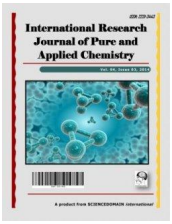

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Hyphenated Techniques in Liquid Chromatography as Current Trends in Natural Products Analysis

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Authors' contributions

This work was carried out in collaboration between both authors. Author NNI designed the study and wrote the first draft. Author SJA reviewed the draft and managed the references. Both authors read and approved the final manuscript.

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ABSTRACT

Historically, drug discovery from natural products has been a time and resource-intensive process. Bioassay-guided isolation of natural products often leads to already known compounds of limited, or no chemical or pharmacological interest. Some bioactive compounds are unstable and separation using the traditional approach is often difficult. Full structural elucidation of pure compounds usually requires milligram quantities of compounds and that may require extracting large kilogram quantities of material especially for minor compounds. Rapid detection of biologically active natural products is desired, and to achieve this, dereplication of crude extracts performed prior to isolation work is of crucial importance for avoiding the isolation of a known constituent. Natural products research, as a strategy in drug discovery, has evolved over the last two decades with technological advances in the tools which are prerequisite in isolation and structural elucidation of compounds. Such is the shift from the classical/traditional stand-alone instrumental analytical approaches to newer hyphenated techniques (LC-UV/DAD, LC-MS and LC-NMR).This review describes the general principles and literature applications of these productivity tools in natural products isolation and structural elucidation and also as assay tools for quality control studies, with a discussion on their successes

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and intrinsic challenges. These hyphenated techniques will advance the course of natural products research and reduce the time and cost invested in the study of natural products, speeding up the drug discovery process.

Keywords: Liquid chromatography; hyphenated techniques; dereplication; natural products research; drug discovery.

1. INTRODUCTION

1.1 Natural Products Research and Its Role in Drug Discovery

Natural products research (NPR) which is primarily concerned with identifying pharmacologically active compounds from natural sources, including plants, fungi, bacteria, and marine organisms, has recorded advances in the drug discovery and development program which has been beneficial to mankind over the last century [1,2] The importance and relevance of NPR as a strategy in drug discovery and development cannot be undermined; 61% of the 877 new chemical entities (with low molecular weights) registered as drugs worldwide during the period of 1981-2002 were natural products (NPs), or were inspired by NPs [3], 19 NP-based drugs were approved for marketing worldwide between 2005 and 2010 [4]. Excellent examples of drugs from natural sources include penicillin, streptomycin, taxol and artemisinin, just to mention a few. Despite the availability of different approaches for the discovery of therapeuticals, the success of NPs can be attributed to their high chemical diversity, biochemical specificity, greater number of chiral centres and increased steric complexity than either synthetic drugs or combinatorial libraries, and the effects of evolutionary pressure to create biologically active molecules by interactions with different proteins and biological targets [5,6]. Going with the proven history of providing medicinal agents, and as an indispensable tool in biomedical research, pharmaceutical companies have over the last decade, renewed their interest in NPR as many plant-derived compounds have been launched in the market or undergoing clinical trials for the potential treatment of various diseases [7,8]. In view of the large number of plant species potentially available for study, it is essential to have efficient systems available for the rapid chemical and biological screening of the plant extracts selected for investigation [9]. Thus, technical improvements in the methods employed in NPR are needed for a simpler, less time-consuming strategy in the drug discovery program.

1.2 Strategies in Natural Products Discovery

1.2.1 Biological screening; activity-oriented separation

One main utility into the extraction of natural products and isolation of secondary metabolites is in the biological activities and suitable bioassays are essential for monitoring the required effects. The search for promising plant extracts and subsequent activity-guided isolation put specific requirements on the bioassays to be used. They must be simple, inexpensive and rapid in order to cope with the large number of samples including extracts from the screening phase and all fractions obtained during the isolation procedure. They must also be sensitive enough to detect active principles which are generally present only in small concentrations in crude extracts. Their selectivity should be such that the number of false positives is reasonably small [10,11].

The classical approach leading up to a hit or lead compound involves extract preparation and pre-fractionation, biological screening in pharmacologically relevant assays, and isolation and characterization of the active compound(s) through bioassay-guided fractionation [12]. Though this approach is more selectively targeted towards the discovery of bioactive compounds, it can be laborious [2] and synergism and competition of constituents in extracts and fractions limit its use, as these factors may give false positive or negative results in the isolation process [13-16]. Microfractionation of extracts as a bioassay strategy represents a modern approach of the classical bioassay-guided fractionation and has the advantage of utilizing less material than the conventional method. Microfractionation with HPLC requires the microfractionation of the crude extract and drying of fractions, usually in 96-well microtitre plates. This can be used for measuring and localizing the bioactive NPs by correlating the observed biological activity in the well plates with the corresponding component in the chromatogram [17] and often incorporating

