPLC columns (50×1.0 mm I.D., 1.7 μm, 300 μL/min) also provided a very rapid separation that was compatible with metabolite fingerprinting

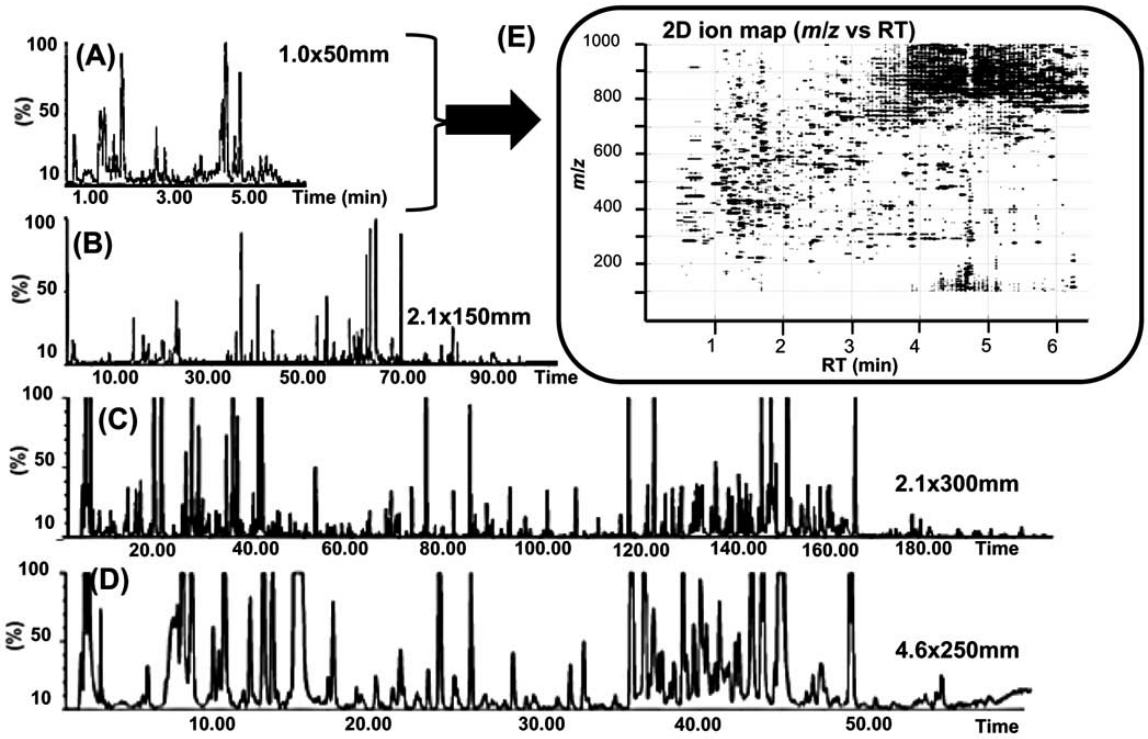

Fig. (1). Differences between HPLC and UHPLC profiling methods and geometrical gradient transfers. Chromatograms obtained by NI UHPLC–ESI-TOF-MS of *A. thaliana* (**A**) on a 1.0×50mm, (**B**) on a 2.1×150mm and (**C**) on a 2.1×300mm columns. Standard NI LC-ESI-TOF-MS profiling on a 4.6×250mm column(**D**). (**E**) 2D ion map of chromatogram (A) *m/z* vs RT (fingerprinting). Adapted from [17] and [16]. Copyright 2008 with permission from Elsevier.

for metabolomic studies at the expense of a reduction in peak capacity but with the benefit of high-throughput capabilities (Fig. **1A**) [17].

2.2. LC-PDA

PDA provides UV spectra directly on-line and is particularly useful for the detection of natural products with characteristic chromophores. For example, polyphenols can be efficiently localised by this method since they exhibit characteristic chromophores. With this type of compounds, UV spectral libraries can be built and used for dereplication, but these compounds have to be analysed in the same LC conditions because the composition of the mobile phase might affect the UV bands slightly. However, compared to LC-MS, this feature is limited since only NPs having characteristic chromophores can be recognised. The same method can also be used to assess peak purity by recording several spectra across given LC peaks and checking for differences between the spectra. More information on the localisation of the hydroxyl groups in polyphenols can be obtained after post-column addition of the UV-shift reagents, commonly used for the structural characterisation of these product in their pure form [19]. Such techniques have been successfully applied for the characterisation of flavonoids [20] and xanthones [21].

2.3. LC-MS

The coupling of HPLC with MS (LC-MS) is extremely powerful in terms of detection, quantification and identification of a wide range of natural products. Hyphenation enables a bidimensional detection where each metabolite is resolved in both chromatographic (retention time) and mass spectrometric (*m/z*) dimensions. In classical profiling LC-MS methods, rather long analysis times for the chromatographic separation are required (typically 120–60 min). As shown in Fig. (**1**) the throughput can, however, be increased using faster techniques such as UHPLC or the LC resolution significantly increased by keeping the same gradient time as for HPLC [17].

The application of LC-MS in the analysis and characterization of natural products has been recognised as a major breakthrough [22]. Indeed, while being expensive, the use of a mass spectrometer as an HPLC detector provides excellent sensitivity and selectivity for the analysis of NPs in complex biological matrices. Furthermore MS detection provides important structural information on-line such as molecular weight, molecular formula and diagnostic fragments, which are crucial for dereplication and rapid on-line characterisations of NPs. The type of structural information provided on-line is however strongly depending on the interface and ionisation method used as well as the type of analyser used.

Historically, a main challenge in LC-MS was that high liquid flows from HPLC were not compatible with the high vacuum required for the mass spectrometer. Since the early 1980s, a number of different interfaces have been developed to address this issue and overcome the difficulties [11, 23]. The overwhelming popularity of LC-MS today is largely due to atmospheric pressure ionisation (API) interfaces including electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) and most of the profiling studies performed concern these two main ionisation methods. For both ESI and APCI, ionisation occurs at atmospheric pressure. Some combination of high voltage and heat is used to provide the ionisation that is needed to produce the ions that are assayed by the MS system. In ESI, the high voltage field (3–5 kV) produces nebulisation of the column effluent resulting in charged droplets that are directed toward the mass analyser. These droplets get smaller as they approach the entrance to the mass analyser. As the droplets get

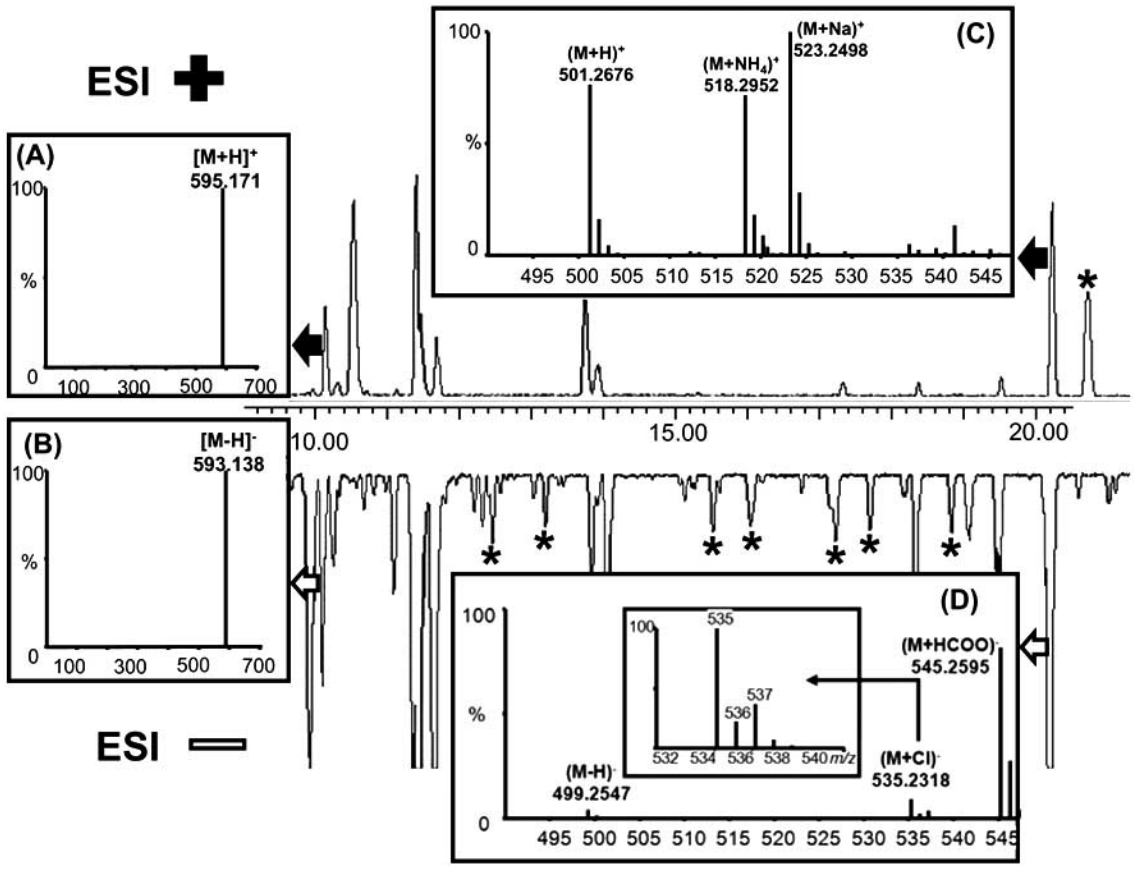

Fig. (2). UHPLC–ESI-TOF-MS reconstructed ion chromatogram of crude extract of *A. thaliana* in PI and NI modes. Peaks labeled with * are detected in one ionisation mode only. Insert (**A**) and (**B**) show protonated and deprotonated molecules for a flavonoid glycoside of MW 594 (**C**) and (**D**) show adducts observed for a monoglycerolipid of MW 500 in PI and NI mode [17]. Copyright (2008) with permission from Elsevier.

smaller, individual ions emerge in a process referred to as 'ion-evaporation'. These ions are then separated by the MS system. In APCI, heat is used to vaporise the column eluent, and then a corona discharge is used to ionise solvent molecules, which then produce the analyte ions via chemical ionisation mechanisms. More recently, a third type of ionisation source, termed atmospheric pressure photoionisation (APPI), has become available. In APPI, heat is used to vaporise the column eluent (similar to APCI), but the ionisation is produced by way of an ultraviolet (UV) lamp that produces 10 eV photons. Depending upon the solvent system used, the 10 eV photons will either ionise the mobile phase solvent or a dopant (e.g., toluene) added to the column effluent; these ions then produce the analyte ions through various ionisation mechanisms including charge or proton transfer [24].

With these three ionisation methods a very large variety of NPs ranging from low molecular weight apolar constituents to high molecular weight polar molecules can be successfully analysed.

These API methods generally provide a soft ionisation and mainly molecular ion species in the form of protonated molecules $[M+H]^+$ (positive ion mode, PI) or deprotonated molecules $[M-H]^-$ (negative ion mode, NI). To obtain a comprehensive profiling, crude extracts generally have to be analysed in both polarity modes. This can be done by two separated analyses or by performing a polarity switching experiments from scan to scan, if the duty cycle of the analyser if fast enough to maintain good detection performance. An example of PI and NI ESI-TOF-MS ion reconstructed chromatograms obtained for profiling the crude extract of *A. thaliana* are displayed in Fig. (**2**). These chromatograms correspond to an enlargement of the 10 to 20 min RT range of Fig. (**1B**).

In Fig. (**2**), the complementarity of both PI and NI detection modes appears clearly. As shown in the chromatograms, some constituents where only detected in PI while others were only visible in NI. Particularly, in the retention time domain displayed, more peaks were detected in the NI mode. For some compounds such as flavonoid glycosides (MW 594), both PI $[M+H]^+$ (*m/z* 595) and NI $[M-H]^-$ (*m/z* 593) molecular ions were recorded (Fig. **2 A** and **B**), which provided a straightforward attribution of the molecular weight as well as the molecular formula from the high mass capabilities of the TOF-MS system. For other constituents such as the monoglycerolipids [25], a series of adducts were recorded in both PI and NI modes, shown Fig. (**2C** and **D**). This type of pattern of molecular ion species renders the determination of the MW more challenging for an unknown constituent. Declustering of the adducts can be accomplished by collision-induced dissociation (CID) either at the API source (in-source CID) or in conjunction with tandem MS (MS–MS) experiments [23].

A systematic LC-MS-ELSD study was conducted on 96 structurally diverse NPs, including phenolics, alkaloids, amides, terpenoids, steroids and polyketides, with a short HPLC generic gradient (C_{18}, 5-95% ACN :H_2O 0.1% formic acid in 10 min). Good overall ionisations of most compounds in both ESI PI and NI modes were obtained [26]. The generic separation used was suitable for the

elution of 86 of the 96 compounds (RT > 1 min <10 min), 75 were well detected in PI and 57 in NI, while all compounds were detected by ELSD (4 µg on-column). Therefore, a relatively universal detection can be obtained if both PI and NI modes are used or alternated during acquisition.

It is important to understand that analyte ionisation is largely compound-dependant and is mainly governed by proton affinity. As a rule of thumb for a good approximation, acidic molecules (e.g., carboxylic acids or phenolics) will mainly produce $[M-H]^-$ in NI, while bases (e.g., alkaloids, amines, etc.) will generate $[M+H]^+$ in PI. Compounds, such as glycosides, will have a high affinity for salts and will tend to form sodium adducts in PI. ESI (PI) is mainly used for alkaloids, APCI (PI) for pigments and carotenoids, ESI (PI/NI) for triterpene glycosides, and APCI or ESI (PI/NI) for phenolics and flavonoids. APCI is generally more suitable for non-polar constituents, while ESI provides a softer ionisation and gives a good response for polar metabolites. Additionally, APCI is generally recognised to be less prone to matrix effects than ESI [27]. Only a few studies have referred to the use of APPI for NP detection [28].

For drug discovery, the performances of APPI, APCI and ESI were evaluated on 106 standards and 241 proprietary drug candidates occupying a wide chemical space [29]. The results indicated that the detection rates in PI were 94% for APPI, 84% for APCI, and 84% for ESI. Combining PI and NI mode detection, APPI detected 98% of the compounds, while APCI and ESI detected 91%. In APPI, for analytes of low polarity and low proton affinity, $M^{+\cdot}$ was the predominant ion species generated, while for high polarity and high proton affinity compounds, $[M+H]^+$ ion species dominated the spectra. APPI is thus regarded by some authors [28] as a more universal detection method for LC-MS than APCI and ESI, but it is still not widely used in natural product analysis.

