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Review

LC–NMR for Natural Product Analysis: A Journey from an Academic Curiosity to a Robust Analytical Tool

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Abstract: Liquid chromatography (LC)–nuclear magnetic resonance (NMR) combines the advantage of the outstanding separation power of liquid chromatography (LC) and the superior structural elucidating capability of nuclear magnetic resonance (NMR). NMR has proved that it is a standout detector for LC by providing maximum structural information about plant originated extracts, particularly on the isolating ability of isomeric (same molecular formula) and/or isobaric (same molecular weight) compounds as compared to other detectors. The present review provides an overview of the developmental trends and application of LC–NMR in natural product analysis. The different LC–NMR operational modes are described, and how technical improvements assist in establishing this powerful technique as an important analytical tool in the analysis of complex plant-derived compounds is also highlighted. On-flow, stop-flow and loop-storage modes, as well as the new offline mode LC–solid phase extraction (SPE)–NMR and capillary LC (capLC)–NMR configurations which avoid the ingestion of expensive deuterated solvents throughout the experiment, are mentioned. Utilization of cryogenic probe and microprobe technologies, which are the other important promising approaches for guaranteeing sensitivity, are also described. Concluding remarks and future outlooks are also discussed.

Keywords: separation technique; spectroscopic technique; hyphenated techniques; LC–NMR; natural products

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1. Introduction

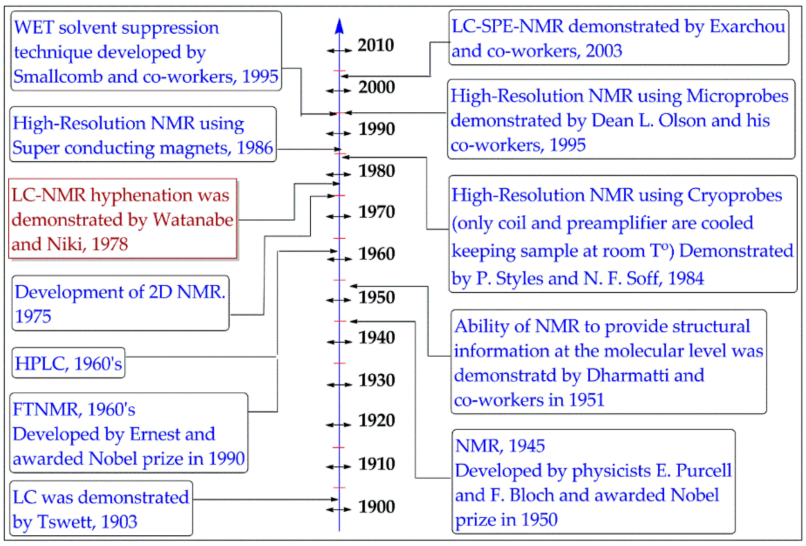
The extraordinary technological advancements and technical improvements achieved so far in innovative analytical hyphenated techniques help to enhance the full chemical characterization of natural products. The study of complex biological matrices such as crude plant extracts needs efficient detection and rapid characterization routes. At the early stage of separation, the initial identification of target constituents is essential for further selective identification procedures [1].

Although, liquid chromatography (LC) was conceived by the Russian botanist Mikhail S. Tswett in 1903, high-performance liquid chromatography (HPLC), comprising a variety of packed columns integrated with high-pressure pumps, was achieved in the late 1960s. An extensive advancement of HPLC, involving an automated computer controlled system with highly sensitive detectors, has extended the limits of detection to the femtogram level. These advances have caused HPLC to become an indispensable analytical instrument with a countless number of applications in both industry and academia [2]. However, the search for a universal detector for HPLC in separating complex mixtures, such as analysis of natural products, can still be challenging and requires extensive work.