analytical tools (LC-UV/DAD, LC-MS, LC-NMR), as shown in the recent study on the extract of *Rhynochosia viscosa* [18]. This target-based technology speeds the drug discovery process and eliminates the laborious, time and resource-costs of the isolation process [18-20]. These methods though, are tedious, as the bioactivity is determined after the off-line evaporation of the mobile phase used in chromatography. To circumvent these difficulties, techniques combining the chromatographic separation with an on-line biological detection step, have been developed. Biochemical detection methods are one such method as this technique based on mechanism-based screening, have become one of the major breakthroughs in drug discovery. Biochemical detection (BCD) assays can be defined as the detection of bioactives based on biochemical reactions or simulated biochemical reactions [21]. BCD methods include enzyme activity/affinity detection (EAD), receptor affinity detection (RAD), metabolite profiling systems, and antioxidant activity assays, as tools to identify bioactives in plant extracts. These methods are used to fast-track identification of bioactives in extracts or mixtures without the need for lengthy separation and isolation procedures [21]. The instrumentation consists of a HPLC-DAD and/or MS apparatus in hyphenation with BCD. Plant extracts are initially separated by HPLC and then a post-column bio(chemical) assay determines the bioactivity of the individual analytes while DAD, MS, or NMR can be used simultaneously as detectors for the identification of the active compounds [22]. The combination of analytical technologies and continuous flow biochemical detection has enabled biological and chemical evaluation of bioactive molecules within a single analysis and profoundly reduced the time required for compound characterization [23]. In a high throughput bioassay, Schobel et al. [24], applied this technique in the preliminary screening of 9888 plant extracts for ERα and ERβ binding activity, and the six most active extracts analysed by information from the MS data. Ingkaninan et al. [25], also employed this method in the isolation of an acetylcholinesterase (AChE) inhibitor from Narcissus 'Sir Winston Churchill'. The alkaloid, identified as ungiminorine exhibited mild inhibitory activity on AChE.

1.3 Chemical Screening in Natural Products Research

Chemical profiling of crude extracts usually involves separation of compounds in the form of chromatography and determination of the structure of compounds in the form of spectroscopy and spectrometry. Chromatography is a separation technique based on the distribution of a compound between two phases- a moving, mobile phase that is passed over an immobile stationary phase. Two types of chromatography are operational; gas chromatography (GC) and liquid chromatography (LC), classified according to their mobile phase.

Gas chromatography is a widely used analytical technique especially for the separation of volatile compounds. The GC system is comprised of the carrier gas system, an injection system, a separation column, splitter and detector and a trap to collect the effluent [26]. In NPR, it is applied for the isolation of low volatility compounds such as terpenes, monoterpenes, sequisterpenes and their oxygenated derivatives which are ubiquitous in and responsible for the characteristic aroma of essential oils, a known important source of natural products with pharmacological activities [27-29].

Liquid chromatography is classified mainly on the basis of the physical arrangement of the system; column chromatography (vacuum LC, flash LC, low-pressure LC, medium-pressure LC and high-pressure LC), planar-chromatography (TLC, paper chromatography), or between two liquid phases (counter-current chromatography). Thin-layer chromatography (TLC) is the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment while the HPLC is the most versatile, robust and widely used technique for the isolation of natural products. Stitcher in his review [30], discussed the liquid-solid and liquid-liquid isolation techniques in their various forms, and their applications in natural product isolation.

Prior to the era of spectroscopy, physical properties such as boiling point, melting point, refractive index, organoleptic tests and various colour reactions were used to obtain some preliminary structural information. These were then compared to similar structures in literature to determine if there were any close matches. Where more than one structure existed, these compounds were converted to a known derivative and properties tested and compared. However with the advent of spectroscopy (NMR, IR, and UV) and spectrometry (MS), an organic natural product chemist is equipped with the technology to identify these metabolites. These

classical instrumental techniques though, present their limitations which include:

1.3.1 Isolation of known metabolites

For drug discovery purposes, the frequent isolation of known metabolites heightens the overall efforts especially in bioassay-guided fractionation, since some common or ubiquitous compounds may confuse the natural products chemist in his search for new compounds [31].

1.3.2 Small sample size

Spectroscopic methods, particularly NMR, usually require 1 mg or more of compound in order to obtain the prerequisite spectra [32]. Whereas microgram quantities of material may be sufficient for assaying for biological activity, milligram quantities are required for structural elucidation. Isolating these quantities requires considerable time, sample handling and effort, and often times, includes recollection of plants or re-fermentation of micro-organisms, and extraction on a large scale which is cost intensive.

1.3.3 Instability of bioactive compounds

Some bioactive compounds occur as geometric and stereo- isomers. In some cases, these compounds even when well resolved using the classical chromatographic methods, become unstable on drying [33]. This may lead to artifact formation or even the complete destruction of the compound. This therefore poses a problem with the traditional approach to structural elucidation.

1.3.4 Environmental hazards

In NPR, the minor compounds are often found to be biologically active. To isolate these compounds in even milligram quantities, large amounts of plant material, 10 kg or more, are often required for extraction. This invariably leads to deforestation and worse still, extinction of some rare medicinal plants [34].

For efficient separation of metabolites, good selectivity and sensitivity of detection, together with the capability of providing on-line structural information, hyphenated liquid chromatographic (LC) techniques are preferred [35]. They play an important role as an analytical support in the work of phytochemists for the efficient localization and rapid characterization of natural products. This chemistry-based approach integrates analytical tools and efficient spectral databases at the fractionation stage, with a focus on dereplication (identification of known compounds present in an extract prior to their isolation) and structural elucidation of novel compounds [36,37].