For LC-MS applications, many types of mass spectrometers can be used. Those that are low resolution (LR), such as the single quadrupole (Q) mass spectrometers, are the most commonly used and least expensive. Those giving high resolution (HR) and exact mass capabilities, such as time-of-flight (TOF) instruments, are becoming increasingly popular. TOF-MS provides resolution up to 20'000 and very good mass accuracy (<5 ppm). They can be operated with a high acquisition frequency over a broad mass range compatible with rapid chromatographic methods and without compromising sensitivity. The high mass accuracy of HR instruments represents an important feature for dereplication because it gives the possibility to measure the molecular formula of all NP in a given mixture. This restricts the number of possibilities when searching a database of natural products, especially when a cross-search involving chemotaxonomical information can be performed.

To obtain additional structure information, molecular ions generated by soft ionisation methods can be fragmented in tandem mass spectrometers using MS/MS or MS^n experiments. In this respect, triple quadrupole (QQQ) MS–MS systems are the most commonly used. They are also particularly useful for bioanalytical assays and for specific quantification purposes in complex matrices through multiple reactions monitoring (MRM) that provide very specific detection [30]. Ion-trap (IT) mass spectrometers have the unique capability of producing multiple stage MS-MS (MS^n) data that may be essential for structural elucidation. In addition to these type of mass spectrometers, there are a growing number of additional varieties, including hybrid systems that combine LR and HR analysers for specific applications [11, 24]. In these MS-MS systems, the fragmentation can be performed by selecting specific molecular ions or by performing data-dependant experiments in which the mass spectrometer automatically selects the most abundant ion at a given retention time for fragmentation.

In natural product chemistry, the MS/MS spectra are mainly useful for the partial determination of sugar sequences of various glycosides, for the classical fragmentation of flavonoids or related compounds for the determination of substituents positions on the A or B rings or for the differentiation of isomers [31]. For other studies, the interpretation of the MS/MS spectra often requires the analysis of many related products to extract structurally relevant information. For the analysis of completely unknown constituents this approach alone cannot record enough information for structural determination.

As an example of the type of information that can be obtained on-line for triterpene glycoside, the LC-ES-MS^n spectra recorded in an ion trap system on a pentaglycosylated saponin in the crude extract of the molluscicidal plant *Phytolacca dodecandra* (Phytolaccaceae) [32]. In this case, multiple scan-dependant MS^n experiments were recorded automatically during the elution of the LC peak from the extract, and the main ion of each spectra was selectively fragmented. By decomposing the resulting fragmentation product (MS^n), sugar sequences were partially elucidated. The deprotonated molecule $[M-H]^-$ (*m/z* 1249) yielded a first fragment at *m/z* 1087 (-Glc: 162 Da) which then cleaved into ions at *m/z* 925 or 941, due to the simultaneous loss of a glucosyl (-162 Da) or a rhamnosyl (-146 Da) unit, characteristic for a branched sugar chain. The ion at *m/z* 779 issued from the fragmentation of that at *m/z* 925 (-Rha) or 941 (-Glc). Finally, the *m/z* 617 ion and the aglycone ion at *m/z* 455 arose from cleavage of that at *m/z* 779. In this case, the LC-MS^n only provided information regarding the number of hexoses (4) and desoxyhexose (1) as well as the MW of the aglycone. These data were sufficient to dereplicate this product with a good confidence based on the literature data for the existing saponins from *Phytolacca dodecandra*, but it would not have permitted a complete structure assignment since the exact nature of the sugars and the aglycones as well as the position of interglycosidic linkages could only be ascertained by NMR measurements.

2.4. LC-NMR

Due to the lack of efficient commercial databases, especially for LC-MS-MS spectral comparison, the dereplication process often requires additional spectroscopic information to ascertain the identity of known natural product or to partially identify unknown metabolites. In this respect, LC-NMR provides important complementary structural information or, in some cases, a complete structural assignment of natural product on-line [33, 34]. The interest of a direct coupling of HPLC with NMR resides in the fact that full structure elucidation and stereochemical information can be obtain (with 2D spectra in the stop-flow mode) but also because it is a highly non selective detection technique which can bring a comprehensive detection of any hydrogen containing compound present in the HPLC eluate [33].

The coupling of HPLC with NMR has started in the early 80's [35] but it necessitated the improvement in probe technology, the construction of high-field magnets and the progresses in pulse field gradients and solvent suppression, to give a new impulse to this technique which has really emerged since the mid-1990s as a very efficient method for the on-line identification of NPs in complex matrices [34]. It took nearly 20 years to establish LC-NMR and this long period can be mainly attributed to the intrinsic low sensitivity of NMR. Even today, the on-line coupling of HPLC with NMR is

insensitive in comparison with LC-MS, but various strategies which involved various operation modes of hyphenated systems and at-line coupling of the NMR to the HLPC with on-line sample method such as SPE-NMR or tubeless technology such as CapNMR has again boosted this powerful identification method. LC-NMR has now evolved in comprehensive and very powerful LC-NMR-MS platforms [36, 37] used for many applications in drug discovery including dereplication [38] rapid *de novo* identification of new natural products [39] and biomarker identification in metabolomics [40].

LC-NMR represents an interesting complementary technique to LC-PDA-MS for on-line identification of LC peaks. Recent applications have demonstrated its usefulness in various fields of analytical chemistry.

2.4.1. On-flow and stop-flow LC-NMR

The principle of coupling an HPLC to a NMR is rather simple and does not require a complex interface such as for LC-MS since the use of solvent is natural for NMR detection which was not the case for MS detection (Fig. **4A**). In this respect continuous flow probes have been designed for cryomagnets with volume ranging between 30-120 µl. In this case, fixed U-type glass tube was fixed in the Dewar of the NMR probe body, breaking the central symmetry of the magnetic field. This design ('saddle'-shaped geometry) showed an excellent resolution, approaching those registered with the rotation of the NMR tube.

The main problem in LC-NMR resides in the fact that deuterated solvent would be ideally needed for an optimum detection of the analyte. However in standard HPLC systems using 4 mm i.d. column and operating at 1 ml/min, this is not economically feasible. Thus for classical LC-NMR operation, standard HPLC grade solvent are used and water is replaced by deuterated water (D_2O). The profiling studies are thus performed with CH_3CN-D_2O and MeOH-D_2O. In these solvent systems the difficulty is to observe the analyte resonances in the presence of the much larger resonances of the mobile phase. This problem is worse in the case of typical LC reversed-phase operating conditions, where more than one protonated solvent is used and where the resonances changed frequencies during the analysis in gradient mode. These problems have been overcome thanks to the development of fast, reliable and powerful solvent suppression techniques such as water suppression enhanced through *T*1 effects (WET) [41], which consists of a combination of pulsed-field gradients, shaped radiofrequency pulses, shifted laminar pulses and selective ^{13}C decoupling. The main drawback of this procedure is that analyte signals localised under the solvent resonances will also be suppressed. This problem can be circumvented by performing the separation in fully deuterated solvents, on micro-columns with minimum deuterated solvent consumption [42-44]. Another problem occurring in LC-NMR is that the chemical shifts recorded in a typical reversed-phase solvent will differ from those reported in standard deuterated NMR solvents. This can be a drawback if precise comparisons with literature data have to be performed.

Concerning sensitivity the LODs of LC-NMR are several orders of magnitude higher than LC-UV or LC-MS. These limits also depend on the type of magnet used (400-900 MHz) and the type of flow probe employed (standard LC-NMR probes 40-120µl, micro-coils 20 nl-1µl or cryo flow probes). The mode of operation also strongly affects sensitivity. LC-NMR can indeed be operated in the on-flow mode, the spectra are recorded on the fly and limited number of acquisition are recorded when the LC peak crosses the flow cell. In the stop-flow mode an additional sensitive method of prior detection, such as LC-UV or LC-MS, is used to trigger LC-NMR data acquisition. In practice, one of these detectors is connected on-line before the NMR instrument, and the signal of the analyte of interest passing through this detector is used to trigger a valve, which will stop the LC flow exactly when the peak reaches the NMR cell after a calibrated delay. The stop-flow mode allows the acquisition of a larger number of transients than in the on-flow mode for any given LC peak, thereby providing satisfactory LC-^{1}H NMR spectra of compounds present in the low-microgram range. The solvent suppression is of a better quality than in the on-flow mode. With a standard LC-NMR flow probe (60 µl on a 500 MHz), the detection limits are about 20 µg of NP injected on-column in the on-flow mode, but they can reach the ng range when LC-NMR is used at-line with sensitive microflow probes [32].

Due to the inherent low sensitivity of LC-NMR, the on-column loading for LC-NMR is of several orders of magnitude higher, compared with LC-UV or LC-MS, for delivering the highest amount of separated analyte in the lowest elution volume possible into the LC-NMR cell [34]. For crude plant extract analysis, for example, the amount injected on-column can be of several milligrams. To cope with these important sample loadings, long HPLC columns (250 mm) or columns with large inner diameter (8-10 mm) are used for standard LC-NMR measurements. Due to the poor solubility of some extracts, dissolution of the sample in deuterated dimethylsulfoxide is often required for injection of such quantities on-column. As a consequence, specific chromatographic conditions have to be developed for LC-NMR to separate large amounts of sample with satisfactory LC resolution and maintain flow conditions compatible with the detection cell used.

LC-NMR, in the on-flow mode, can provide ^{1}H-NMR spectra of the main constituent of a crude extract rapidly without the need of complex automation. This type of information, in addition to other on-line spectroscopic data (e.g., PDA and MS), may ascertain the structural determination and facilitate the dereplication process. Examples of on-flow LC-NMR analyses for crude extract profiling are manifold (e.g., for flavonoids, alkaloids, terpenes, carotenoids) [32,33,38], although a relatively limited number of NPs, essentially only the very major ones, are detected in this way. The technique is also extremely useful for detecting compounds that are labile or might epimerise or interconvert as a result of their isolation [45].

As shown in the case of the on-line determination of main alkaloids of *Erythroxylum vacciniifolium* on-flow conditions enabled the recording of ^{1}H-NMR spectra of the main constituents **24** (Fig. **3B**); however, minor compounds were not detected in this way. In this case, stop-flow analysis of the minor compound **7** was performed and a good quality of ^{1}H NMR spectra was obtained (Fig. **3C**) [46].

2.4.2. Alternative Methods SPE-NMR and CapNMR

As shown in Fig. (**4**), one possibility for overcoming the limitation of on-flow LC-NMR (Fig. **4A**) is to use preconcentration methods prior to NMR detection. This involves performing NMR detection at-line after HPLC separation with either trapping the LC-peaks on SPE (LC-SPE-NMR) [13, 47, 48] or with HPLC microfractionation, drying and reinjection of the concentrated LC-peak in deuterated solvent in micro-flow capillary LC-NMR probes (CapNMR) [14, 37, 49].

LC-SPE-NMR has become very popular for a further increase in LC-NMR detection. Indeed, the advantage of this technique is

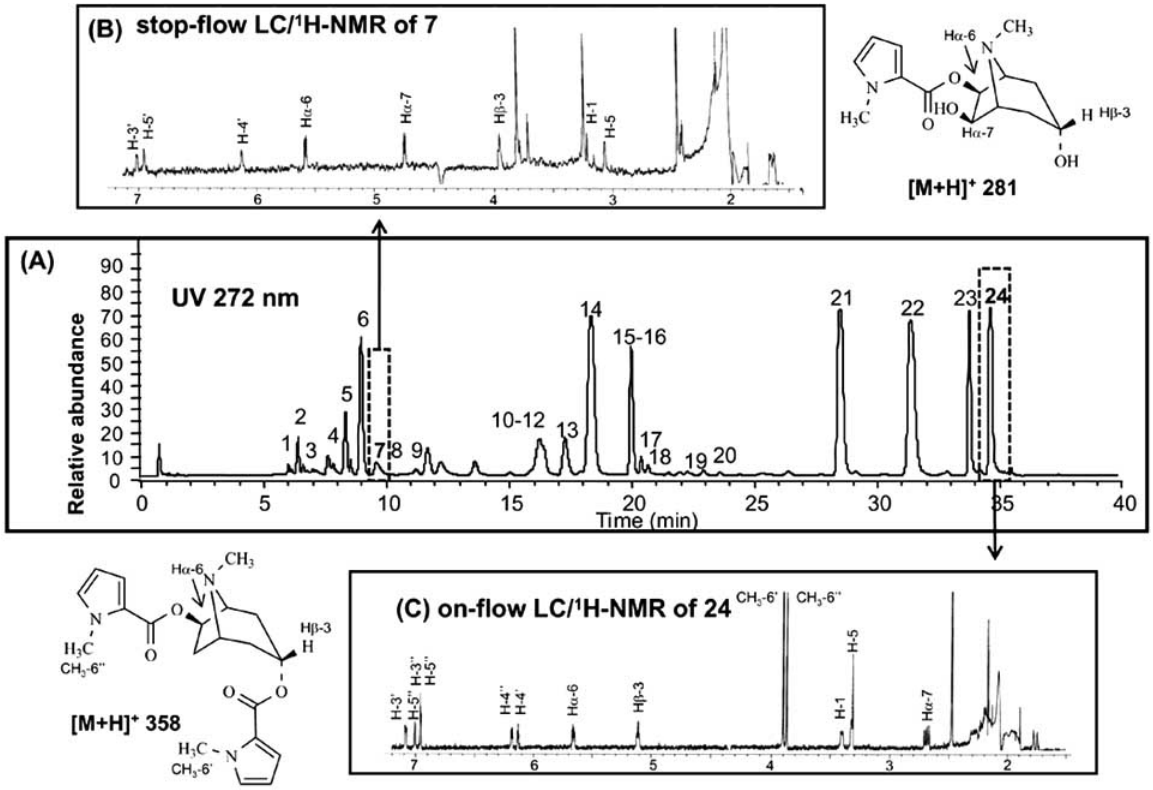

Fig. (3). (**A**) LC-UV trace (272 nm) of the alkaloid extract of *Erythroxylum vacciniifolium* (Erythroxylaceae). Amount injected: 3 mg. HPLC conditions: C-18 column, Macherey-Nagel CC Nucleodur 100-5 (125 mm 8.0 mm i.d.); MeCN (2 mM NH_3):D_2O (2 mM NH_3 (5: 95–100: 0; 80 min) gradient including five isocratic elution steps; 1.2 ml/min. LC–NMR conditions: 32 scans/increment (on-flow); 60 µl flow cell (3 mm i.d.); 500 MHz. (**B**) stop-flow LC/^{1}H-NMR of minor compound 7 ; (**C**) on-flow LC/^{1}H-NMR of the major compound 24. Adapted from [46]. Copyright (2009) with permission from Elsevier.