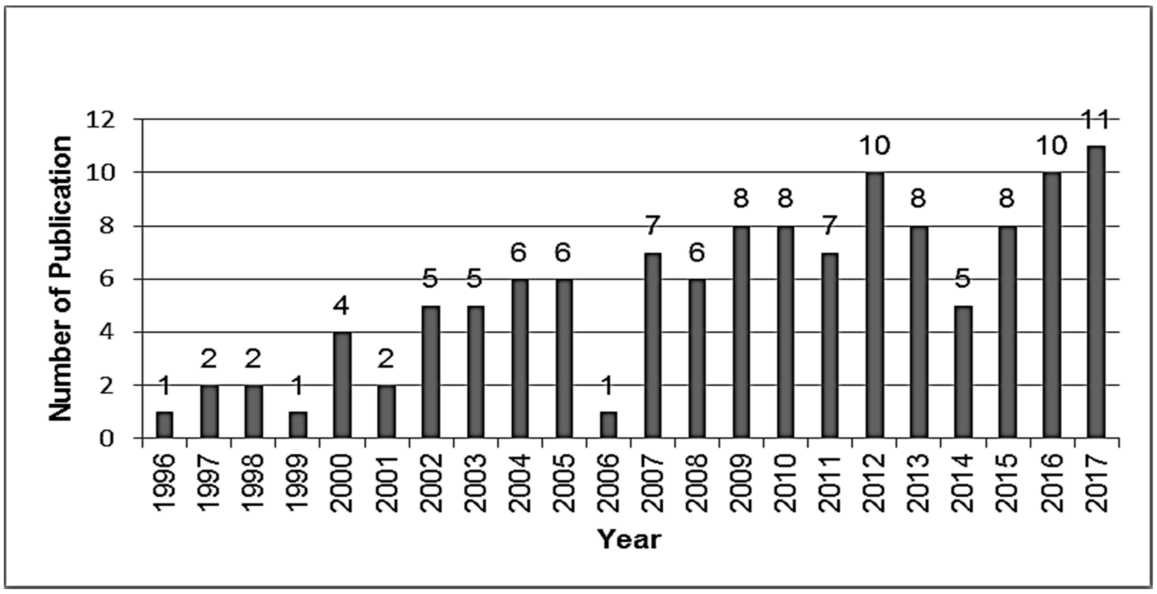
Initially, the nuclear magnetic resonance (NMR) spectroscopic technique was discovered in 1945 by physicists Edward Purcell of Harvard [3] and Felix Bloch of Stanford University [4] to determine the magnetic moments of all elements; however, the ability of NMR to provide structural information through chemical shifts at the molecular level was demonstrated in 1951 by Dharmatti, an organic chemist, and coworkers [5] in structure elucidation using the organic molecule ethanol. Later on, this discovery was rapidly implemented by organic chemists in elucidating the chemical structure of natural products and their metabolites as well as other complex chemical matrices.

Chemists have long searched for alternative detectors that can replace the traditional LC UV detector in natural product analysis that leads to the renaissance of hyphenated techniques. While UV detectors are very sensitive, they only provide limited information on the structure of the eluting compound. Hence, an extended development of verities of hyphenated techniques have rapidly emerged and been integrated as analytical tools for complicated mixtures such as crude plant extracts [6,7].

In chemical analysis, Infrared-spectroscopy (IR) and Mass Spectroscopy (MS) detectors, which provide better sensitivities and selectivities, would be preferred [8]. However, these spectroscopic detectors do not permit a full identification, except for some well-known natural products. Hence, a harmonized online detection technique is needed to analyze complex mixtures such as crude plant extracts. In this regard, coupling NMR as a detector with HPLC, generally abbreviated as LC–NMR, would be a sounding hyphenated technique for getting detailed online structural information [6,9,10]. Indeed, the increasing developmental progress in NMR technology has provided new motivation for the use LC–NMR [11], as shown in Scheme 1, which presents the timeline of major events associated with the development of LC–NMR. Therefore, it can be seen that the developmental progress of LC–NMR is steadily increasing. This is depicted in Scheme 2, which was compiled from the Sciencedirect website (among the leading platforms of peer-reviewed literature). Sciencedirect was used as a representative scientific website to examine the developmental trend of the hyphenated technique in the specified period of time. Hence, in this review, the developmental trends and applications of different modes (continuous flow mode alternatively called measurement under dynamic conditions; stop-flow as well as loop/cartridge storage modes collectively and alternatively called measurement under static conditions) of operation of LC–NMR were discussed with illustrative examples and practical applications in natural product analysis available in the literature.