2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Two major goals of NPR are 1) to isolate a compound responsible for a particular biological activity and, 2) to purify a sufficient amount of that compound to characterize it partially or fully [38]. In isolating natural products from complex crude extracts, a lot of pre-fractionation and fractionation techniques are employed, sometimes yielding very little success. To achieve efficient isolation of natural products from these complex matrices without the need for complex sample preparation, the high performance (or high pressure) liquid chromatography (HPLC) has become one of the most robust and versatile techniques used for the analysis of multicomponent samples on both an analytical and preparative scale [39,40]. It is routinely used in phytochemistry to pilot the preparative isolation of natural products and to control the final purity. HPLC is the most widely used analytical separation technique for the qualitative and quantitative determination of compounds in natural product analysis. The advantages over most other separation techniques include convenience, speed, choice of column stationary phases, high sensitivity, applicability to a broad variety of sample matrices and ability to hyphenate the chromatographic method to spectroscopic detectors [5]. Many bench top HPLC instruments now are modular in design and comprise a solvent delivery pump, a sample introduction device such as an auto-sampler or manual injection valve, an analytical or preparative column (stationary phase), a guard column, detector and a recorder or a printer. A schematic/ operational illustration of an HPLC system is provided in Fig. 1. In his review [39], Stead extensively discussed the principal separation modes used in HPLC, column-packing materials and solvents, preparative HPLC instrumentation and detection methods, chromatographic method development, and the practicalities of carrying out a preparative HPLC-based natural product isolation.

Over the years, there have been improvements on the performance of the HPLC in terms of resolution, speed and reproducibility. This is attributed to technological advances in some basic conditions for the operation of the HPLC. Such is the new invention, ultra high pressure liquid chromatography (UHPLC) which has been introduced in NP research. The UHPLC which has been applied in the analysis of complex mixtures like plant extracts, operates at very high pressures, uses a stationary phase of less than 2 µm and mobile phases at high linear velocities, allows for a remarkable decrease in analysis time, increase in peak capacity, sensitivity and reproducibility compared to the conventional HPLC [41]. The UHPLC and other newly developed fast-LC approaches such as monolithic columns and high-temperature liquid chromatography (HTLC), have been qualitatively and quantitatively evaluated and found to decrease analysis time and maintain good efficiency in LC, while been equivalent to conventional LC from a quantitative point of view in terms of trueness, precision and accuracy [42].

The end requirement of the pure compounds determines the size and scale of the isolation technique. Analytical scale HPLC systems with column internal diameter (i.d) usually around 4.6 mm, are employed for studies which involve microgram quantities such as in initial bioassay studies and for obtaining information about sample mixtures without relying on their recovery. Laboratory preparative (prep-) scale HPLC systems are employed for isolation studies involving structural elucidation (especially carbon and 2D NMR experiments) and in-vivo studies requiring milligram quantities of pure compound, and the column i.d range from 10 to 100 mm [17,40].

The HPLC has become very popular in the natural product laboratories and has found successful application and often used in combination with other types of column and planar chromatography, for the separation of complex mixtures and is generally the final purification step in natural product isolation [44-48]. It also finds application in standardization of herbal medicines as it is routinely used for the analysis of many phytopharmaceuticals and documented as a quality control technique in most pharmacopoeias [49-52].

The development of measurement techniques coupled (hyphenated) to HPLC has provided powerful tools such as LC-ultraviolet-photo diode array detection (LC-UV-DAD), LC-mass spectrometry (LC-MS), LC-multiple stage MS (LC-MS"), LC-nuclear magnetic spectrophotometry (LC-NMR) and LC-infrared spectrophotometry (LC-IR). These hyphenated systems generate multi-dimensional data (chromatographic and spectroscopic) for online identification and dereplication purposes.

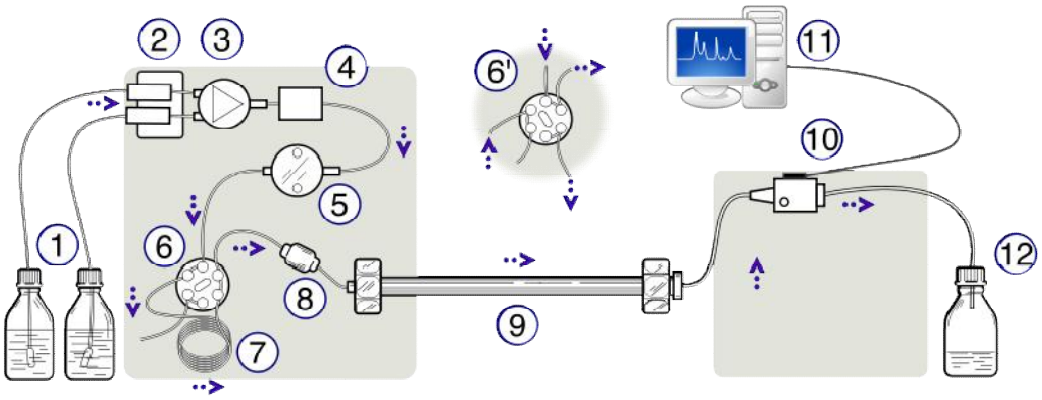

Fig. 1. Schematic representation of a High Performance Liquid Chromatograph created by Yassne Mrabet and reprinted from Ref. [43]

(1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) UV-Detector, (11) Data acquisition, (12) Waste or fraction collector