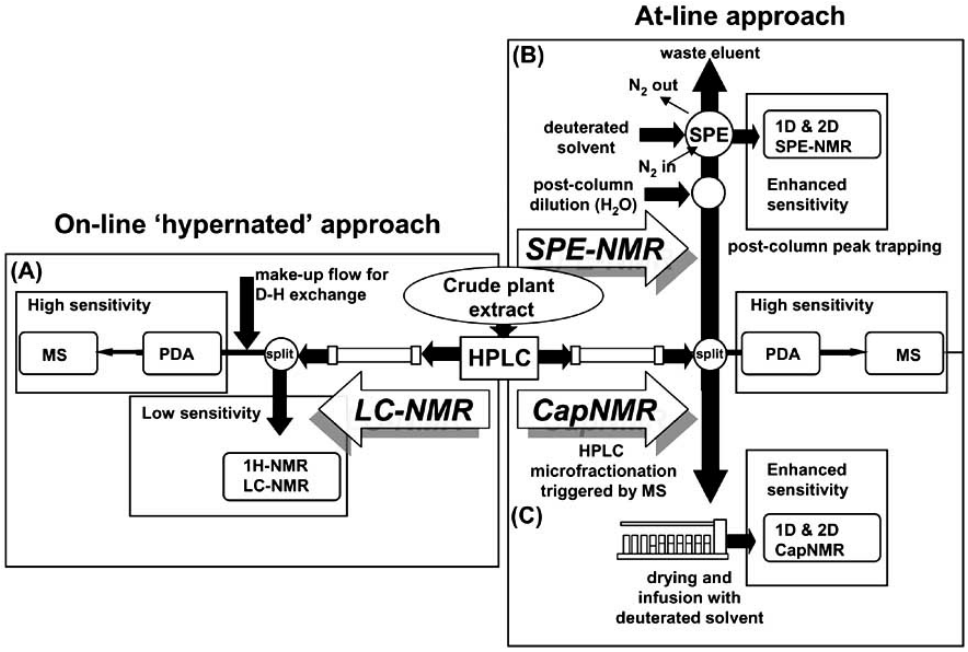

Fig. (4). Summary of the different ways to hyphenate NMR with HPLC either on-line or at-line. (**A**) On-flow LC-NMR in a hypernated system with PDA and MS detection. (**B**) LC-SPE-NMR with post-column addition of H_2O for trapping drying and reinjection in a LC-NMR flow cell. (**C**) CapNMR detection after HPLC microfractionation.

that it is able to couple NMR with on-line SPE, which is a powerful technique for reproducible, rapid and selective preparation. In LC-SPE-NMR, the separated peaks are diluted post-column with water and trapped automatically on SPE [15]. After a drying step with nitrogen to remove all solvents used for chromatographic separation, the analytes are transferred with the deuterated solvent of

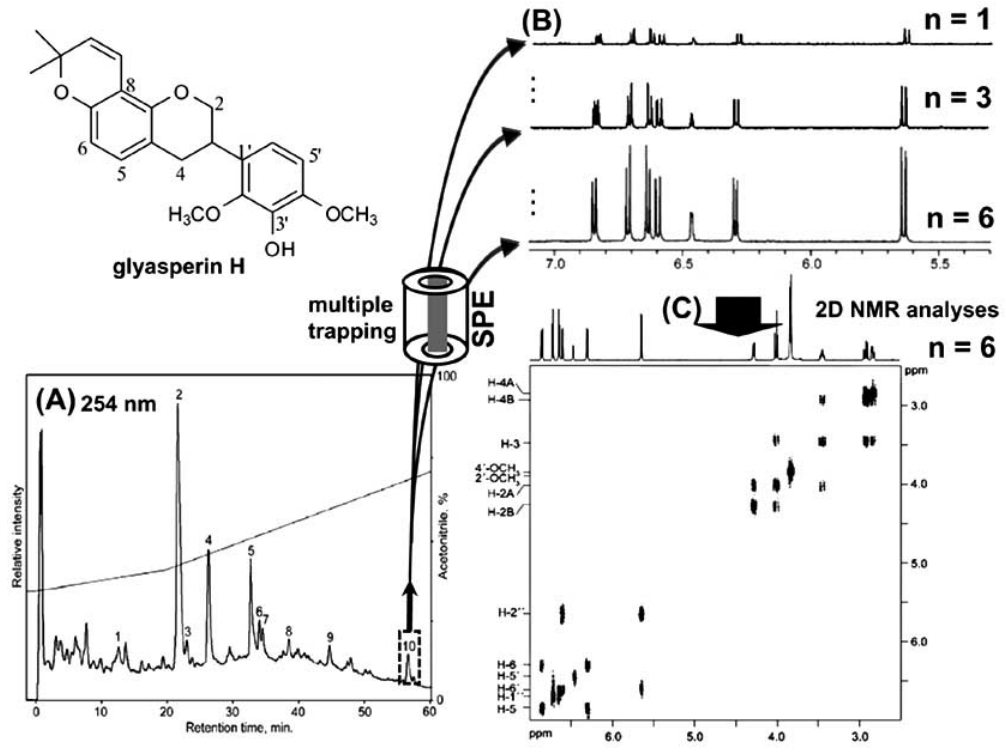

Fig. (5). (**A**) Chromatogram of the ethanolic extract of *Smirnowia iranica* roots on a C_{18} column; acetonitrile gradient profile in waters at 254 nm. (**B**) multiple trapping of peak 10 on LC-SPE-NMR. (**C**) COSY spectrum with peak 10 after 6 trappings. Adapted from [53]. Copyright (2005) American Chemical Society.

choice to the NMR flow-cell probe (Fig. **4B**) [50]. This technique presents the advantage that the separation can be carried out with normal protonated solvent and, moreover, multiple trapping of the same analyte from repeated LC injection circumvents problems of low concentration. The operation can be fully automated on state-of-the-art systems, but a good optimisation of the trapping conditions is required for optimum performances [48, 51]. In this respect, the use of either semi-preparative or analytical columns with and without multiple trapping has been investigated [52]. It was found that the choice of the optimum separation strategy is mainly related to the polarity of the analyte of interest.

An example of the effect of multiple trapping on sensitivity is illustrated using the analysis of isoflavonoids from *Smirnowia iranica* in Fig. (**5**). The ^{1}H-NMR spectrum of glyasperin H (peak 10), a representative compound present in small amounts, is presented. As is shown, up to six repeated trappings of peak 10 resulted in a linear increase of the signal-to-noise ratio in the NMR spectra (Fig. **5B**) which enable the recording of informative 2D NMR spectra such as the ^{1}H-^{1}H COSY spectra (Fig. **5C**) [53].

Compared to stop-flow LC-NMR, the increased sensitivity provided by analyte focusing multiple trapping and the advantage of fully deuterated solvent operation, give the ability to perform the full set of 2D correlation experiments on the analytes, including HMBC, in a reasonable analysis time. A typical LC-SPE-NMR, which would need a full set of 2D spectra, will require about 120 µg in a 60 µl cell on a 600 MHz equipped with a room temperature probe head. For a secondary metabolite (MW < 700 Da), a series of high-quality homonuclear and heteronuclear 2D spectra were recorded overnight. Single ^{1}H-NMR spectra can be obtained with about 10 nmol of substances within a reasonable timeframe [54].

The sensitivity of the flow cell can be enhanced by using a cryogenated probe by a factor of roughly 3 to 4 [47]. For LC-NMR, cryofit inserts for the adaptation of 30 µl probes in a 3 mm i.d. inverse cryoprobe have been successfully used for the analysis of very limited amounts of plant material [55].

One of the last improvements in flow cell design has been the introduction of reduced-diameter solenoid NMR coils (capillary NMR: CapNMR), which represents a particularly attractive approach to enhance NMR sensitivity for mass-limited samples [14]. Similar to LC-SPE-NMR, CapNMR represents another alternative for the indirect hyphenation of NMR with HPLC. For analysing extracts, the same microfractionation procedure as that described for LC-SPE-NMR can be used; however, in this case, the LC peaks are not trapped on SPE cartridges but simply collected into vials or in 96-well plates and dried usually by speedvac evaporation. Each sample can be dissolved in a volume slightly exceeding the volume of the flow cell (6.5 µl for a 5 µl flow cell volume), filtered, injected into the probe, and parked in the centre of the microcoil by pushing it with an adjusted push volume (typically 8-10 µl) (Fig. **5C**) [56, 57]. The disadvantage of the work at-line is that more sample handling is needed compared with the on-flow approach. However, NMR spectra of higher quality are obtainable in standard deuterated solvents at low cost, whereas other information such as UV and MS data can be obtained on-line during the microfractionation. Automation for direct infusion of 10-µl sample from a low-volume 384-well plate has recently been demonstrated [58] and has been optimised for injection volumes in the microliter scale for LC-MS-CapNMR plateform using the microdroplet NMR approach [37]. This type of approach is also very important for checking the chemical integrity of natural products in a high-throughput pharmaceutical screening program. These at-line measurements also have the considerable advantage of providing spectra recorded in standard deuterated solvents with the highest possible concentration for a given amount of compound. Furthermore, access to ^{13}C-NMR

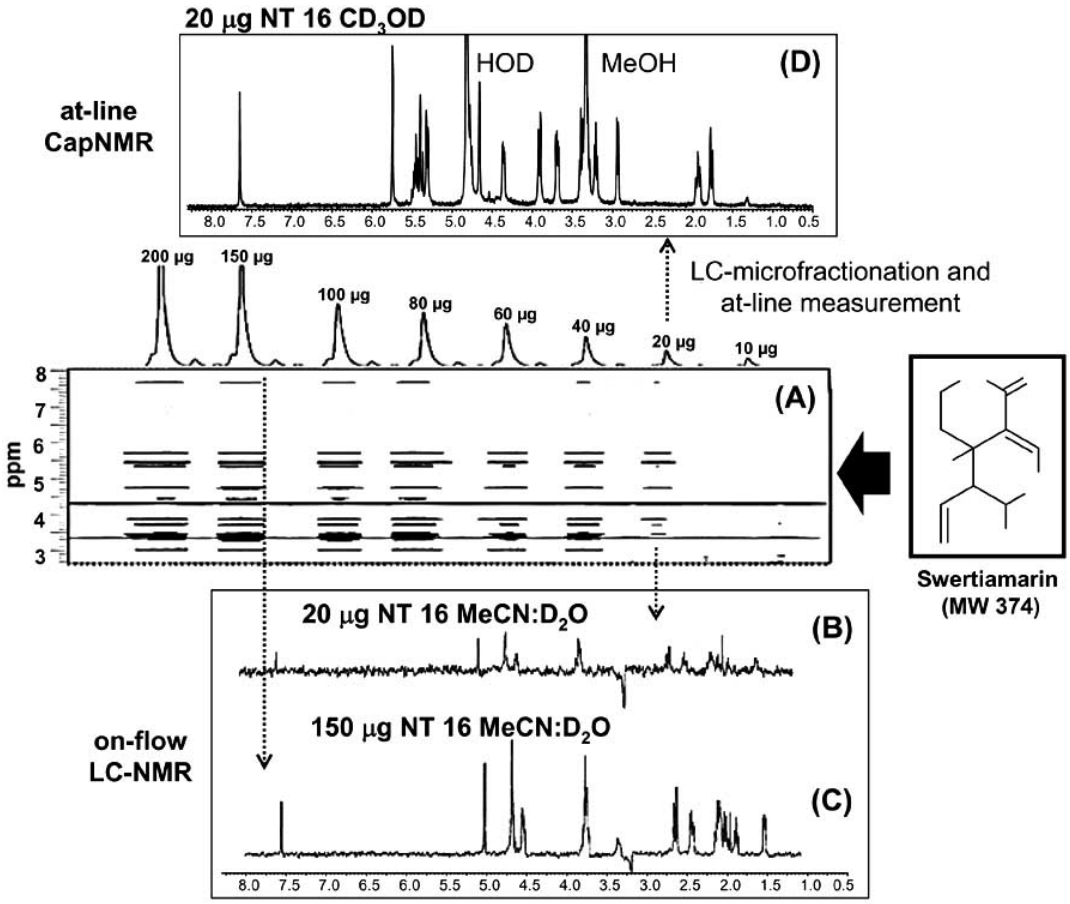

Fig. (6). Estimation of sensitivity and the quality of NMR spectra recorded either by on-flow LC-NMR (500 MHz, flow cell 60 µl active volume) or at-line CapNMR (500 MHz, flow cell 1.5 µl active volume) by injection of different amount of swertiamarin (2) always with the same number of transients (NT=16). Adapted from [32]. With the permission of Taylor & Francis Group.

information deduced indirectly from inverse detection (HSQC and HMBC) experiments is feasible, providing detailed structural information for *de novo* structural determination of natural products.

An example of the type of spectra that can be recorded by CapNMR for swertiamarin, a secoiridoid of molecular weight 374, after microfractionation is illustrated in Fig. (**6D**) and compared to that obtained by repeated injections at different concentration in on-flow LC-NMR (Fig **6A** on-flow 2D plot). With a standard LC-NMR flow probe (60 µl on a 500 MHz), the detection limits were about 20 µg on-column in the on-flow mode (Fig. **6B**), whereas a spectrum of good quality was obtained with about 150 µg (Fig. **6C**). If the same amount of compound (20 µg) was injected in a volume corresponding to that of a CapNMR probe (5 µl on a 500 MHz), a much better S/N ratio was obtained thanks to the probe design and the effect of the increase in concentration (Fig. **6D**).

With an automated microflow NMR platform, interpretable ^{1}H-NMR spectra were obtained from analytes at the 200 ng level in 1 h/well [37]. With a more classical CapNMR approach, typically, a few tenths of µg (depending on the magnet strength and MW) are needed to obtain a full set of 2D correlations overnight. On a 600 MHz system, on a routine basis, COSY NMR spectra were reported to be acquired with less than 10 µg in ca. 1 hour, 30 µg were needed for HSQC in 5 hours, and 70 µg for HMBC in ca. 15 hours. Direct ^{13}C-NMR data can already be obtained with 200 µg of NPs [57].

3. PROFILING WITHOUT HYPHENATION

With the important development of multivariate data analysis techniques, the spectroscopic methods used for HPLC detection (mainly NMR, IR and MS) are more frequently applied to the analysis of crude extracts without prior HPLC separation for a rapid evaluation of the composition, especially in the quality control of herbal medical products and metabolomics [59].

3.1. Direct NMR Profiling

The profiling of many crude extracts can be performed by direct NMR analysis. This method is simple, comprehensive, reproducible for both long and short term studies and requires only very limited sample preparation, it has been applied for many applications related to quality control and metabolomics.