Scheme 1. Timeline of major events connected with the development of Liquid chromatography (LC)–nuclear magnetic resonance (NMR).



Scheme 2. Number of articles published on LC–NMR in 1996–2017 in all application areas with "LC-NMR" as a searching key word (available online on Sciencedirect website: https://www.sciencedirect.com/ (accessed on 20 December 2018, 6:24 P.M.)).

2. LC–NMR Instrumentation and Principal Modes of Operations

2.1. LC–NMR Instrumentation

Analytical methods that integrate or connect chromatographs with spectrometers online are called hyphenated techniques [12], and they have attracted attention in recent years as high-throughput analytical methods that provide separation of mixtures as well as the spectra of the structural or compositional of various components at the same time. The term hyphenation or coupling also refers to the studies that have been conducted to combine these techniques, which resulted in improved standards that are unreachable with the individual techniques. Consequently, LC–NMR is a hyphenated technique that merges LC (separation technique) with NMR (structurally informative spectroscopic detection method). The general instrumentation of an LC–NMR system is given in Figure 1. The main component of an LC–NMR is the isolation zone (column), interface zone and the detection zone—i.e., probe for recording NMR spectra. The HPLC is directly connected to the NMR under a computer controlled data acquisition system with automated harmonization of the different operations. A sensitive detector, such as UV and/or MS, is usually coupled in parallel with a proper splitting ratio in order to monitor the main detector (NMR) measurements [13].

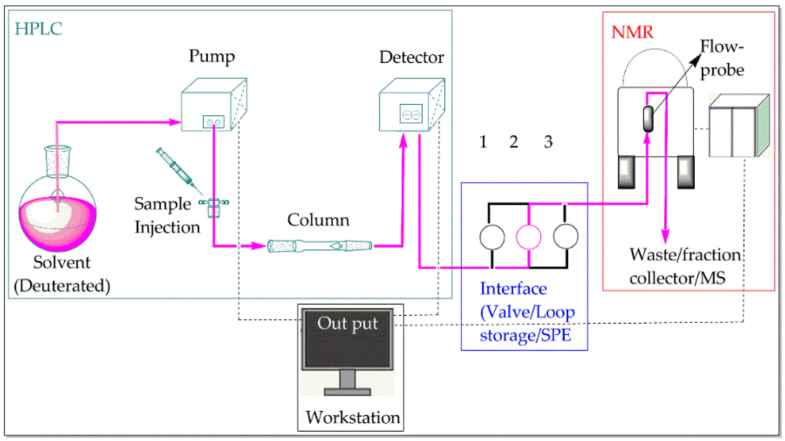


Figure 1. The instrumental setup of LC–NMR system.

2.2. LC–NMR Principal Modes of Operation

Several operational modes of LC–NMR have been employed in order to deal with the major sensitivity difficulties of NMR. Generally, LC–NMR experiments can be performed mainly in three modes—namely, continuous flow (measurement under dynamic conditions), direct stop-flow and loop/cartridge storage (measurement under static conditions) (Figure 2) [14]. All these modes of LC–NMR have been employed in several studies depending on the interest of the analyst and type of analysis where they have their own advantages and disadvantages, as described below.