2.1 HPLC Hyphenated Techniques in Natural Product Analysis

Hyphenated techniques are methods combining two or more analytical techniques (usually a separation and a spectroscopic technique) into one integrated technique described by the use of a hyphen e.g. GC-MS, LC-MS, LC-NMR, and LC-UV-DAD. The "hyphen" refers to the process and hardware used to make these techniques compatible, and turns the entire hyphenated system into a single instrument [53]. Gas chromatography–mass spectrometry (GC–MS) was the first widely used hyphenated technique, and is one of the most accurate and efficient tools in chemical analysis, though restricted, for analyzing volatile organic samples [54]. In the last two decades, hyphenated liquid chromatography techniques particularly with the HPLC, have recorded much success in natural products research. The combination of the high separation efficiency of HPLC with these different detectors has made possible the acquisition of on-line complementary spectroscopic data on an LC peak of interest [19] (Fig. 2). The coupling of various hyphenated methods together (hypernation) has the advantage that all structural information is obtained within one single analysis, and this has been demonstrated in an on-line HPLC-UV(DAD)-FT-IR-NMR-MS analysis of natural products, where two compounds, 20-hydroxyecdysone and polypodine B, were identified [55,56]. This is not an easy task to perform though, since operation conditions that are compatible (compatibility of the eluent composition, sensitivity and dynamic range of the methods being hypernated) with all of them, have to be found [19].

Some advantages of HPLC hyphenated techniques include:

a) Identification of known compounds present in an extract prior to their isolation (dereplication). Dereplication strategies employ a combination of separation science, spectroscopic detection technologies and on-line database searching. Whereas LC-UV-MS [57], with information from the database search engine [58], provide the preliminary structural information needed for dereplication, LC-NMR provides complimentary information in cases where there is some difficulty in identifying the compounds and for detailed online structural analysis [59]. Time and resource investment in an often tedious preparative isolation process is reduced, with more concentration of resources on the elucidation of novel chemical entities [60]. This makes the production and analysis of large natural product libraries more efficient in practical applications.
b) Comprehensive metabolite profiling especially in the ongoing field of plant metabolomic research. Metabolite profiling which refers to the detection and identification of plant metabolites, is very difficult to achieve with crude extracts due to their complex nature. A metabolite database employing LC-MS was used in determining the metabolomics of tomato fruit, *Solanum lycopersicum* [61]. This way, several novel compounds not previously reported for tomato fruit were identified in this manner and added to the database.
c) Compounds with very similar polarity can be more easily separated and identified which would otherwise be more tedious if preparative purification of individual components on a sufficient scale for bioactivity testing was to be achieved by employing the traditional approach [62].
d) Structural investigation of labile or unstable natural products. A typical example is the investigation of the methanol extract of an African Scrophulariaceae species *Jamesbrittenia fodina*. In this study, one of the fractions contained two isomeric peaks in the same proportions, suggesting that the constituents were probably unstable upon drying of the fraction. This was analyzed using stop-flow LC/^{1}H- NMR [33]. Similarly, Schaller et al. [63] employed the on-flow and stop-flow LC-NMR to isolate two unstable diterpenes from a Zimbabwean tree, *Bobgunnia madagascariensis* and study their interconversion reactions.
e) Chemical fingerprinting and quality control of herbal drugs. One of the challenges experienced with natural products research is the inconsistent reproducibility of the crude extract against a biological target. Apart from biological assay protocols, factors such as time of collection, habitat and mode of growth of plant (wild, cultivated), mode of collection, drying of plant are also considered in standardization of herbal medicines. Fingerprinting analysis is used to evaluate the quality of the herbal material both

qualitatively and quantitatively, and relate it to the observed biological activity [64]. In their study, Montoro et al. [65] employed an LC/MS/MS method to quantify glycyrrhizic acid in *Glycyrrhiza glabra* root samples and found the Chinese roots to contain the highest amounts of glycyrrhizic acid, followed by those from Italy. The chromatographic fingerprint of *Desmodium styracifolium* using HPLC-DAD-MS^n was also reported by Zhou et al. [66] in which 20 common peaks were identified.

2.1.1 Liquid Chromatography- Ultraviolet–Diode Array Detector (LC/UV-DAD)

The single wavelength detector has long been replaced by the photodiode array detector (DAD or PDA) for online structural determination. A photodiode array is a lined array of discrete photodiodes on an integrated circuit (IC) chip for spectroscopy. It is placed at the image plane of a spectrometer to allow a range of wavelengths to be sensed concurrently. Unlike the single wavelength detector where the sample must be injected numerous times with changing wavelength, DAD can be programmed to accommodate a wavelength range so that all molecules absorbing within the range can be identified in a single analysis [67]. DAD detection provides UV/VIS spectra directly online and is particularly useful for the detection of natural products possessing characteristic chromophores (e.g. polyphenols, polyketides, phenolics and alkaloids). All wavelengths are stored during analysis, and thus, multiple wavelengths can be monitored at the same time for detection of different classes of compounds. Combined with post-column addition of UV shift reagents classically used for the structural characterization of flavonoids, more information is obtained on the precise localization of the hydroxyl groups on the polyphenols as this is achieved by comparing the genuine and shifted online DAD spectra online [5,6,37]. The incorporation of reference compounds in databases provides spectral libraries which has facilitated dereplication studies as computer matching can be realized automatically. However, the compounds being analyzed must be subjected to the same HPLC conditions, as the composition of the mobile phase may affect the UV bands slightly [5]. The utility of hyphenation of the HPLC with DAD is limited as the technique requires mobile phases with low cut-offs and it is not applicable to organic compounds without chromophores. Politi et al.[68] employed the HPLC-UV/PAD for the partial dereplication of vismiones, anthraquinones, flavonoids, benzoquinones and xanthones and partial identification of two new bianthrones from the leaf and root extracts of *Vismia guineensis*. In a quality control assay of herbal medicines, 12 authentic and 26 commercial samples of *Fructus Aurantii immaturus* were quantitated and identified by HPLC-DAD combined with chemometric methods [69].