The ^{1}H-NMR spectrum provides a wealth of chemical information regarding a crude extract. With the aid of chemical shifts and coupling constants, some metabolites can be easily identified. The observed chemical shift positions and spin-spin coupling pattern for each proton provide information regarding the types of protons 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 integration of the signals in the spectra. There is no need for calibration curves to convert signal intensity into concentration, as is used in other methods [60]. For reproducible measurements, only a few parameters need to be op-

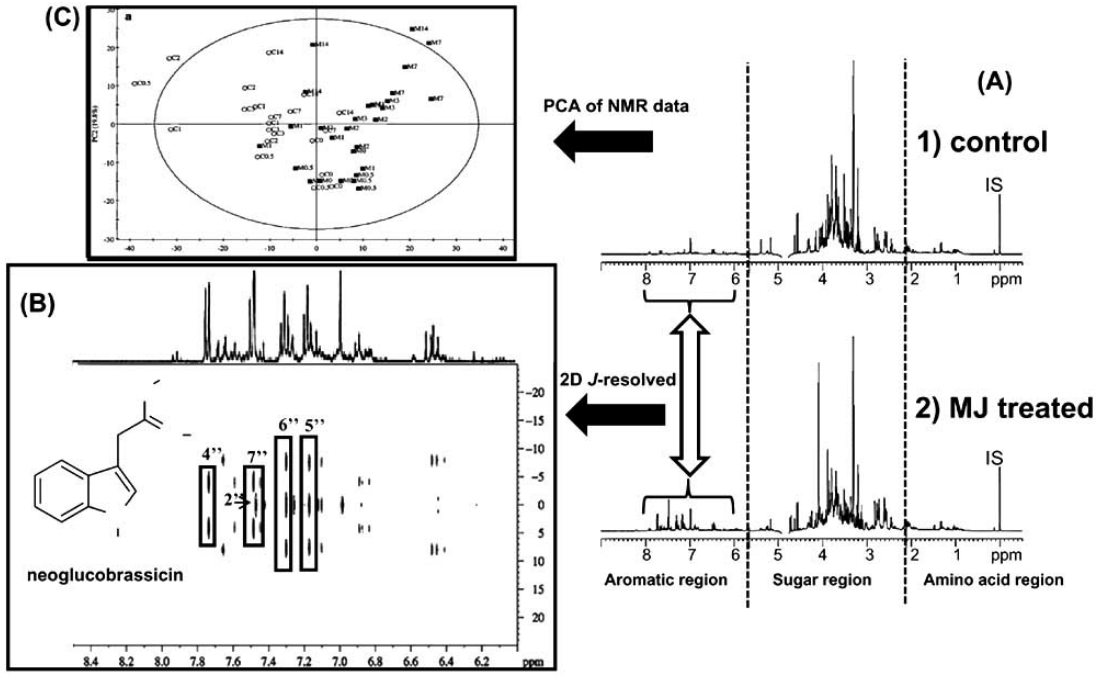

Fig. (7). (**A**)^{1}H NMR spectra of methanol-d4/D_2O extraction of control (1) and MJ treated (2) *Brassica rapa* leaves (14 days after treatment). IS, internal standard (TSP). (**B**) *J*-resolved NMR spectra of MJ treated *Brassica rapa* leaves in the region of δ 8.5–6.0 and peak assignments of neoglucobrassicin. (**C**) Score plot of PCA of *J*-resolved NMR data of (*Brassica rapa*) Control plants are shown as open circle (○) and MJ treated plants are shown as solid box (▪). The number after symbol shows the time (day) after MJ treatments. Adapted from [162]. Copyright (2009) with permission from Elsevier.

timised: relaxation delay, pulse width, acquisition time and suppression of residual water signal. A single deuterated solvent or a combination of them are sometimes used with a buffer for sample extraction. For example, a mixture of methanol-d4 and KH_2PO_4 buffer in D_2O (pH 6.0) have been reported to extract a wide range of metabolites including amino acids, carbohydrates, fatty acids, organic acids, phenolics and terpenoids in a single step [61].

When samples are compared either in metabolomics or quality control assessment studies, MVDA is a key step for sorting the discriminating signals from the complex spectra. Prior to data mining, the spectra have to be digitalised by a procedure called bucketing, which divides the spectrum into small bins (typically bins of 0.02 to 0.04 ppm) and sums all intensities in each bin [60]. Full-resolution NMR data can also be used for MVDA, which gives more precise information about the constituents responsible for clustering [62]. Because fluctuations in chemical shifts can be recorded, even with a fixed temperature and pH [63], alignment methods have been developed to improve the separation of NMR data for further statistical analysis [64].

Direct NMR profiling is very powerful for recording important changes in composition within a mixture. One of the main drawbacks of the approach is related to the intrinsically low sensitivity of NMR, and such approaches are not well adapted if minor changes in metabolite composition have to be assessed.

The interesting work of Verpoorte and co-workers on the metabolic changes of *Brassica rapa* (Brassicaceae) leaves after methyl jasmonate (MJ) treatment exemplify this metabolomic strategy [61]. Application of *J*-resolved ^{1}H-NMR (Fig. **7B**) on crude CD_3OD-D_2O extracts combined with multivariate data analysis (Fig. **7C**) allowed for detecting differences in metabolite profiles between controls and MJ-treated *B. rapa* (Fig. **7**). The *J*-resolved ^{1}H-NMR experiments have the advantage over standard direct ^{1}H-NMR that *J* coupling constants are displayed in the second dimension (Fig. **7B**) and the 1D trace of this 2D experiment is simplified showing only single peaks for each proton without their multiplet patterns. An enhanced resolution it thus provided. In this study as shown in Fig. (**7A**), major changes in the aromatic region of the NMR spectra recorded on *B. rapa* after 14 days of MJ treatment clearly appeared. All NMR signals that were affected were highlighted based on the MVDA treatment and the corresponding score plot obtained (data not shown) [61]. Additionally, 2D NMR data provided structural information on the metabolite changes produced by MJ treatment, involving a wide range of compounds such as glucosinolates, hydroxycinnamates, sugars and amino acids. Quantitative analyses of few compounds was also possible using the relative peak area of corresponding signals to that of an internal standard (TSP: trimethyl silyl propionic acid sodium salt). This direct-NMR method applied for a crude plant extract demonstrates the wide applicability of an integrated fingerprinting approach without hyphenation.

3.2. Direct MS Fingerprinting

If a more sensitive detection of individual metabolite is required, profiling of crude extracts can also be performed by direct mass spectrometry (DIMS) methods, either by infusion of the mixture in a LC-MS interface like ESI or by application of desorption techniques directly on the biological matrix such as Matrix-Assisted Laser Desorption/Ionisation (MALDI) or Desorption Electrospray Ionisation DESI. These methods provide total mass spectra (TMS) of the extracts. In the datasets recorded, the ions need to be separated with high resolution to obtain a very detailed profiling since no orthogonal dimension is provided by orthogonal methods such HPLC. Thus, in addition to TOF-MS, which is mainly used for LC-MS, high-resolution (HR) MS instruments are also used for direct MS fingerprinting without separation. In this respect, mass spectrometers such as Orbitrap (resolution of 100'000, accuracy <

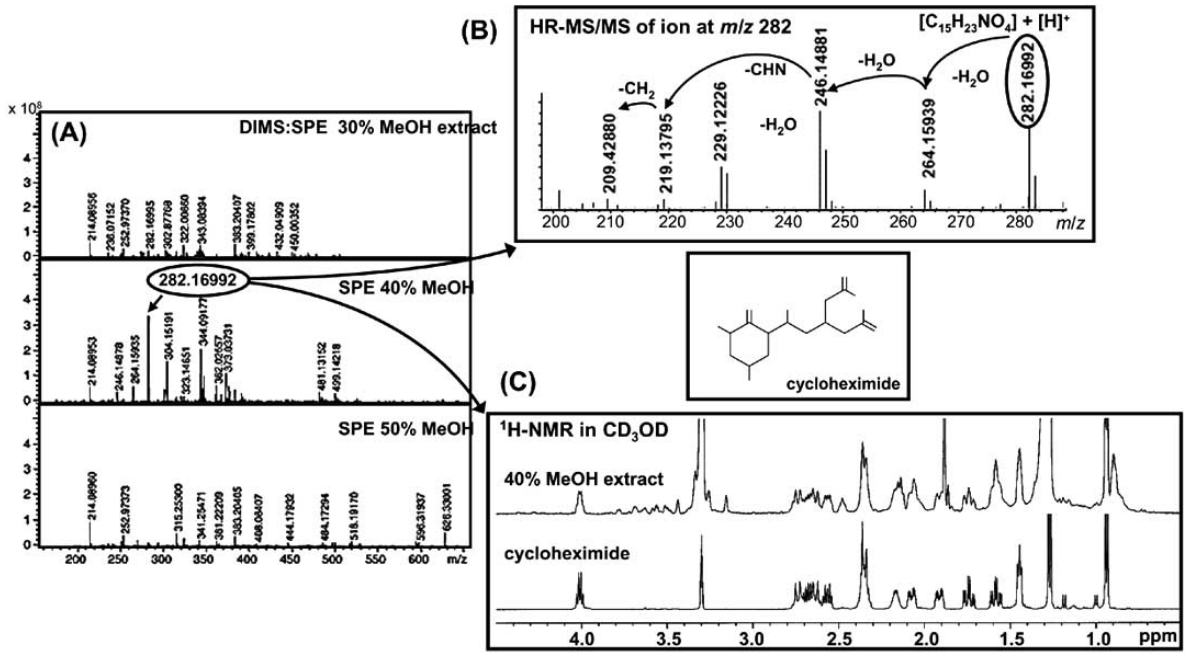

Fig. (8). (**A**) PI ESI-FT-ICR-MS spectra for three methanolic water fractions (from bottom: 30%, 40%, 50% MeOH). Dominant peak *m/z* 282.1699 in fraction 40% is indicated by an arrow. (**B**) Collision induced dissociation (CID) of the isolated mass 282 with corresponding neutral losses which correspond to CID fragmentation of commercial standard cycloheximide. (**C**) Comparison of ^{1}H NMR spectra of cycloheximide standard and the bioactive culture supernatant fraction obtained from eluting with 40% aqueous methanol. Adapted from [68] Copyright (2009) with permission from Elsevier.

1ppm) or Fourier Transform Ion Cyclotron Resonance (FTICR) (resolution > 100'000, accuracy < 1ppm), although expensive, are now becoming reference instruments for numerous studies.

DIMS enables rapid and high-throughput screening of hundreds of samples, mainly for metabolite fingerprinting in metabolomic studies. The speed of the analysis and the greater sensitivity make DIMS a valuable tool compared to direct-NMR fingerprinting. However, DIMS has limited quantification capabilities and metabolite identification is difficult based on accurate mass alone, particularly for isomeric compounds. Another drawback comes from the ionisation suppression effect, which arises from competitive ionisation with other components in the matrix, such as ionic compounds, charged organic compounds, organic acids/bases and hydrophobic compounds. DIMS analyses have to be done in both PI and NI modes to obtain a representative fingerprint of the sample.

Politi and co-workers compared the direct-NMR and direct-MS profiling for quality control of herbal medicinal tinctures. ^{1}H-NMR spectra of an out-of-date *Echinacea purpurea* (Asteraceae) tinctures and that of a new one present almost the same profile. However, the old batch showed a characteristic broadening of the proton signals that could be related to degradation phenomena of the plant metabolites. DIMS provided valuable information in PI mode which reveals major differences between *m/z* 500 to 750, while in NI mode, mass spectra showed the same pattern [65]. This study demonstrates that none of the direct methods are able to describe the entire metabolic profile of a complex natural product mixture. However, each detector presents their own advantages and limitations; consequently a combination of both represents a valuable approach for obtaining a faithful fingerprint.

Another original MS method is MALDI-TOF, which has been used for direct profiling without a separation step on several different samples [66]. Analysis of *A. thaliana* leaves resulted in the detection of 108 monoisotopic peaks; among them, 56 were identified with a limit of detection of a few picomol.

DIMS is mainly used to obtain a fingerprint of the metabolome and represents a valuable tool for phenotyping studies. An interesting work by Oikawa and co-workers which discriminated *A. thaliana* samples treated with different herbicidal chemical classes illustrates this strategy [67]. Methanol extracts were directly injected in a FTICR-MS detector. This allowed separation of treated samples after mass error correction and statistical analysis. Moreover, the extensive use of a database (KNapSAcK) and MS/MS structural analyses allowed the identification of a few candidate metabolite markers. The Kanaya group used the same metabolomic strategy to analyse the metabolic profile of different growth stages in *E.coli*. The direct injection in FTICR-MS and further MS/MS experiments in NI mode allowed the assignment of 72 metabolites based on KNapSAcK prediction.

These high-resolution methods have been applied by the Haesler group to study interactions between the oomycetous root rot pathogen *Phytophtora citricola* and its antagonistic microorganism *Kitasatospora* sp [68]. Three MeOH/H_2O extracts of a *Kitasatospora* culture (30%, 40%, 50% methanol) were profiled by DIMS with a FTICR-MS. Inhibition of *P. citricola* could only be observed for the 40% methanol/water fraction (Fig. **8A**), while all other fractions were inactive. A rapid separation through an SPE column (increasing stepwise by 10% up to 100% methanol, 1 mL for each fraction) and further bioassays led to the isolation of the active fraction. The FTICR-MS spectra of this fraction showed three major peaks at *m/z* 282.1699, *m/z* 304.1519 and *m/z* 344.0918, not or scarcely present in the other fractions. The signal at *m/z* 282.1699 showed the highest intensity and peak *m/z* 304.1519 corresponded to the sodium adduct of this mass (Fig. **8A**). ChemIDplus database interrogation showed 31 possibilities for the ion at *m/z* 282 and no substance for *m/z* 344.0918. Further MS^2 experiments demonstrated the same fragmentation pattern as cycloheximide commercial standard (Fig. **8B**). As the mass analysis and the inhibition test indicated cycloheximide to represent the biocontrol active substance,

^{1}H-NMR pattern of the reference cycloheximide was compared to a methanol-d4 microbial extracts and confirmed the presence of cycloheximidine in the crude extract (Fig. **8C**).

4. TYPE OF STUDIES PERFORMED USING PROFILING METHODS

As shown, different complementary profiling methods exist in combination or not with chromatographic methods. They have all been applied to crude plant extracts or complex biological mixtures of natural product from other origins. Most of the profiling studies can be classified as one of three main types: dereplication, quality control or metabolomics.

Currently, two complementary approaches are predominantly used: metabolite profiling and metabolite fingerprinting. For the latter, the intent is not to identify each observed metabolite, but to compare patterns or 'fingerprints' of metabolites that change from a given type of sample to another. Metabolic profiling focuses on the analysis of a group of metabolites either related to a specific metabolic pathway or a class of compounds [3].