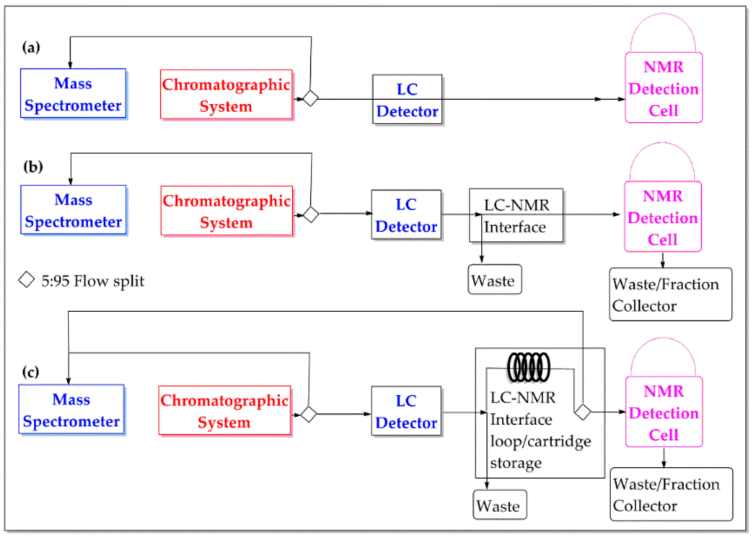


Figure 2. Schematic setups of the different LC–NMR working modes with parallel mass spectrometer (MS) detection: (**a**) online/continuous-flow mode, (**b**) stop-flow mode and (**c**) loop/cartridge storage mode.

2.2.1. Online-Flow Mode

In the online-flow mode, the outlet of the LC detector is connected directly to the NMR probe (Figure 2a) and while the compound peaks are eluting, the spectra are continuously acquired. The chromatographic system is used to separate and move the components through the NMR probe for detection. Continuous flow is the easiest setup since it does not involve any synchronization between the separation and the detection system, and thereby maintains a good separation resolution. However, it has a poor sensitivity due to the fact that the eluted peaks have a short exposure time in the detection cell—i.e., NMR flow cell [15]. Additionally, the chemical shifts of the sample as well as the solvent depend on the solvent properties which may cause shifting of the positions of NMR peaks, while the solvent composition is changing during elution [16].

2.2.2. Stop-Flow Mode

Similarly, in the stopped-flow mode, the NMR probe is directly connected to the outlet of the LC detector at the interface (Figure 2b). It has a better sensitivity as compared to the online-flow mode with a better signal-to-noise ratio as it is clearly indicated in the illustrative example given in Figure 3 [1]. It also permits the detection of only selected peaks of interest with the help of the valve found in the interface. After the eluted peaks are detected by the LC detector and have reached the NMR detection cell, the chromatographic working conditions (such as pump and gradient) should be stopped until the NMR signals are acquired, then the chromatographic working conditions continue until the next peak reaches the NMR detection cell again [14]. The time-slice mode is a modified extension of the stopped-flow mode, where the flow is stopped at regular and programed intervals. An LC–NMR experiment involving this type stopped flow have been also employed in several studies [17,18]. The major advantage of time-slice mode is that it can be used when the

separation is poor—for example, in identifying two analytes having closer retention times. Generally, the major disadvantage of the stop-flow mode may be that it is dependent on separations resolved for >2 min retention time [19].