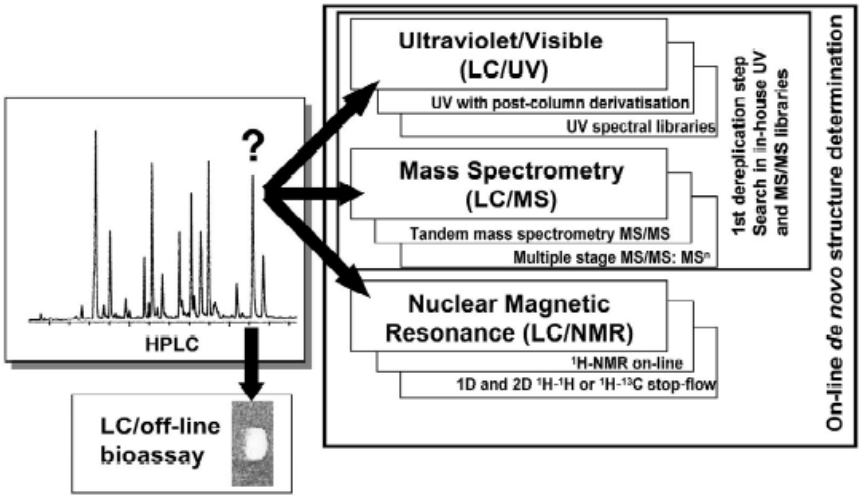

Fig. 2. Schematic diagram of type of information that can be obtained on a given LC peak with different LC-hyphenated techniques available designed by Wolfender et al. Reprinted from Ref. [19]

2.1.2 Liquid Chromatography- Mass Spectrometry (LC-MS, LC-MS-MS^n)

Mass spectrometry (MS) is one of the most sensitive methods of molecular analysis. It has the ability to determine the molecular weight and to obtain structural information of the analyte. Due to its high power of mass separation, very good selectivities can be obtained [9]. The LC-MS has become the most generic hyphenated technique used today, separating the crude extract using reversed-phase HPLC, and the eluent post-column split into a mass spectrometer and a fraction collector. The coupling between LC and MS had been difficult because of the incompatibilities between HPLC (liquid-phase operation, high pressures, high flow rates, and relatively low pressures) and MS (high vacuum, high temperatures, gas-phase operation, and low flow rates), but to overcome these challenges, different LC-MS interfaces were developed [35]. The most popular interfaces used in LC-MS are based on atmospheric pressure ionization (API) techniques and the two most commonly used are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). APCI is suitable for low and moderate polarity compounds while ESI is most widely used for the analysis of polar molecules [70]. Herderichet al.[71], discussed the potentials and limitations of these two techniques for the analysis of natural products. These API techniques which require some combination of high voltage and heat, is used to provide the ionization that is needed to produce the ions that are assayed by the MS system. Analyte ionization is largely compound dependent and is governed mainly by proton affinity. Soft ionization of the analytes is produced to obtain molecular mass information, thus molecular ion species are mainly recorded in the form of either protonated molecules $[M+H]^+$ (positive-ion mode, PI), deprotonated molecules $[M-H]^+$ (negative-ion mode, NI)[72].Different adducts (e.g., $[M + Na]^+$ (PI) or $[M + HCOO]^-$ (NI) are also produced, depending on the solutes and modifiers used. Compounds such as glycosides will have a high affinity for salts and will tend to form sodium adducts in PI [5]. Adduct formation and in-source fragmentation (for example, the loss of water) may complicate the process of identifying the molecular ion. Since only restricted fragment information is provided by this ionization technique, complimentary structural information on the molecular fragmentation can be generated by in-source collision-induced dissociation (CID) in HPLC-MS-MS or MS^n experiments. The term "MS-MS" refers to the coupling of two mass analyzers and an inert target gas, which is introduced between the two mass filters, enhancing fragmentation [73]. In the two-stage MS experiment, an ion of interest (the precursor ion) is selected in the first stage and then fragmented, and the masses of the fragments are then measured, constituting the second stage. The generated CID spectra are, however, not comparable to those recorded by EI, and this hampers a direct use of the standard EI-MS natural products libraries for dereplication purposes [5]. For performing automated dereplication procedures, specific LC/MS-MS libraries have to be built based on standards available in a given laboratory, which consequently limits this approach [19]. Another challenge experienced with the LC-MS is in finding an optimal ionization condition suitable for analyzing a crude extract. A crude extract may be viewed as a complex mixture of different secondary metabolites with molecular weights and in different concentrations, with different physical and chemical properties, and it would be difficult for each of the ionization techniques to efficiently analyze the extract. This could be overcome by analyzing the crude extract using different ionization modes [74]. Several types of mass analyzers are used in the HPLC-MS application. These include those of low resolution, such as the single quadrupole (Q) mass spectrometers, those giving high resolution and exact mass capabilities, such as time-of-flight (TOF) and the Fourier transform-based (FT) mass spectrometers, the triple-quadruple (QQQ) MS-MS systems for measuring structurally relevant fragments or for very specific detection and the ion-trap (IT) mass spectrometers which have the unique capability of producing multiple stage MS-MS (MS^n) data that may be essential for structural elucidation purposes [5]. The TOF and QQQ mass analyzers are more readily compatible with UHPLC than IT and FT mass spectrometers [75]. Funari and co-workers [76], recently applied the UHPLC-MS as a dereplication tool for the metabolite profiling of Brazilian *Lippia* species in which fourteen compounds were unambiguously identified. Fu et al. [77] applied a combination of HPLC/ESI-TOF-MS and HPLC/ESI-IT-MS^n to the screening of phenolic and other polar compounds in olive leaf extracts, allowing for high resolution acquirements, accurate mass measurements and complimentary structural information. HPLC-MS is commonly used as multi-hyphenated with UV-DAD for dereplication purposes as demonstrated in the study by Ferrari et al. [78]