4.1. Dereplication

Dereplication is the process of differentiating those natural product extracts that contain nuisance compounds, or known secondary metabolites, from those that contain novel compounds that are of interest [69]. Such a process represent an important step in drug discovery programs because the early structural determination of known natural products avoids time consuming isolation, rationalisation and optimisation of bioactive guided isolation procedures [70].

The dereplication methods based on the use of LC-MS and LC-MS/MS are very sensitive and provide structural information (low or high-resolution molecular mass and molecular formula), which is searchable in most commercial databases, such AntiBase, Dictionary of Natural Products, MarinLit, KNapSAcK and, with limitations, SciFinder Scholar and STN International (CAPlus)). The disadvantages of these methods, which can lead to false compound identification, are the uncertainty regarding the apparent molecular ion species, which cannot be easily attributed to adduct ions (see for example Fig. **2**) or the presence of interfering ions from minor components that ionise more readily than the component of interest [69].

In many cases, cross-searching with chemotaxonomical information helps to reduce the number of possibilities. To ascertain the structural assignment complimentary LC-PDA and/or LC-NMR data are often needed [71], and *de novo* structure determination can be obtained mainly based on at-line NMR methods [56].

4.2. Quality Control and Standardisation

Crude plant extracts sold as phytopharmaceutical or nutraceutical require that their composition is assessed with precision and kept constant. For this, a usual way to standardise an extract is to quantify the active(s) principle(s). Often, however, the active principles are not clearly defined and the standardisation can be made on a compound characteristic of a given plant, which serves as a marker but is not directly linked to the biological activity of the extract.

In order to obtain an overall idea of the composition, several new methods of standardisation of crude extracts involve metabolite profiling or fingerprinting in combination or not with MVDA (metabolomics). Such methods enable definition of the degree of similarity between extracts (phytoequivalence). They typically involve LC-UV methods with peak identification based on LC-MS [22] or direct NMR profiling methods [72].

4.3. Metabolomics

The term metabolomics can be considered as a large scale analysis of metabolites in given organisms in different physiological states. It has recently emerged with other 'omics' technologies in biological research [73] and represents an important aspect of systems biology [5]. Indeed profiling the metabolome may actually provide the most functional information of the 'omics' technologies [74] by giving a broad view of the biochemical status of an organism that can be used to monitor significant variations of metabolites that are the end products of the cellular regulatory processes. With the recent important development of analytical methods and data mining, metabolomics has rapidly evolved to provide a global picture of molecular organisation in plants at the metabolite level. In plant science, a very important increase in studies related to plant metabolomics has been recoded over the last years [75]. In phytochemistry and phytotherapy, metabolomics can be used for classification and characterisation of different species of medicinal plants for quality control purposes, especially when the active principles are unknown. Such methods may also provide a better understanding of the mode of action of herbal medication in relation to systems biology [76]. Further reports have confirmed the use of this approach to tackle issues related to the localisation of active principles in medicinal plants by a combination of metabolic profiling and multivariate analysis [77].

5. STUDIES ON NATURAL PRODUCTS USING LC-PDA-MS AND UHPLC-PDA-MS

LC-MS covers the ionisation of the majority of NPs, provided that appropriate conditions are used. Of course, the applications to NPs are manifold, and more details can be found in dedicated reviews [22, 23, 78, 79]. Concerning the detection of NPs in biological fluids, an interesting review covers the aspects related to sample preparation and quantification of flavonoids, alkaloids, saponins and sesquiterpenoids [80].

In Table **1**, the most recent applications for metabolite profiling and fingerprinting using LC-MS or direct MS methods are summarised. As shown, this approach is widely used in the fields of dereplication, quality control and metabolomics.

5.1. Dereplication

Dereplication by LC-MS has been successfully applied to several classes of natural products including flavonoids [81], polyphenols [82], polyprenylated acylphloroglucinols [83], polyprenylated xanthones [84] and anthocyanins [85]. These methods usually involve the hyphenation of LC-PDA for complementary structural information related to the characteristic chromophore of various NPs. When metabolite identification is needed, extensive use of UV profiles with MS/MS fragmentation patterns and/or HRMS spectra and subsequent database interrogations represent the most common strategy.

Parallel use of multiple detectors and detection modes can adequately reveal the complexity of a crude extract. Mohn and coworkers have used various hyphenated techniques to establish a comprehensive metabolic profile of *Isatis tinctoria* (Brassicaceae). Both dichloromethane and methanol fractions have been profiled

Table 1. Recent Application (2006-2009) of Metabolite Profiling and Fingerprinting Using LC-MS or Direct MS

Metabolite Identification/Dereplication				
Source	***Application***	***Method***	***Instrumentation (Ionization mode)***	***Ref.***
Glycine max	flavonoid profiling	LC-MS/MS	ESI-QLITMS (+)	[90]
Lotus japonicus	flavonoids profiling	LC-MS-MS/MS	ESI-FTICRMS (+)	[91]
Arabidopsis thaliana, Lupinus angustifolius	flavonoid glycosides profiling	$LC\text{-}MS^n$	ESI-ITMS (+/–)	[92]
Medicago truncatula	polyphenol profiling	LC-UV-MS/MS, LC-MS, GC-MS	ESI-ITMS (+/–), ESI-QTOFMS (+), EI-QMS	[81]
Lychnophora sp.	polyphenol profiling	LC-PDA-MS-MS/MS	ESI-QTOFMS (+/–)	[82]
Garcinia xipshuanbannaensis	xanthones profiling	UHPLC-MS/MS/MS	ESI-QTOFMS (+)	[84]
Tropaeolum tuberosum	anthocyanins profiling	LC-MS/MS	ESI-QQQMS	[85]
Colchicum crocifolium	colchicinoids profiling	LC-UV/PDA, LC-MS	APCI-ITMS (+)	[120]
Garcinia sp.	Phloroglucinols derivatives profiling	UHPLC- MS/MS	ESI-QTOFMS (+)	[83]
Lychnophora ericoides	metabolite profiling	LC-PDA-MS-MS/MS	ESI-QTOFMS (+/–)	[121]
Aspergillus parasiticus, Aspergillus flavus	metabolite profiling	LC-MS	ESI-TOFMS (+)	[122]
Arabidopsis thaliana, Drosophila melanogaster, Acyrthosiphon pisum, and human blood	metabolite profiling	MALDI-MS	MALDI-TOFMS (–)	[66]
Isatis tinctoria	metabolite profiling	LC-PDA-MS, LC-MS/MS, LC-ELSD	ESI, APCI-TOFMS (+/–), ESI-ITMS (+/–)	[86]
Curcuma domestica	metabolite profiling	CapHPLC-MS/MS, LC-MS/MS, GC-FID, GC-MS, HR-GC-MS	ESI-OTFTMS (+), ESI-QQQMS (–), EI-QMS, EI-MMS	[87]
Quality assessment				
Angelica acutiloba	metabolite fingerprinting	UHPLC-MS	ESI-TOFMS (+/–)	[123]
Panax sp.	saponins profiling	UHPLC-MS/MS, LC-MS	ESI-QTOFMS (+)	[124]
Panax ginseng	saponins profiling	LC-UV-MS	ESI-ITMS (–)	[95]
Camelia sinensis	flavonoids profiling	LC-MS	ESI-ITMS (+/–)	[125]
Zingiber officinale	gingerols profiling	LC-MS/MS	ESI-ITMS (+/–)	[98]
Naodesheng (TCM)	metabolite profiling	LC-UV-MS	APCI-QMS (–)	[96]
Danshen dropping pill (TCM)	metabolite profiling	LC-UV-MS	ESI-QMS (–)	[100]
Gan-Lu-Yin (TCM)	metabolite profiling	LC-UV-MS	ESI-QMS (+), ICPMS	[99]
Metabolomics: Stress/interactions				
Arabidopsis thaliana	oxylipin profiling	UHPLC-MS	ESI-TOFMS (–)	[25]
Arabidopsis thaliana	metabolite profiling	Direct MS	ESI-FTICRMS (+/–)	[67]
Arabidopsis thaliana	metabolite profiling	Direct MS, UHPLC-MS	ESI-TOFMS (–)	[101]
Arabidopsis thaliana	metabolite fingerprinting	LC-MS	ESI-TOFMS (–)	[126]
Arabidopsis thaliana	metabolite profiling	LC-MS, GC-MS	ESI-ITMS (+), EI-QMS	[127]
Medicago truncatula	metabolite profiling	LC-MS, GC -MS	ESI-FTICRMS (+), EI-TOFMS	[128]
Medicago truncatula	flavonoid profiling	LC-UV-MS	ESI-QTOFMS (+)	[129]
Stereum hirsutum, Coprinus micaceus and C. disseminatus	metabolite profiling	GC-MS	EI-TOFMS	[130]
Meatbolomics Genotype/taxonomy				
Taxus sp.	metabolite fingerprinting	LC-MS	ESI-QMS (+ / –)	[131]
Arabidopsis thaliana	flavonol profiling	UHPLC-PDA-MS-MS/MS	ESI-QTOFMS (+)	[132]
Citrumelo, Carrizo citrange, Cleopatra mandarin	metabolite fingerprinting	GC-MS, LC-MS	EI-ITMS, ESI-QTOFMS (+ / –)	[133]
Drosophila melanogaster	metabolite profiling	LC-MS	ITFTMS (+)	[134]
Wines	anthocyanins profiling	Direct MS	ESI, APPI-QTOFMS (+)	[135]

Table 1. contd…

Metabolite Identification/Dereplication				
Source	***Application***	***Method***	***Instrumentation (Ionization mode)***	***Ref.***
Arabidopsis thaliana	metabolite profiling	CapHPLC-MS-MS/MS, Direct MS	ESI-QTOFMS (+), ESI-FTICRMS (+)	[136]
Brassica oleracea	metabolite fingerprinting	LC-PDA-MS, Direct MS	ESI-QMS (+/–)	[137]
Escherichia coli	metabolite profiling	Direct MS	ESI-FTICRMS (–)	[138]

ESI: electrospray ionization; EI: elcetronic impact; MALDI: matrix-assisted laser desorption/ionization; ICP: inductively coupled plasma; APCI: atmospheric pressure chemical ionization; Q: quadrupole; QQQ: triple quadrupole; QTOF: quadrupole time of flight; IT: ion trap; OT: orbitrap; FTICR: fourier transform ion cyclotron resonance; QLIT: quadrupole-linear ion trap; M: magnetic sector; (+): positive mode ionization; (–) negative mode ionization; ELSD: evaporative light scattering detector; FID: flame ionization detector; LC: high performance liquid chromatography, UHPLC: ultra high performance liquid chromatography; CapHPLC: capillary HPLC; GC: gas chromatography UV: ultra violet; PDA: photodiode array; TCM: traditional chinese medicine

through a combination of several detectors such as PDA, ESI-MS and APCI-MS in both PI and NI modes. The parallel use of the unspecific evaporative light scattering detector (ELSD) ensured that all separated compounds were assigned to at least one mass spectrometric detection mode. Additional structural information was obtained by MS/MS experiments and by high-resolution mass spectra recorded by ESI-TOF-MS. More than 65 compounds belonging to various structural classes such as alkaloids, flavone glycosides, glucosinolates fatty acids, porphyrins, carotenoids, lignans, cyclohexenones and phenylpropanoids were identified [86]. Another broad metabolic profile of the widely used Turmeric (*Curcuma domestica*) has been established for both polar extract (methanol) and non-polar extract (hexane) by extensive use of GC-HRMS and LC-HRMS. This multiple hyphenation method has allowed for the characterisation of 61 polar compounds and 11 non-polar metabolites by means of their retention time and fragmentation patterns [87].

Dereplication of a particular class of compounds, such as polyphenols, in which important information can be interpreted from PDA spectra, usually requires less spectroscopic information and LC-PDA-MS-MS have been routinely used. Since flavonoids exhibit characteristic UV spectra it is also possible to obtains structurally relevant information by simple LC-PDA-UV with concomitant use of classical UV shift reagents that will provide complementary information regarding the position of free hydroxyl groups [21].

These methods are well illustrated with the examples of flavonoid profiling. The high diversity of these constituents (more than 7000 have been reported) is mainly related to the oxygenation pattern of their aglycones and the diversity and position of the glycosylated moieties attached to them. For such compounds, well established rules concerning their fragmentation have been published [88] as well as related to their UV spectra [89]. Several studies report the use of these hyphenated methods for extensive flavonoid profiling in plants [81, 90-92].

Laganà and co-workers have established the flavonoid profile of soybeans by LC-ESI-MS/MS in PI mode using a gradient elution profile within 44 minutes [90]. This allowed for the identification of 66 compounds, including aglycones, mono-, di- and triglycosides and acetylated and malonylated glycosides based on literature data and comparisons with standards.

An integrated approach utilising LC-PDA-ESI-MS and GC-MS was used by the Sumner group for the large scale identification of polyphenols in *Medicago truncatula* (Fabaceae) roots and cell cultures [81]. In a first step, IT-MS detection (PI an NI mode) based on 70 minutes linear gradient, was used to obtain MS and MS^n spectra of each of the compounds and their fragmentations patterns and their characteristic UV spectra. A total of 25 polyphenols were observed in NI ESI-MS which provided greater molecular ion abundance while the PI yielded more fragmentation information but only 21 peaks were detected. In a second step, LC-ESI-QTOF-MS in PI mode was used to acquire high mass accuracy data for the on-line determination of the molecular formula. In a third step, GC–MS was used to unambiguously characterise the aglycone and sugar constituents of each flavonoid after enzymatic hydrolysis and derivatisation. This multiple hyphenated strategy allowed the identification and quantification of 35 polyphenol derivatives in roots and cell culture extracts.

Another profiling method based on HR-MS/MS was developed by Suzuki *et al*. for flavonoid profiling in *Lotus japonicus* (Fabaceae). LC-PDA-FTICR-MS with a 45 min gradient enabled the detection of 61 flavonoids including compounds that have not been reported previously [91]. Among them, fourteen were completely identified by comparison with authentic compounds. The high accuracy of *m/z* values (< 1ppm) between observed and theoretical values, allowed prediction of molecular formulas of unknown compounds with the help of isotope peak information for determination of chemical composition.

As discussed in section 2.1.1, improvements in liquid chromatographic techniques allow for very rapid profiling. This is well illustrated by the work of Yonekura *et al.* who used UHPLC-MS to identify the flavonoids produced by *Arabidopsis thaliana* wild-type and flavonoid biosynthetic mutant lines. In this cased the PI UHPLC-ESI-QTOF-MS method enabled the separation and identification of 32 flavonols within 10 minutes. Further fragmentation pattern studies and co-elution with standards permitted structural confirmation.