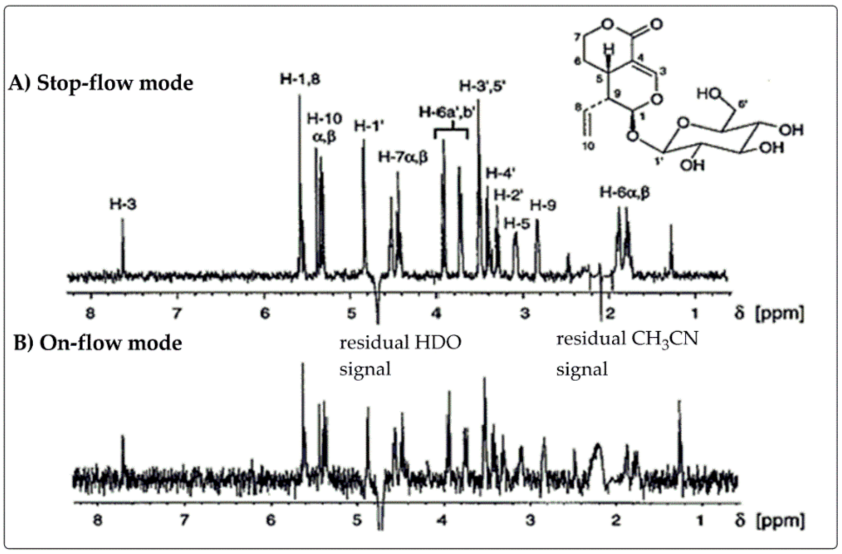


Figure 3. The comparison of stop-flow (**A**) and online-flow (**B**) modes of LC–1H–NMR spectra of sweroside in a dichloromethane extract of *Swertia calycina* depicting S/N ratio enhancement in stop-flow mode (reproduced from Ref. [1] with permission. Copyright © 1997, John Wiley & Sons, Ltd.).

2.2.3. Loop/Cartridge Storage Mode

In this type of mode, the outlet of the LC detector must be connected directly to sample storage loops or cartridges—i.e., loop storage or cartridge (solid phase extraction; SPE) storage (Figure 4). Usually, an LC detector (commonly UV and/or MS) is employed to monitor eluted peaks from the column in parallel. Then, the detected peak is directed and collected in either the sample loops or SPE cartridges with the help of the chromatographic working conditions. When the separation is completed, the LC pump can be used again to push the precollected peaks into the NMR flow cell by using a valve as shown in the general instrumentation block diagram in Figure 2c. In the new offline mode (SPE/cartridge storage mode)—also called LC–SPE–NMR, which was demonstrated by Exarchou and co-workers in 2003 [20]—nondeuterated solvents are used in the HPLC system and the separated peaks will be collected in SPE cartridges. After the SPE cartridges are dried using nitrogen, the deuterated solvent is used to push it to the NMR flow cell. Loop storage gives better peak resolution as compared to direct stop-flow mode (Figure 5) [16]. Several research groups have used the LC–SPE–NMR mode in the identification of natural products—for instance, Lambert and his coworkers reported ten new isoflavonoids in addition to the seven previously reported constituents from the roots of *Smirnowia iranica* (*S. iranica*) and two compounds are described in Figure 6 as illustrative examples to show the practicability of this mode [21]. Even though the loop/cartridge storage mode avoids the consumption of expensive deuterated solvents throughout the experiment, there might be sample decomposition/alteration during storage and also it requires additional special pumping equipment to elute samples from temporary storage [19]. Based on the current and future needs of the end-user, a literature survey and our opinion, a strong emphasis will be placed upon the LC–SPE–NMR mode due to its cost effectiveness, achieved by avoiding consumption of expensive deuterated solvents throughout the experiment.

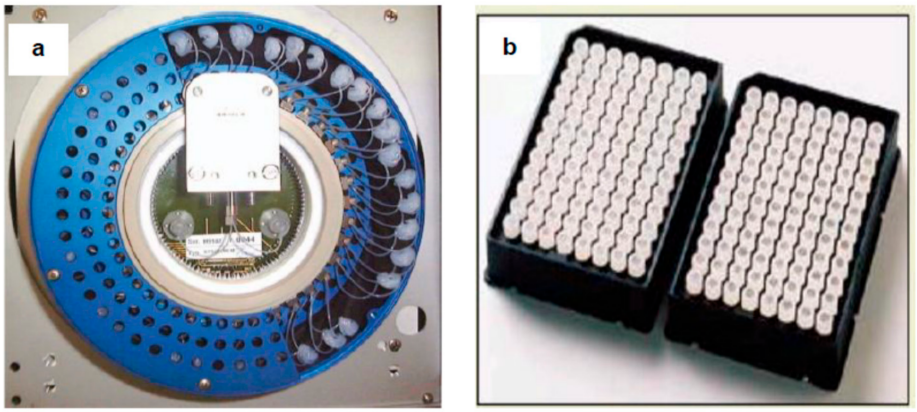


Figure 4. (**a**) Removable loop cassette for 36 sample loops (the position of eluted peaks in each loop is monitored by memory board sits in the center); (**b**) two units of Spark Holland solid phase extraction (SPE) Unit with 96 SPE cartridges (robot gripper is used to monitor the eluted peaks position in SPE cartridges).