where three C-glycosyl flavones were identified on-line by LC-UV-APCI-MS^n analysis of the crude extract of *Gnidia involucrate*. Six xanthones and secoiridoids were also identified in a chemical screening by LC with online UV photodiode array detection (LC-UV-DAD) and thermospray (TSP) mass spectrometric detection (LC-TSP-MS) on the crude extracts of *Swertia calycina* [79]. In an assessment study by Simirgiotis [80], the phenolic profile of two edible plants, *Cryptocarya alba* and *Crataegus monogyna*, called peumo in Chile, were investigated by HPLC-DAD-ESI/MS-MS, and the HPLC fingerprints used to authenticate and differentiate the two species.

2.1.3 Liquid Chromatography- Nuclear Magnetic Resonance (LC-NMR)

The coupling of high liquid chromatography with nuclear magnetic resonance, LC-NMR is one of the most powerful techniques in the isolation and structural elucidation of unknown compounds in crude mixtures [81]. 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. LC-NMR represents a potentially interesting complementary technique to LC-UV-MS for detailed on-line structural investigation of compounds presenting original structural features or displaying interesting activities after LC-bioassays [59]. It is a non-destructive technique and this allows for the full recovery of all components for further studies such as bioassays or mass spectrometry [60,82]. LC-NMR is the most versatile of hyphenated techniques, as full structural and stereochemical information can be obtained (by use of 2D NMR). It also has the advantage of being a highly non-selective detection technique as ^{1}H-NMR spectroscopy will detect any hydrogen-containing compound present in the HPLC eluatein a sufficient amount regardless of its structure. Integration of LC-NMR with other known LC-coupled techniques provides a powerful tool for on-line de novo elucidation of compounds of interest [83].

The HPLC-NMR hyphenation involves two main modes of operation, the direct and indirect HPLC-NMR methods. Direct HPLC-NMR methods are those in which the NMR data acquisition is performed on the HPLC column eluate, in the solvent used for chromatography. There are three major modes of operation in the direct HPLC-NMR method; continuous (on)-flow, stop-flow, and loop storage modes. The HPLC system is connected directly to a flow-cell of the NMR spectrometer operating in any of these modes. The indirect HPLC-NMR method is the HPLC-SPE-NMR mode. A few examples of these modes of operation, and their applications in natural product analysis, are provided in Table 1.

As the name suggests, the continuous-flow experiments the NMR spectra are acquired continuously, while the chromatography is running. Due to this, the residence time of the analyte in the NMR flow-cell is limited, also limiting the acquisition time, and affecting the spectral quality [81]. Continuous flow experiments provide only preliminary information about extract or fraction components, normally restricted to major components. To increase measurement sensitivity and perform two-dimensional NMR experiments or 1D experiments with small amounts of the analyte which require a longer acquisition time, the flow must be stopped when the chromatographic peak of interest reaches the NMR cell. In the stop-flow mode, selected chromatographic peaks can be trapped in the HPLC-NMR for a period, thereby allowing for extended acquisition times and consequently greatly improved sensitivity and resolution in the resulting ^{1}H-NMR spectra [53]. Depending on the chromatographic conditions and acquisition period with stopped flow, there may be a diffusion-mediated band broadening of analytes within the column which can be manifested both as a decrease in concentration and as collapsing peaks [84].To avoid this, a device was developed that directs individual chromatographic peaks into capillary loops, and controls the transfer of loop contents, one-at-a-time, into the NMR flow-probe [82]. In the loop-storage technique, the loops are made of capillaries and not cavities as the NMR flow-cells, and only minimal peak broadening occurs. More so, the flow-cell can be extensively washed between transfers of individual peaks stored in the loops, and thus analyte cross-contamination can be avoided. The only drawback with this technique may be the increased chance of decomposition of unstable compounds [53]. It can be regarded as an intermediate between direct and indirect HPLC-NMR methods because though acquisition of NMR data can be physically and chronologically disconnected from the HPLC separation, the NMR data are still obtained in the solvent used for HPLC.