5.2. Quality Control

LC-UV is the most widely employed method for the quality control and the quantitative analysis of herbal products with the MS detectors mainly used for qualitative evaluation [22]. Many studies have benefited from the use of LC-MS for quality control.

For the well-studied medicinal plant Ginseng more than 30 papers have discussed LC-MS detection either in plant extracts or in body fluids [93]. Wang *et al.* reported a quantification method in MS-MS in the single reaction monitoring mode (SRM) for a very selective dosage of Rb1, Rc and Re [94]. In this case, both $[M+Na]^+$ and $[M+H]^+$ ions were observed, but the CID MS-MS spectra of the $[M+H]^+$ species were found more informative than those of the sodiated adducts. Recently, a method to detect and quantify the nine major ginsenosides of *P.ginseng* has been established. ESI (NI) allowed a LOD of 10 pg/µL and UV quantification provides an acceptable linearity (>99%) with an RSD less than 3% [95]. Yu *et al.* used APCI (NI) for the quantification of 6 ginsenosides as well as other products in a complex TCM preparation (Naodesheng in-

jection) containing five plants, including *Rhizoma Notoginseng* [96]. In this case, the addition of 0.1 % formic acid into the mobile phase ensured the formation of stable $[M+HCOO]^-$ molecular ions. The detection of these ions in the SIM mode produced data with acceptable linearity, repeatability, precision and accuracy (LOQs : 0.02—0.2 μg, linearity coefficient > 0.9900, RSD < 3 %). It is also noteworthy to mention that Ginsenosides have been analysed and quantified in body fluids with similar LC-MS methods [97]. Thus, as shown here, depending on the conditions and the matrix studies both APCI and ESI (PI and NI) enable the quantification and identification of Ginsenosides provided that a correct optimisation is insured.

LC-MS ionisation is also instrument dependant and constitutes a major drawback for the production of standard mass spectrum libraries. This is exemplified by the work Gang and co-workers on the fragmentation patterns of gingerol derivatives. Precursor ions and their subsequent fragments were analysed in PI and NI ESI-MS/MS with different instruments. The NI ESI-MS profiling analysis of extracts from ginger rhizomes on ThermoElectron LCQ advantage failed to detect the predicted precursor ions $[M-H]^-$ although UV detection clearly showed the presence of gingerols. Indeed, injection of pure standard compounds produced covalent dimers of the respective gingerols $[(2M-2H)-H]^-$. On an Agilent LC-MSD-Trap-SL ion trap, the predicted monomer precursors $[M-H]^-$ were observed. In PI mode, the results were quiet similar with formation of covalent dimers $[(2M-2H)+H]^+$ with different adducts formed with an LCQ advantage, while the LC-MSD instrument produced the protonated monomer $[M+H]^+$ and other adducts. Tests on a ThermoElectron LCQ Classic ion trap in NI mode presented both covalent dimers $[(2M-2H)-H]^-$and monomers $[M-H]^-$. Interestingly, MS^n experiments on these instruments showed the same fragmentation patterns in both PI and NI modes. This clearly shows that the formation of precursor ions is instrument dependant whereas the corresponding fragmentation behaviours are not [98].

A more challenging analytical problem is presented by traditional Chinese medicine (TCM) formulations consisting of several medicinal herbs. A mixture of herbs presents a very complex chemical profile of thousands of constituents. Several TCM formulations have been profiled using LC-MS [99, 100]. Gan-Lu-Yin is a TCM formulation consisting of ten herbs. It relieves side effects of radiation therapy in the treatment of nasopharyngeal cancer and may be used as a health tonic. The organic fraction was analysed by LC-PDA-ESI-MS in PI mode. Several LC-MS fingerprints were obtained using total ion chromatogram (TIC) and selected ion chromatogram (SIM) techniques, with the latter giving a much better signal-to-noise ratio. In total, 15 marker compounds were identified on the basis of their retention time and mass spectra in comparison with reference standards. In addition, the distribution of trace metal elements were established by inductively coupled plasma MS after microwave-assisted digestion of organic compounds [99].

Fan *et al*. developed multiple chromatographic fingerprints for the "Danshen dropping pill" (DSDP) that comprises *Salvia miltiorrhiza* and *Panax notoginseng*. DSDP is a common herbal medicine used for the prevention and treatment of coronary arteriosclerosis, angina pectoris and hyperlipaemia. Two extraction methods were used to obtain the sample solutions I (water extract) and II (4% ammonia solution followed by SPE on C_{18}). These two sample solutions were used to develop corresponding fingerprints by LC-PDA-ESI-MS (NI). The main characteristic constituents of DSPD in fingerprint I were identified as depsides, the active pharmacology compounds of *S. miltiorrhizia*, while saponins of *P. notoginseng* were characterised in fingerprint II. In total, 26 marker compounds were identified on the basis of their retention time and UV and MS spectra in comparison with reference standards [100].

5.3. Metabolomics

In metabolomics, it is important to obtain very reproducible LC-MS or DIMS data sets with high throughput and high resolution, for precise comparison and detailed metabolome composition coverage. Many studies report the use of these methods for stress/interactions or genotype/taxonomy and in some case for quality control purposes (see Table **1**).

Most of these studies enable the detection of relevant features (*m/z* at a specific RT for LC-MS, or HR-*m/z* for DIMS) related to a given genetic modification or stimuli, but they sometimes failed to provide *de novo* identification of the biomarkers. In this respect, full elucidation regarding the configuration of the substituents on the skeletal structure usually requires complementary information from NMR experiments, which can be facilitated using the LC-NMR on-line or at-line techniques [38].

This strategy is well illustrated by the example of a plant stress response study. Investigations of wound biomarkers involved the use of UHPLC-TOF-MS fingerprinting followed by statistical analysis and subsequent profiling studies to target metabolites of interest. A scaled-up final step allowed for structural characterisation by capillary NMR (CapNMR) (Fig. **9**). Briefly, in a first step, extracts of unwounded and wounded *A. thaliana* were analysed by LC-TOF-MS using very short columns (20 mm, 5 μm). The data generated by this approach were analysed similarly to a direct infusion experiment (DIMS)[101]. Total mass spectra (TMS) were generated for each sample and data mining tools were applied in an attempt to discriminate the wounded samples from the unwounded ones. In a second step [17], UHPLC-TOF-MS was integrated into the methodology, enabling the metabolite fingerprinting of numerous specimens harvested at various time points after wounding. Data were treated by taking advantage of both chromatographic and accurate mass information. An example of the 3D data generated for one of the leaf samples analysed is illustrated in Fig. (**9A**). Numerous fingerprints in the form of 2D ion maps (*m/z* vs. RT) were treated by MVDA (Fig. **9B**). Unsupervised (Fig. 9C, PCA) and supervised data treatment methods provide a list of significant metabolites (*m/z* vs. RT) (putative biomarkers) (Fig. **9D**) which represent the most contributive variables for the separation of the wound states. Some of these compounds were dereplicated, based on their molecular formula, additional MS/MS experiments and comparison with standards, such as known signalling molecules like jasmonic acid (JA) and other related oxylipins [102]. Other biomarkers were evidenced as a single *m/z* 225 ion. They occurred as close positional isomers and diastereoisomers and, as a result, could not be identified based on MS data alone. Thus, in a third step [40], high resolution UHPLC using long gradient coupled to TOF-MS was used to precisely localise the unknown markers of interest. The conditions used were then transferred to a semi-preparative HPLC scale to purify the compounds. Four wound-induced hydroxylated jasmonates sharing the same molecular formula and MS fragmentations were efficiently separated by UHPLC-TOF-MS and were clearly differentiated by capNMR [40]. The CapNMR spectra of one of these isomers is shown in Fig. (**9 E**) together with its structure determined *de novo*. The developed approach proved to be a

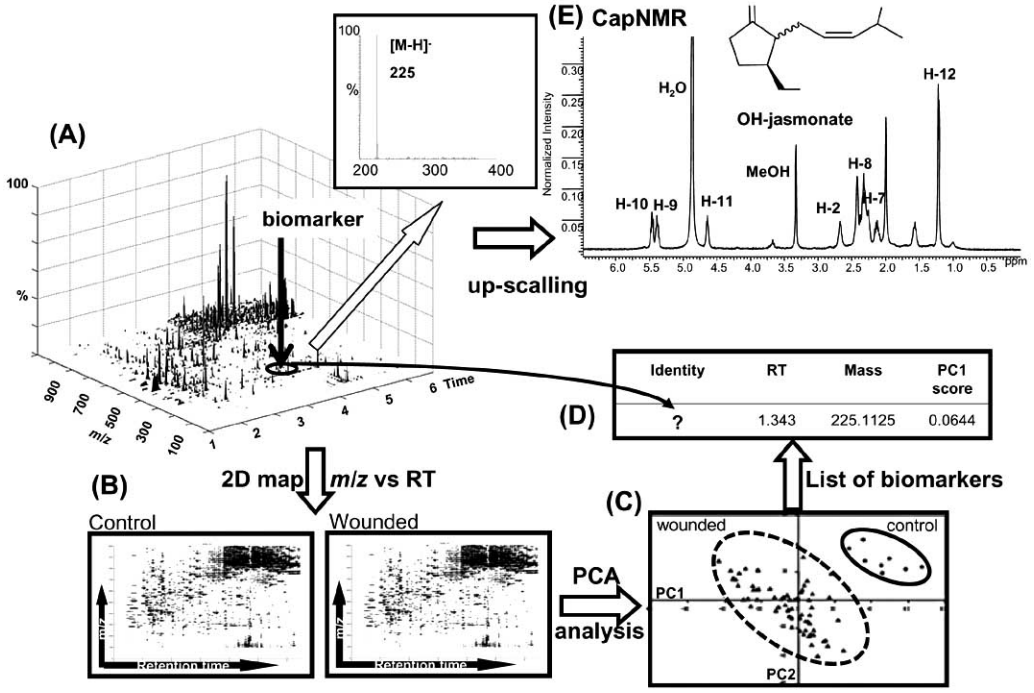

Fig. (9). (**A**) UHPLC-ESI-TOF-MS metabolite fingerprinting in the form of 3D ion maps of an *A. thaliana* extract obtained 90 minutes after wounding. (**B**) Generation of 2D ion maps (RT vs *m/z*) from UHPLC-TOF-MS data on wounded and control pants. (**C**) PCA score plot obtained on the basis of numerous UHPLC-ESI-TOF-MS metabolite fingerprintings on control and wounded plants. (**D**) List of ions with PC1 score for the ion at *m/z* 225. (**E**) Identification of ion at *m/z* 225 by CapNMR after up-scaling. ^{1}H-NMR of hydroxylated-jasmonate. Adapted from [17, 40, 101]. Copyright (2008) with permission from Elsevier.

powerful tool for marker discrimination and identification and has been successfully utilised in other subsequent studies [25, 39, 103].

6. STUDIES ON NATURAL PRODUCTS USING LC-NMR, CAPNMR AND SPE-NMR

In crude extract profiling studies, LC-NMR or the other at-line methods have been predominantly use for dereplication studies or for targeted *de novo* identification of given bioactive metabolites or biomarkers issued from metabolomic studies. Various facets of the use of hyphenated methods for rapid structural determination have been illustrated. The number of applications of rapid on-line identification of natural products is in a constant growth. In Table **2**, a summary of the most recent applications (2006-2009) is presented, including the type of experiments and the probe used. For earlier applications, the reader can refer to other reviews [13, 32, 33, 56, 104].

6.1. Dereplication Studies

As shown in Table **2**, an important number of applications in the field of natural products have been performed by the LC-SPE-NMR while applications of CapNMR are also rapidly growing. The interest for these at-line methods reside mainly in the quality of the spectra that are generated compared to classical LC-NMR as discussed previously. In most applications, beside the recording of high quality 1D-NMR with the at-line methods, 2D-NMR experiments such as ^{1}H-^{1}H (COSY, TOCSY) as well as ^{1}H-^{13}C data (HSQC and HMBC) using a few micrograms of the analyte are acquired for in-depth structural investigations.

The first LC-SPE-NMR analysis on natural products was carried out on an extract of Greek origano and led to the successful identification of various flavonoids, rosmarinic acid and monoterpene carvacrol based on a combination of UV, MS and NMR spectra [47]. In 2007, the technique was applied in the separation and structural verification of the major known constituents present in Greek *Hypericum perforatum* extract. The chromatographic separation was performed in a C18 column using a mixture of MeCN-H_2O as a mobile phase. For the on-line NMR detection, the analytes eluted from the column were trapped one by one in separate SPE cartridges, and thereafter transported into the NMR flow-cell. LC-PDA-SPE-NMR and LC-PDA-MS allowed for the characterisation of constituents of *H. perforatum*, mainly naphtodianthrones, phloroglucinols and phenolic acids. Two phloroglucinols (hyperfirin and adhyperfirin) were detected for the first time, which have been previously reported to be precursors in the biosynthesis of hyperforin and adhyperforin [72].

Recently, this technique has been employed in the identification of alkaloids form *Lobelia siphilitica* and *Hippobroma longiflora* (Campanulaceae) and sesquiterpene lactones from *Distephanus angulifolius* (Asteraceae) [105, 106]. Another interesting study has demonstrated its use for the identification of iridoid and caffeoyl glycosides from *Pinguiluca lusitanica* (Lentibulariaceae) [107].