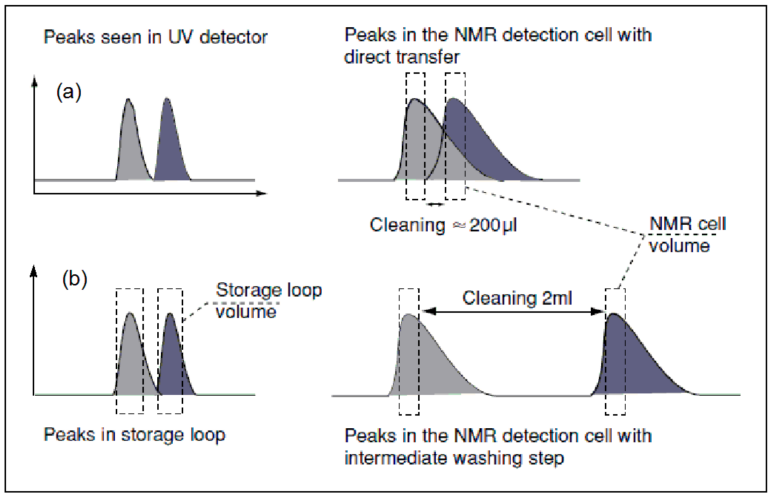


Figure 5. (**a**) Comparison of peak resolution of the direct stop-flow and (**b**) loop-storage/loop transfer procedures (reproduced from Ref. [16] with permission. Copyright © 2002, John Wiley & Sons, Ltd.).

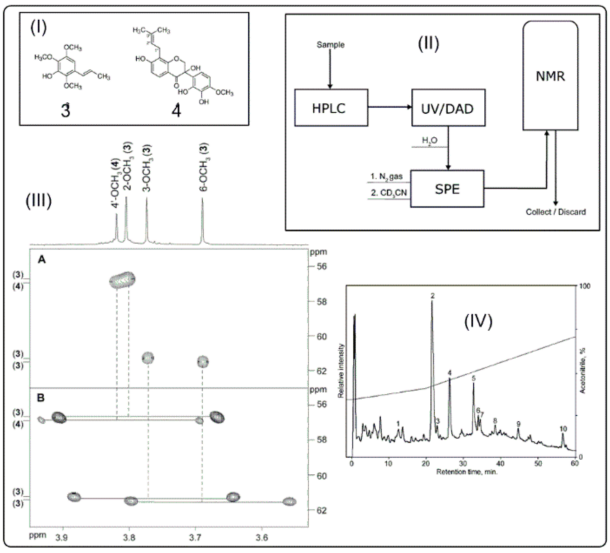


Figure 6. (**I**) Structure of extracted compounds of 3 and 4. (**II**) Instrumentation diagram of the high-

performance liquid chromatography (HPLC)–SPE–nuclear magnetic resonance (NMR) used. (**III**) HPLC–SPE–NMR experiments heteronuclear correlations spectra of compounds 3 and 4 fragments obtained from peak 2 where (**A**) heteronuclear single quantum correlation (HSQC) spectrum (total acquisition time 9 h 40 min) and (**B**) One-bond correlations in heteronuclear multiple bond correlation (HMBC) spectrum (total acquisition time 15 h 7 min). (**IV**) HPLC chromatogram of compounds 1–10 from the ethanolic extract of *S. iranica* roots on a C18 column; acetonitrile gradient profile in water with average absorbance at 254 and 300 nm (reproduced from Ref. [21] with permission. Copyright © 2005, American Chemical Society).

3. Technological Progresses and Limitations of LC–NMR

The complex nature of natural products and the isolation and quantification of their metabolites has led to the development of LC–NMR. After demonstrating that NMR can be used as a detector for HPLC at the end of the 1970s [22], continuous technological improvements have been made. Recently, LC–NMR has become among the most powerful analytical methods for the separation and structural elucidation of unknown compounds in mixtures. The major technological advancements of LC–NMR, such as use of superconducting magnets [23], solvent suppression [24], strong field superconducting magnets [25], microprobes [26] and cryoprobes [27] technologies, help to improve the acquired sensitivity and resolution [26–28]. Undeniably, the current advances and improvements of NMR play an important role in making LC–NMR a practical and useful method.