Table 1. Different NMR hyphenated modes of operation and their application in natural product analysis

Mode of operation	Plant	Class of compounds characterized	Reference
On- flow	*Orophea enneandra*	Lignans, tocopherol, polyacetylene	Cavin et al. 1998, [94]
	Belamcanda chinensis	Isoflavonoids	Kang et al. 2008, [95]
	Ancistrocladus guineensis	Alkaloids	Bringmann et al. 1998, [96]
Stopped-flow (including loop storage)	*Monotes engleri*	Prenylatedflavanones	Garo et al. 1998. [97]
	Schizanthus grahamii	Tropane alkaloids	Bieri et al. 2006,[98]
	Swietenia macrophylla	Limonoids	Schefera et al. 2006, [99]
HPLC-SPE-NMR	*Hypericum perforatum*	Naphtodianthrones, phloroglucinols, flavonoids, phenolic acids	Tatsis et al. 2007,[100]
	Warburgia salutaris	Sequisterpenes	Clarkson et al. 2007, [62]
	Smirnowia iranica	Isoflavonoids	Lambert et al. 2005, [101]
	Neolitsea sericea	Flavonoid glycosides, Isoquinoline alkaloids	Lam et al. 2008, Lee et al. 2008, [102,103]

Apart from the general lower sensitivity of the NMR detector, the main drawback of the direct HPLC-NMR methods is the solvent used for chromatographic separation; since most solvents contain hydrogen, they will give rise to an NMR signal much stronger than that of the analyte. This results in the difficulty of observing analyte resonances in the presence of the much larger resonances of the mobile phase, thus the need for elaborate solvent suppression [5]. Suppression of solvent brings about distortion of the spectral region where the solvent resonances were present, and distorts or removes nearest analyte resonances. The use of deuterated solvents for chromatographic separations can be used to minimize solvent resonances, but the cost is very high [81].

Due to some of these challenges experienced with the direct NMR hyphenation, the indirect HPLC-NMR, often known as HPLC-SPE-NMR which is based on post-column analyte trapping by solid-phase extraction, was developed. In this approach, chromatographic peaks eluted from a reversed-phase HPLC column are passed one by one through small solid-phase extraction columns (SPE cartridges) in order to remove the analyte from the HPLC mobile phase. The SPE cartridges are subsequently dried with nitrogen gas, and the analytes desorbed with a deuterated solvent for NMR spectroscopy. The use of solid phase extraction (SPE) interface between the NMR spectrometer and the chromatograph, enables analyte focusing, change from a non-deuterated HPLC solvent to a deuterated NMR solvent, and multiple SPE trapping for increasing sensitivity [85]. The combined benefits of analyte concentration and accumulation and use of deuterated solvents enable acquisition of high quality 2D NMR data (COSY, gHSQC and gHMBC) from multiple peaks, including minor peaks present in very complex chromatograms, without compromising the chromatographic separation process [86]. Major classes of secondary metabolites have been isolated by this technique as shown in Table 1, and the hyphenation of HPLC, PDA, MS, SPE and NMR, and often to on-line determination of bioactivity, has emerged as an attractive strategy in the dereplication, structural elucidation of new compounds and drug discovery process [87-89].

Cryogenic technology and capillary (Cap) LC-NMR are the other important developments in NMR spectroscopy. Cryogenic cooling of the NMR radio frequency coils and electronics and the development of new miniaturized probe

technologies give the possibility to preconcentrate the samples, with the aim of increasing sensitivity and reducing costs [90]. Miniaturization of separation techniques is potentially important in hyphenated NMR, particularly for mass-limited samples in drug discovery [82]. The introduction of miniaturized solenoidal micro coils with an active detection volume of 1.5 µl allow detection limits in the low-nanogram range for low molecular weight metabolites [91,83]. The sensitivity of miniaturized separation techniques hyphenated to NMR is higher, since capillary separations give rise to better resolved peaks. In addition, the challenges of solvent suppression are overcome because the solvent consumption for separations are low, which makes the use of fully deuterated solvents economically feasible [92]. Although capillary NMR can be successfully hyphenated to the matching HPLC equipment, it is mostly used in an off-line mode. Dried samples are taken up in few microliters of NMR solvent and introduced into the probe manually or by the aid of a syringe pump [85]. The off-line combination of HPLC-SPE and Cap NMR was employed, to isolate and characterize five sequisterpene lactones and four phenylpropanoids from the crude extract of *Thapsia garganica* [93].

3. OTHER LC TRENDS IN SEPARATION TECHNIQUES

3.1 Counter-current Chromatography

One separation method gaining wide acceptance in natural product chemistry is the counter-current chromatography (CCC). This technique, which has been found comparable though not as efficient as HPLC, can be explored for fractionation and isolation purposes [104,105]. Separation of compounds is based on the principle of liquid-liquid partitioning chromatography; continuous partitioning between two immiscible liquid phases, without a solid support. One of the liquid phases of the two phase system is used as the stationary phase using either gravitational or centrifugal force to retain the stationary force in the column while the other liquid phase is used as a mobile phase pumped through the stationary phase. The immiscible phases usually have an auxiliary solvent, miscible in both phases, aiding in the partitioning of the analytes between two immiscible phases. The use of the two-phase solvent system results in a good number of combinations of solvents to choose from, enabling separation of compounds with a wide range of polarities. The separation of the solutes is achieved as a function of the specific partitioning coefficient (Kd) of each solute between the mobile and stationary phases [106].