As an example, the on-line identification of antioxidant compounds using DPPH and ABTS radical scavenging techniques from the methanolic extracts of *Tilia europea*, *Urtica dioica*, *Lonicera periclymenum* and *Hypericum perforatum* was performed using the combination of LC-MS and LC-UV-SPE-NMR as well as on-line bioassays. To increase the sensitivity of LC-UV-SPE-NMR, multiple trapping was used, which allowed larger quantities of the compound to accumulate on the corresponding SPE cartridges from three successive LC runs similarly to what was demonstrated in Fig. (**5**) for the analysis of glyasperin H. The trapped compounds were extracted from the corresponding SPE cartridges with 560 µl of acetonitrile-d_3 into the NMR flow-cell. The screening of the four

Table 2. Recent Applications (2006-2009) of Direct NMR, LC-NMR, LC-SPE-NMR and CapNMR for On-Line or at-Line Metabolite Fingerprinting or Profiling

Metabolite Identification/Dereplication					
Source	***Application***	***Method***	***Experiment***	***Instrumentation***	***Ref.***
Haplophyllum acutifolium	alkaloids profiling	LC-PDA-MS-SPE-NMR	^{1}H-NMR, COSY, HSQC, HMBC, NOESY	ESI (+); 600 MHz, 30 μL	[139]
Machilus philippinensis	flavonoids glycosides profiling	LC-MS, HPLC-SPE-NMR	^{1}H-NMR	ESI (+), 400 MHz, 30 μL	[140]
Distephanus angulifolius	sesquiterpene lactones characterization	LC-MS-SPE-NMR	^{1}H-NMR	ESI-IT-MS (+ / –), 600 MHz, 5 mm	[105]
Lobelia siphilitica, Hippobroma longiflora	alkaloid identification	LC-MS-SPE-NMR	^{1}H-NMR	600 MHz, 5 mm	[106]
Harpagophytum procumbens	metabolite profiling	LC-MS, HPLC-SPE-NMR	^{1}H-NMR, ^{13}C, COSY, TOCSY, HSQC, HMBC	ESI, APCI, 600 MHz, 30 μL	[141]
Neolitsea sericea var. *aurata*	flavonoids glycosides profiling	LC-SPE-NMR	^{1}H-NMR	400 MHz, 30 μL	[142]
Trina glauca and other *Apiaceae* spp	polyacetylenes profiling	LC-SPE-NMR	^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR	500 MHz, insert 60 μL	[143]
Penicillium roqueforti	sesquiterpenes identification	LC-MS-SPE-NMR	^{1}H-NMR, ^{13}C-NMR, COSY, TOCSY,NOESY, gHSQC, gHMBC	ESI-IT-MS, 600 MHz, 5 mm	[109]
Pinguiluca lusitanica	iridoid and phenylpropanoid glycosides profiling	LC-MS, LC-SPE-NMR	^{1}H-NMR	ESI-TOF-MS (–), 600 MHz	[107]
Neolista serica var. *aurata*	isoquinoline alkaloids profiling	LC-SPE-NMR	^{1}H-NMR, COSY, NOESY	600 MHz , 30 μL	[144]
Blepharis aspera	phenylpropanoids profiling	LC-MS, LC-SPE-NMR	^{1}H-NMR	ESI-IT-MS (+), 600 MHz , 30 μL	[145]
Corydalis solida	alkaloids profiling	LC-MS^{n}, LC-SPE-NMR	^{1}H-NMR, COSY, HSQC, HMBC	ESI-IT-MS (+), 600 MHz, insert 30 μL	[55]
Thespesia danis	gossypol profiling	LC-MS-SPE-NMR	^{1}H-NMR	ESI-IT-MS (+ / –), 600 MHz, 30 μL	[146]
Tilia europea, U. dioica, Lonicera periclymenum, H. perforatum	antioxydant metabolite profiling	LC-MS, LC-SPE-NMR	^{1}H-NMR	ESI (–), APCI (+ / –)-IT-MS, 400 MHz, 120 μL	[108]
Phyllanthus reticulatus	flavonoid glycosides profiling	LC-SPE-NMR	^{1}H-NMR	400 MHz, 120 μL	[147]
Schizanthus grahamii	tropane alkaloids profiling	LC-NMR, stop-flow and LC-SPE-NMR	^{1}H-NMR ^{1}H-NMR , COSY	600 MHz, insert 60 μL. 600 MHz, 30 μl	[113]
Swietenia macrophylla	limonoids profiling	LC-NMR, stop-flow	^{1}H-NMR, TOCSY, HSQC, gHMBC	600 MHz, 120 μL	[112]
Erythrophleum fordii	amides diterpenoids	LC-NMR, stop-flow	^{1}H-NMR, COSY	500 MHz, 60 μL	[148]
Chamomilla recutita	metabolite profiling	LC-MS/MS, LC-NMR, stop-flow	^{1}H-NMR	ESI-IT-MS (+ / –) 400 MHz, , 60 μL	[149]
Bixa orellana	carotenoids profiling	LC-MS, CapHPLC-CapNMR, stop-flow	^{1}H-NMR, COSY	APCI (+), 600 MHz, 2 μL	[44]
Sepedonium chrysospermum	metabolite profiling	LC-bioactivity profiling, MS, CapNMR	^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC	ESI-TOF-MS (+), 500 MHz, 1.5 μL	[110]
Psorothamnus arborescens	isoflavones profiling	LC-MS, CapNMR	^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC	ESI-TOF-MS (+ / –), 600 MHz, 5μL	[150]
Thapsia garganica	sesquiterpene lactone and phenylpropanoids profiling	LC-MS, LC-SPE-CapNMR	^{1}H-NMR,COSY TOCSY, HSQC, HMBC	ESI-IT-MS (+ / –), 500 MHz, 1.5 μL	[151]
Muehlenbeckia sp., *Aspergillus* sp.	metabolite profiling	LC-MS, CapNMR	^{1}H-NMR, COSY, HSQC, HMBC	ESI-TOF-MS (+), 500 MHz, 1.5 μL	[69]
Fischerella ambigua	metabolite profiling	LC-MS-MS/MS, CapNMR, stop-flow	^{1}H-NMR	ESI-IT-MS (+), 500 MHz, 1.5 μL	[37]
Dactylospongia sp.	metabolite profiling	LC-NMR on flow, LC-NMR stop flow	^{1}H-NMR, ^{13}C, gCOSY, gHSQC, HMBC	500 MHz, 60 μL	[114]

Table 2. contd…

Metabolite Identification/Dereplication					
Source	***Application***	***Method***	***Experiment***	***Instrumentation***	***Ref.***
Belamcanda chinensis	isoflavonoids profiling	LC-MS, LC-NMR on flow	^{1}H-NMR	ESI-IT-MS (+), 500 MHz, 60 μL	[111]
Nandina domestica	aporphine alkaloids profiling	LC-NMR, stop-flow	^{1}H-NMR	500 MHz, 60 μL	[152]
Lysidice brevicalyx	metabolite profiling	LC-MS^{n}, LC-NMR stop flow	^{1}H-NMR, dqfCOSY	ESI-IT-MS (+ / –), 500 MHz, 60 μL	[153]
Olea europaea	metabolite profiling	LC-MS, LC- SPE-NMR	^{1}H-NMR	ESI-IT-MS (–), 500 MHz, 60 μL	[154]
Spider venom	metabolite profiling	Direct-MS , Direct-NMR	^{1}H-NMR, dqfCOSY, NOESY, HMQC, HMBC	ESI-QMS (+ / –), 600 MHz	[155]
Sisymbrium officinale	metabolite profiling	LC-PDA, Direct-NMR	^{1}H-NMR, DOSY, TOCSY	600 MHz	[156]
Foliar endophytes of *Picea glauca*	polyketides profiling	LC-UV-NMR, stop-flow	^{1}H-NMR	600 MHz, LC-NMR insert 60 μL	[157]
Metabolomics: Quality assessment					
Hypericum perforatum	metabolite profiling	LC-MS/MS, LC-SPE-NMR	^{1}H-NMR, COSY, TOCSY	ESI-IT-MS (–), 400 MHz, 120 μL	[72]
Tomato juice, palm oil, mandarin juice	carotenoids profiling	LC-NMR stop flow	^{1}H-NMR, gCOSY,	500 MHz, 60 μL	[117]
Cannabis sativa	metabolite profiling	Direct-NMR	^{1}H-NMR	500 MHz	[116]
Ginseng	metabolite fingerprinting	Direct-NMR	^{1}H-NMR, *J*-resolved, COSY, HSQC, HMBC	400 MHz	[158]
Solanum lycopersicum	metabolite profiling	LC-MS, Direct-NMR	^{1}H-NMR	ESI-QTOF-MS (–), 500 MHz	[159]
Echinacea purpurea, Hypericum perforatum, Ginkgo biloba, Valeriana officinalis	metabolite fingerprinting	Direct-NMR, Direct-MS	^{1}H-NMR	500 MHz ESI-qqqMS (+ / –)	[65]
Artemisia annua	metabolite profiling	Direct-NMR	^{1}H-NMR	500 MHz	[160]
Panax ginseng	metabolite profiling	Direct-NMR	^{1}H-NMR, DOSY, HMBC	500 MHz	[115]
Galphimia glauca	galphimine profiling	Direct-NMR	^{1}H-NMR	500 MHz	[77]
Metabolomics: Stress/interactions					
Arabidopsis thaliana	oxylipins profiling	LC-MS, CapNMR	^{1}H-NMR, COSY	APCI-QQQMS (–), 500 MHz, 1.5 μL	[161]
Arabidopsis thaliana	jasmonate derivatives profiling	UHPLC-MS, CapNMR	^{1}H-NMR, TOCSY	ESI-TOF-MS (–) 500 MHz, 1.5 μL	[40]
Botryosphaeria obtusa and *Eutypa lata*	metabolite profiling	UHPLC-MS, CapNMR	^{1}H-NMR,gCOSY TOCSY, NOESY, gHSQC, gHMBC	ESI-TOF-MS (+ / –) 500 MHz, 1.5 μL	[39]
Brassica rapa	metabolite fingerprinting	Direct-NMR	^{1}H-NMR	400 MHz	[162]
Brassica rapa	metabolite fingerprinting	Direct-NMR	^{1}H-NMR, *J*-resolved, COSY, HMBC, HSQC	500 MHz	[119]
Brassica rapa	metabolite profiling	Direct-NMR	^{1}H-NMR, *J*-resolved, COSY, HMBC, HSQC	400 MHz	[118]
Silybum marianum	metabolite fingerprinting	Direct -NMR	^{1}H, COSY, HSQC, HMBC	400 MHz	[163]
Populus tremula	lignin and polysaccharides profiling	Direct-NMR	2D ^{13}C-^{1}H HSQC	600 MHz, 5mm	[164]
Kitasatospora	metabolite profiling	Direct-MS, Direct-NMR	^{1}H-NMR	ESI-FTICRMS, 500MHz, 5 mm	[68]
biotransformation by *Corydalis* and *Macleaya* cell	isoquinoline alkaloids profiling	LC-MS/MS, LC-NMR, stop-flow	^{1}H-NMR	APCI-QQQMS (+), 500 MHz, 60 μL	[165]

Table 2. contd…

Metabolite Identification/Dereplication					
Source	***Application***	***Method***	***Experiment***	***Instrumentation***	***Ref.***
Metabolomics: Genotype/taxonomy					
Galphimia glauca	metabolite fingerprinting	Direct-NMR	^{1}H-NMR	500 MHz	[77]
Dilatris pillansii, D. corymbosa, D. viscosa	metabolite fingerprinting	LC-UV, Direct-NMR	^{1}H-NMR	500 MHz, 5 mm	[166]
Brassica rapa	metabolite profiling	Direct-NMR	^{1}H-NMR, *J*-resolved	500 MHz	[167]

COSY: correlation spectroscopy; DEPT: distortionless enhancement by polarisation transfer; HMBC: heteronuclear multiple-bond correlation; HSQC: heteronuclear single quantum correlation; NOESY: nuclear overhauser effect spectroscopy; DOSY: diffusion ordered spectroscopy; TOCSY: total correlation spectroscopy; CapNMR: Capillary NMR; SPE: solid-phase extraction; ESI: electrospray ionization; APCI: atmospheric pressure chemical ionization; Q: quadrupole; QQQ: triple quadrupole; QTOF: quadrupole time of flight; IT: ion trap; FTICR: fourier transform ion cyclotron resonance; (+): positive mode ionization; (–) negative mode ionization; LC: high performance liquid chromatography, UHPLC: ultra high performance liquid chromatography; UV: ultra violet; PDA: photodiode array.

methanolic extracts allowed for the identification of 14 different antioxidants using exclusively chromatographic hyphenated techniques. Structure elucidation revealed that the antioxidant activity of the extracts was mainly attributed to flavonoid glycosides and caffeoylquinic acids [108].

To improve sensitivity, LC-SPE-NMR spectra were also acquired using cryogenic flow probes. For example, the analysis of the *Penicillium roqueforti* using LC-SPE-NMR allowed the identification of three eromophilane sesquiterpenes, one of which was described for the first time [109]. The structural determinations were based on complete gHSQC, gHMBC and NOESY experiments in combination with high-resolution ESI-MS.

A significant number of recent applications (Table **2**) are performed using capillary NMR. For example, the carotenoids of *Bixa oreollana* has been studied using a capillary LC-NMR system equipped with a home-built double-resonant solenoidal NMR microprobe. For the unambiguous assignment of natural carotenoid stereoisomers, the authors used a closed-loop LC-NMR technique on-line. The technique showed important advantages in comparison to the procedure of at-line microisolation since often this strategy results in carotenoid isomerisation and degradation. Using this system, the authors were able to determine the structure of the main bixin stereoisomers [44].

Munro and co-workers used the CapNMR instrumentation for the identification of active compounds in the mycoparasitic fungus *Sepedonium chrysospermum* [110]. This dereplication approach allowed the rapid recognition of a new peptaibol using only 700 μg of crude *S. chrysospermum* extract. The high-quality 1D and 2D homonuclear ^{1}H NMR and ESI-MS/MS data obtained on the isolated sample of chrysaibol (33 μg) were sufficient for the definition of a planar structure, while the absolute configurations of the amino acid residues were defined by Marfey's method. Chrysaibol was cytotoxic against the P388 murine leukaemia cell line (IC_{50} 6.61 μM) and showed notable activity against *Bacillus subtilis* (IC_{50} 1.54 μM).

The use of the capillary NMR technique on an automated platform has been extensively optimised for sample-limited investigations by Lin and co-workers that use a microscale LC-MS-NMR platform (Fig. **10A**) to identify active Cyanobacterial metabolites [37]. The system combined two innovations in microscale analysis, nanoSplitter LC–MS and microdroplet NMR. The nano-Splitter provided the high sensitivity of nanoelectrospray MS, allowing 98% of the HPLC effluent from a LC column to be collected and concentrated for NMR analysis. Microdroplet NMR is a microfluidic droplet NMR loading method that provided several-fold higher sample efficiency than conventional flow injection methods. Interpretable 1D NMR spectra were obtained from analytes at the 200-ng level, in 1 h/well automated NMR data acquisitions. The system offered impressive LODs, at the 50-ng level for NMR, excellent reproducibility (RSD: 1.17%), and sample recovery on the order of 93%.The applicability of this system to the investigation of a bioactive cyanobacterial extract was demonstrated. The platform recognised four known natural products, ambiguine A, I, E and hapalindole H, from a single 30-μg LC injection of cyanobacterial crude extract LC (Fig. **10B**) and, most impressively, identified one LC-MS peak as a novel bioactive compound (Fig. **10C**) with spectral quality. Taxol (300 ng) was spiked to this mixture to give an idea of the sensitivity of the method developed (Fig. **10B**).

As mentioned under 2.4.2, CapNMR was also found essential for *de novo* identification of trace biomarkers in the frame of MS-based metabolomic studies [3, 40, 103]. These examples illustrate the potential for sample limited application in both natural product discovery as well as in metabolomics. The methodology can be applied in other fields requiring trace analysis of components of complex mixtures.