3.1. NMR Flow-Probe Design

The most significant technical amendment carried out was fabricating a new probe called a flow cell which has different radio frequency (RF) coils and a different probe structural geometry from the conventional probe. In conventional experiments, the sample in the NMR probe rotates inside a Helmholtz coil to avoid magnetic field inhomogeneity (Figure 7A). A new and improved design with an optimum geometry with continuous flow cell, where both ends are open (Figure 7B), was fabricated in cooperation with Bruker Company, Karlsruhe, Germany [8,23]. The flow cell was designed with a U-type glass tube shape elongated by polytetrafluoroethylene tubing called a "saddle"-shaped geometry to compromise the hyphenation between LC and NMR. A practical demonstration of this type of improved design using superconducting magnets was published during the 1980s [25,29].

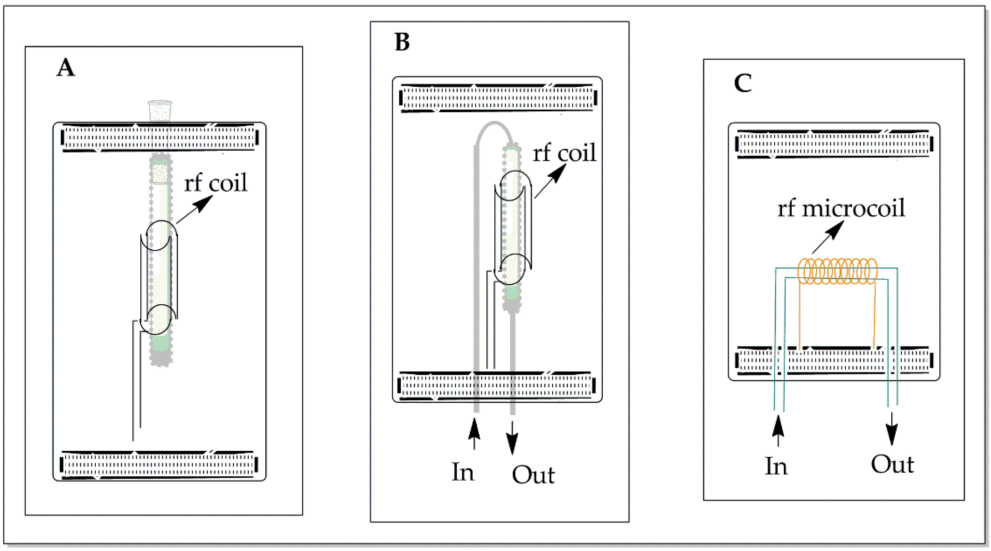


Figure 7. Schematics of NMR cryoprobes: (**A**) conventional and (**B**) saddle/U-shaped continuous-flow NMR probes and (**C**) solenoidal design called Microprobe with microcoil.

As another modification of the NMR flow probe, a novel type of flow cell called cryoprobes has been introduced by a nitrogen/helium cooling system to reduce the resistance of RF coils and preamplifiers by lowering the temperature cryogenically. Cryogenic cooling also helps to increase the sensitivity in detection of trace quantities of components [26,27]. Hence, this new cryoprobe (e.g., Cryoprobe Prodigy, Bruker Biospin) is commonly used in many laboratories and several reports in the analysis of phytochemical profile of natural products have appeared in recent decades [30,31].