Two types of CCC systems are commercially available; the hydrodynamic and hydrostatic machines. The hydrodynamic counter-current chromatography apparatuses use a planetary rotation movement around two rotating axes and are referred to as CCC instruments while the hydrostatic counter-current chromatography apparatuses employ a rotary movement of the column which rotates around one single rotation axis, usually referred to as CPC instruments [30]. In his review article, Ito [107] explained the practical CCC procedures and how to use the instrument under optimal conditions. The main advantages of CCC over the more traditional liquid-solid separation methods are (i) There is no irreversible adsorption; (ii) Low risk of denaturation of the sample due to interaction with silica; (iii) The technique is very economical as it requires low solvent consumption and no expensive column packing material (iv) High performances with purity > 99.9 % and recovery > 90 %; (v) Ability to reverse the flow direction and interchange the mobile and stationary phases during a given chromatographic process [108]. One of the major challenges faced with CCC, is the selection of a suitable two-phase solvent system for method development, as this is very critical for a successful isolation and separation. In a review by Yoon et al. [109], different solvent systems and methodologies for the isolation of natural products were discussed. CCC finds applications in bioassay-directed drug discovery efforts as the major advantage evolves from the complete lack of a solid stationary phase, thereby providing a means of loss-free fractionation [105,110] and metabolic studies [111].

Technological improvements on mechanical parts of older CCC instruments like coil planet countercurrent chromatography [112], droplet countercurrent chromatography (DCCC) [113] and rotation locular counter-current chromatography (RLCCC) [114] have led to the development of newer and more efficient CCC apparatus such as high speed countercurrent chromatography (HSCCC) and high performance (or fast centrifugal) partition chromatography (HPCPC or FCPC) [115]. HSCCC is a CCC method radically improved in terms of resolution, separation time and sample loading capacity.

HSCCC is an efficient preparative technique, yielding multi-gram quantities of samples and widely used for separation and purification of natural products. However, it requires some simple but specific technical knowledge, since the selection of experimental conditions and the practical separation procedure are quite unique [112]. Applications of this technique include the isolation of six isoflavones from a crude extract of *Pueraria lobata* [116], chromone from the extract of *Aleovera* leaves [117], flavonol glycosides from the extract of *Trollius ledebouri* [118] and polyphenols from the extract of Chinese green tea, *Camellia sinensis* [15]. A novel elution mode called pH-zone refining CCC was developed after HSCCC and advantages of this method over the conventional HSCCC include higher loading capacity resulting in higher yield, monitoring of elution peaks of compounds with no chromophores with a pH meter, and highly concentrated fractions near saturation levels. The technique has been successfully applied to the separation of natural products [119].

Hyphenated CCC methods have also been developed for online detection and this has improved the efficiency of the technique as they (HSCCC, CPC) are now used in tandem with MS (ESI, APCI), HPLC- DAD and ESLD. The strategy was demonstrated with the preparative isolation and purification of hyperoside from *Hypericum perforatum* by HSCCC-HPLC-DAD with a two phase solvent system composed of ethyl acetate-ethanol-water (5:1:5 v/v) [120]. In a related approach, CPC-HPLC-DAD-MS was employed in the isolation, purification and identification of xanthones from *Garcinia mangostana* using a biphasic solvent system of heptane/ethyl acetate/methanol/water (2:1:2:1 v/v) [121]. Separation and characterization of flavonol glycosides from apple extract was achieved using CPC/ESI-MS. The CPC protocol used a biphasic solvent system composed of ethyl acetate/ethanol/water (4.5:1:4.5) in isocratic mode and acquisition of the MS data of the isolated compounds was performed in the negative mode [122]. A recent comprehensive review by Michel et al. [109] gave an overview of more applications of direct coupling of CCC or CPC with MS in natural products analysis.

4. CONCLUSION

Chemical screening of crude extracts using hyphenated techniques allows for the efficient targeted isolation of new types of constituents with potential activities as a complimentary approach to bioassay-guided fractionation. LC-hyphenated methods provide some good preliminary information on the nature of constituents of the extract with sample quantities required for structural elucidation significantly reduced. With the structural information, once the novelty or utility of a given constituent is established, a scale up of the chromatographic process of fraction can then be done to obtain a good yield of the constituent for full structure elucidation, biological and pharmacological testing. This way, the isolation of common compounds of little interest is avoided. The integration of HPLC with structurally informative spectroscopic or spectrometric detection techniques such as UV/DAD, MS and NMR, thus allows for crude extracts to be screened not just for biological activity, but also for structural classes. For natural products research to remain competitive as a drug discovery strategy, there must be continuous technological advances in dereplication, isolation, structural elucidation of novel compounds and efficient bioassay techniques. The integration of dereplication technologies with highly sensitive bioassays, either coupled directly (on-line) or indirectly (at-line), will advance the course of natural products research as these new perspectives will reduce the time and cost invested in the study of natural products, and speed up the drug discovery process. Technological advancements are needed for the detection methods to meet with the speed of separation provided by the sub 2-µm columns for HPLC. It is also hoped that in the near future, the challenges experienced with hypernation will be overcome as acquisition of all data in a single run makes processing more efficient and easier to perform. But before then, given the array of technologies, conventional and non-conventional, online and offline, and depending on their availability and feasibility in the laboratories, the natural products chemist has in his armory, a wider range of tools for the analysis of natural products.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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