Classical LC-NMR profiling is also still performed to support dereplication studies. For example, Kang *et al.* applied LC-NMR in the identification of isoflavonoids from *Belamcanda chinensis* [111]. The LC-NMR data were acquired using a 500 MHz NMR system equipped with an LC-NMR cold flow probe with a 60 µl flow cell. ^{1}H-NMR spectra of the five main peaks were obtained in on-flow mode. The WET solvent suppression was used to eliminate the acetonitrile and residual water peaks signals. The use of LC-NMR in drug discovery has also been used in the study of limonoids of *Swietenia macrophylla*. Separations of limonoids was carried out using reversed-phase gradient HPLC elution coupled to an NMR (600 MHz) spectrometer equipped with a 120 µL flow cell in stopped-flow mode. Structure elucidation of the limonoids was attained using the ^{1}H NMR, TOCSY, gHSQC and gHMBC spectra [112]. Christen and colleagues compared two fully automated HPLC-NMR methods for the structural elucidation of four isomeric tropane alkaloids from the stem-bark of an endemic Chilean plant, *Schizanthus grahamii* (Solanaceae). The first approach interfaced a conventional HPLC column to NMR by means of a loop storage unit. After elution with a mobile phase consisting of deuterated water and standard protonated organic solvents, the separated analytes were momentarily stored in a loop cassette and then transferred one-at-a-time to the NMR flow probe for measurements. The second strategy combined HPLC with parallel ion-trap MS detection and NMR spectroscopy using SPE-NMR. The SPE cartridges were dried under a gentle stream of nitrogen and analytes were sequentially eluted and directed to a cryogenically cooled flow-

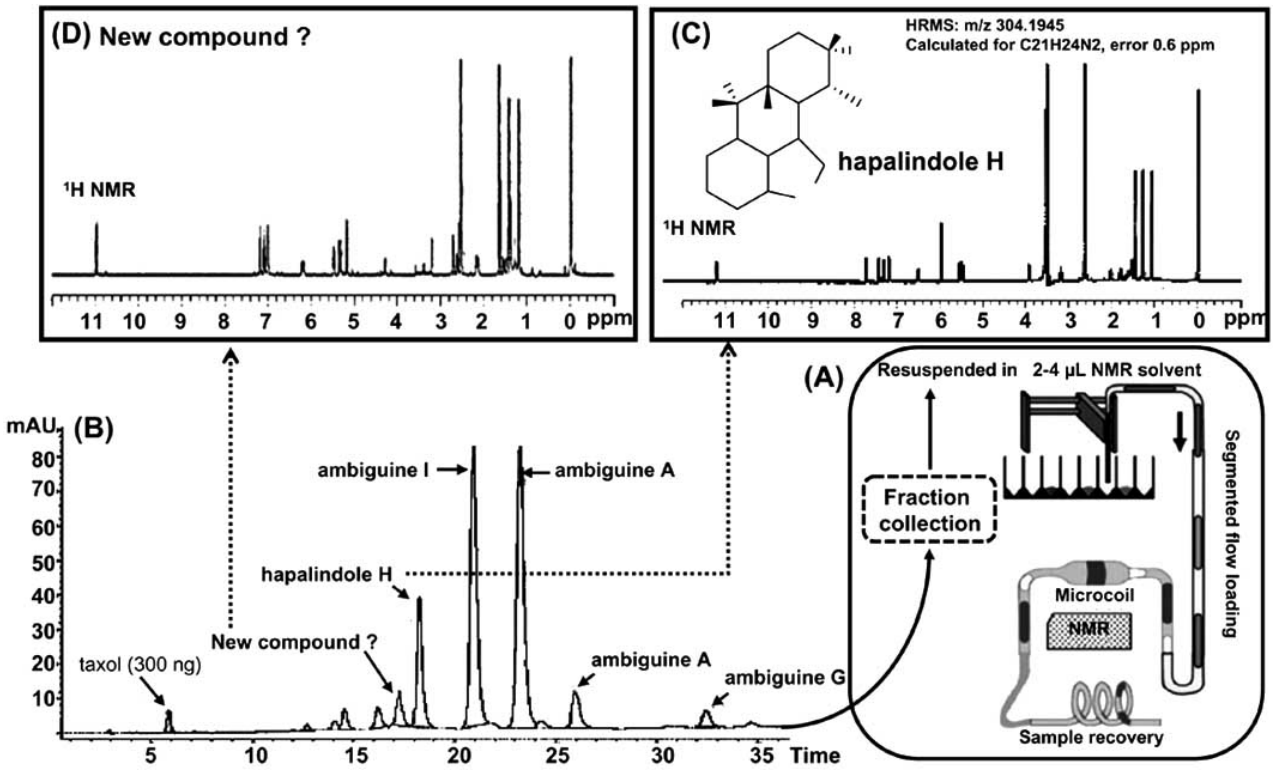

Fig. (10). (**A**) Schematic diagram of the microdroplet autosampler, as applied to natural product discovery. Microfractions are resuspended in a small volume (2–5 μL) of deuterated solvent and loaded into a microcoil NMR probe, with an observed volume of 1–2 μL, using microplate automation. Samples are recovered after NMR analysis for additional analyses, archival or bioassay. (**B**) UV chromatogram of the separation of a bioactive cyanobacterial extract analyzed with the LC-MS-NMR system, indicating known and unknown compounds found. (**C**) ^{1}H NMR spectra of LC peak at 18.3 min identified from the literature as hapalindole H and (**D**) NMR spectra of the 17.3 min peak not found in the in the literature or natural product databases. It was therefore prioritised for detailed structure studies by scale-up purification. Adapted from [37]. Copyright (2008) American Chemical Society.

probe with deuterated solvents. The structures of the four isomeric alkaloids were unambiguously determined by combining NMR assignments with MS data [113].

Recently, the chemical profile of the antitumor active extract of the marine sponge *Dactylospongia* sp. was performed using a combination of both at-line (HPLC followed by NMR and MS) and on-line (on-flow and stop-flow LC-NMR) techniques. On-flow LC-NMR analysis was employed to initially identify compounds present in the dichloromethane extract, while stop-flow LC-NMR experiments were then conducted on the major components present, resulting in partial identification of pentaprenylated *p*-quinol. The complete structure was achieved by 2D-NMR spectra after isolation by preparative HPLC. This study represents one of the few reports describing the application of LC-NMR to chemically profile secondary metabolites from a marine organism [114].

6.2. Quality Control

Quality control assessment is typically performed by direct NMR profiling (Table **2**) since the method is simple and reproducible. NMR-based metabolic fingerprinting has been applied to diverse fields of plant research. Classification and identification of adulterated plant products are among the major interests. Direct NMR analysis has been applied for the comparison of metabolic profiles and fingerprinting of a number of plant extracts used in phytotherapy, such as *Echinaceae purpurea, Gingkgo biloba, Valeriana officinalis and Panax ginseng* [60, 65, 115].

Politi and co-workers applied the direct NMR analysis of hot and cold water extracts as well as ethanol/water mixtures (tinctures) of *Cannabis sativa* to better understand how these extracts differ in their metabolic profile. NMR analysis and *in vitro* cell assays of crude extracts and fractions were also performed. The method uses the diffusion-edited ^{1}H NMR (1D DOSY) and ^{1}H NMR with suppression of the ethanol and water signals. The authors observed that temperature and polarity of the solvents used for the extraction to be two factors that affect the total amount of Δ^9-THC in the extracts and its relative quantity with respect to Δ^9-THC-acid and other metabolites. With this method it was possible, without any evaporation or separation step, to distinguish between tinctures from different cannabis cultivars. This approach opens an interesting path to the direct analysis of plant tinctures without any pre-treatment [116].

In quality control, the hyphenated NMR methods are mainly used for peak identification and not for comparison purposes. In this respect SPE-NMR was used establish the chemical profile extracts of *Hypericum perforatum* [72]. LC-NMR was also applied in this field for the analysis of carotenoids in foods such as tomato and mandarin juice and palm oil. The analyses were performed in stop-flow LC-NMR. This method has also been very useful for efficient analysis of carotenoids that are fragile and sensitive to light and air [117].

6.3. Metabolomics

To date, no LC-NMR or at-line NMR methods have been used for comparison of crude plant extracts. All studies involved either

the use of direct NMR profiling with adequate MVDA or at-line NMR methods such as CapNMR to confirm the identity of biomarkers highlighted by MS-based metabolic approaches. This latter application has been discussed in section 5.3.

Beside the application of direct NMR profiling related to the investigation of *Brassica rapa* leaves treated with methyl jasmonate in section 3.1, many other studies discussing stress/interaction and genotype/taxonomy have been reported. For example the metabolic alterations of *Brassica rapa* leaves attacked by larvae of the specialist *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) and the generalist *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae) were investigated with NMR, followed by a multivariate data analysis [118]. The overlapped ^{1}H NMR spectra made it difficult to identify discriminating metabolites. To overcome the spectral complexity, several two-dimensional NMR techniques were applied. Of those evaluated, *J*-resolved spectroscopy, which affords an additional coupling constant, provided a wide range of structure information for differentiating the metabolites. Based on the *J*-resolved spectra combined with PCA, the major signals contributing to the discrimination were alanine, threonine, glucose, sucrose, feruloyl malate, sinapoyl malate and gluconapin. Recently, the metabolomic changes induced by metal ions such as copper, iron and manganese were evaluated in the same plant. ^{1}H NMR and two-dimensional NMR spectra coupled with principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied. The ^{1}H-NMR analysis followed by the application of chemometric methods revealed a number of metabolic consequences. Among the metabolites that showed variation, glucosinolates and hydroxycinnamic acids conjugated with malates were found to be the discriminating metabolites as were primary metabolites like carbohydrates and amino acids [119].

Metabolomics has also proved to be very effective for assessing bioactivity. Takeda *et al*. used direct ^{1}H-NMR profiling and multivariate data analysis for the investigation of crude extract of *Galphimia glauca*, a plant popularly employed in Mexico for the treatment of central nervous system disorders. The crude extracts from wild plant populations, collected from six different locations were investigated, to differentiate their chemical profile. Pharmacological and phytochemical studies led to the identification of the anxiolytic and sedative compounds consisting of a mixture of norsecofriedelanes, named the galphimine series. Principal component analysis (PCA) of the ^{1}H-NMR spectra of 39 crude extracts revealed clear differences among the populations, with two populations out of the six studied manifesting differences when the main constituents were analysed (PC1 and PC2). These two PCs permitted the differentiation of the various sample populations, depending on the presence of galphimines. This information consistently correlated with the corresponding HPLC analysis and with the neuropharmacological activity. The results of this investigation confirm the advantages of using metabolic profiling for the *in silico* analysis of active principles in medicinal plants [77]. The strategy applied in this study opens new possibilities for the correct selections of plant populations with suitable metabolic and pharmacologic profiles for the development of standardised herbal medicines.

7. CONCLUSION AND PERSPECTIVES

As discussed, various analytical strategies can be used for an efficient profiling of crude extracts of plants or other organisms. Without any doubt, hyphenated HPLC methods are playing an increasingly important role in this respect. With the important progresses made in terms of chromatographic resolution and speed of separation by the introduction of UHPLC, the separation of several hundreds of constituents in complex matrices can be attained. This provides an efficient tool for deconvolution of many natural products and for obtaining high quality profiles.

The progresses made in mass spectrometers in terms of sensitivity and resolution facilitate the extraction of the molecular formula of each constituents over an important dynamic range, while MS-MS experiments generate useful complementary structural information for a wide range of natural products. Following different strategies, that also involve the acquisition of PDA spectra, many compounds can be dereplicated also by additional cross-searches based on chemotaxonomic information and searches in spectral databases.

NMR detection in complement with the other LC-hyphenated techniques, either used on-line or at-line, is playing an increasingly important role as a strategic tool to support profiling studies. Even if relatively insensitive compared to the other analytical detectors, NMR remains the only technique that can unambiguously solve structural identification issues especially for *de novo* metabolite determination. According to the needs, as shown, NMR can be hyphenated to LC systems in different ways. In this field, on-line LC-NMR is now being gradually replaced by alternative at-line approaches such as LC-SPE-NMR or CapNMR. These latter methods do not require compromises to be made and provide high quality 1D and 2D NMR, even for the minor constituents of an extract, and they can now be fully automated. However, for labile constituents, on-line LC-NMR methods remain mandatory and still provide a rapid and efficient means of evaluating the nature and the real proportion of the main constituents in a crude extract. These hyphenated methods have evolved in sophisticated LC-NMR-MS platform that are becoming essential in many drug discovery studies.

At the same time, the development of powerful MVDA methods for analysing direct NMR and direct MS or LC-MS profiling data have given a strong impetus to metabolomics. All these important technological advances in biological sciences have notably strengthened the emerging field of systems biology. Although the complete understanding of living organisms at the molecular system level is far from reality, the comprehensive investigations of living organisms such as plants with the different 'omics' techniques represent an important step forward.

It is a safe bet to assume that profiling methods will continue to evolve and provide even more sensitivity and high resolution in both chromatographic and spectroscopic dimensions. The bottleneck of the approach will however continue to reside in the unambiguous identification of the metabolites detected. In this respect, spectral databases of natural products have to be constituted and open to scientists at large in combination with software that enable partially automated MS fragmentation prediction and aid in NMR interpretation. The development of such tools should accelerate the pace at which natural product chemists will discover promising leads from natural sources or interrogate biological systems from a holistic perspective.

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LIST OF ABBREVIATIONS

APCI	=	Atmospheric Pressure Chemical Ionisation
API	=	Atmospheric Pressure Ionisation
APPI	=	Atmospheric Pressure PhotoIonisation
CapNMR	=	Capillary NMR
CID	=	Collision Induced Dissociation
ELSD	=	Evaporative Light Scattering Detection
ESI	=	ElectroSpray Ionisation
FT	=	Fourier Transform
HPLC	=	High Performance Liquid Chromatography
HR	=	High Resolution
IT	=	Ion Trap
LLE	=	Liquid Liquid Extraction
LOD	=	Limit Of Detection
LOQ	=	Limit Of Quantification
LR	=	Low Resolution
MS	=	Mass Spectrometry
MS/MS	=	Tandem mass spectrometry
MS^n	=	Multiple stage mass spectrometry
MVDA	=	MultiVariate Data Analysis
MW	=	Molecular Weight
NI	=	Negative Ion
NMR	=	Nuclear Magnetic Resonance
NPs	=	Natural products
PCA	=	Principal Component Analysis
PDA	=	PhotoDiode Array
PI	=	Positive Ion
Q	=	Quadrupole
QQQ	=	Triple quadrupole
SPE	=	Solid Phase Extraction
TOF	=	Time Of Flight
UHPLC	=	Ultra High Pressure Liquid Chromatography
UV	=	Ultraviolet

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