Additionally, the reduction in the NMR probe's volume is another appropriate milestone in the modification of flow-probe design to increase mass sensitivity. This improvement was achieved by introducing flow cells called microprobes, where their actual volume is typically between 30 and 120 µL. Further modification was also achieved by employing microprobes with capacities of several microliters (<5 µm) [15,26,32,33]. These types of probes are also structurally different from the saddle-shaped or standard Helmholtz probes because of their probe and RF coil geometries. Their design is called solenoidal-shaped capillary probes where the coil is wrapped directly on the capillary probe (Figure 7C) and these geometrical and size improvements makes them more sensitive than the saddle-shaped flow cells [34]. This kind of approach is called capillary LC–NMR (capLC–NMR) and a number of reports have proven that it is becoming a promising approach for the analysis of high-throughput natural products and their metabolites with a better spectral quality when compared to the other LC–NMR modes [35,36]. Nowadays, commercial microprobes are available at a reasonable cost which is comparable with other probes. However, the application of microprobes is also limited to small volumes and fully soluble compounds and, on such occasions, the utilization of cryoprobes could be a better solution.

3.2. Solvent Suppression

In LC–NMR experiments, sometimes the solvent signal is much larger than that of the sample and this signal must be suppressed. For example, in the case of solvents such as acetonitrile (CH3CN or CD3CN), the solvent peaks are higher than the sample signals which is why a solvent suppression technique is required. After the optimized solvent suppression technique, called the Watergate Excitation Technique (WET), which was developed by Smallcombe and co-workers [24], the quality of LC–NMR spectra was greatly improved. Practically, the WET method is a standard technique for LC–NMR due to its capability to suppress solvent peaks efficiently, as compared to other solvent suppression techniques such as presaturation and excitation sculpting [37]. Their merits and demerits are summarized in Table 1. The general drawback of all solvent suppression methods are, when the analyte signals are overlapping with the solvent signal, they are also suppressed together. A possible remedy for this type of problem is to execute the isolation process by using fully deuterated solvents in microcolumns to avoid consumption of excess deuterated solvents [38].

Table 1. The advantage and limitation of the common solvent suppression methods.

Solvent Suppression Method	Operating Setup	Benefits	Drawbacks
Presaturation	Irradiation of solvent signal for a period of time with a continuous wave RF field	Easy setup and effective for eliminating single solvent signal	Not easy to suppress multiple solvent peaks simultaneously
Excitation sculpting	Extraction of the solvent signals with selective pulses	Could be automated and multiple solvent signals can be suppressed	The pulse sequences need to be optimized and could suppress analyte signals that overlap with the solvent
Watergate excitation technique (WET)	The solvent signals are suppressed at the beginning of the experiment	Could be automated and multiple solvent peaks can be suppressed	Suppresses analyte signals that overlap with the solvent.

4. Application of LC–NMR in Natural Product Identification

NMR is the technique of choice for the rapid determination of the complete structure of natural compounds. The coupling of this technique with HPLC with high separating abilities allows an online and complete identification of compounds in a plant extract [15,39–41]. LC–NMR provides that information without needing handling to treat the eluted peak for an NMR analysis, which could be used for identification of labile compounds. This is why LC–NMR is a technique under dynamic development since its first application was demonstrated at the end of the 1970s [22]. Several publications demonstrated the suitability of LC–NMR to examine different complex mixtures of natural products; however, the first application of LC–NMR to natural product extracts in a chemotaxonomic investigation was presented by Spring and coworkers in 1995 with the characterization of sesquiterpene lactones from the Mexican plant *Zaluzania grayana* and then a new lactone was already identified in on-flow mode [41].

The first LC–NMR practical application for natural product analysis was reported in the mid-1990s. Since then, several applications to characterize natural product extracts have been presented. Different researchers have investigated various plant originated natural product compounds such as isoflavonoids from roots of *Smirnowia iranica* H., [21] prenylated flavonones from whole plants of *Munotes engleri* GILG, [9] secoiridoids from whole *S. calycina* and *G. ottonis* plants, [1] pyrrolizidine alcaloids from *Senecio vulgaris* L., *Senecio mariettea* M. and *Senecio venosus* H. extracts [42], naphthoquinones from roots of *Cordia linnaei* Stearn [43] and isoindoline alkaloid as well as lignanamide from root bark of *Cordia alliodora* [44,80]. Other groups have also reported different natural products such as: antibacterial sesquiterpene lactones, [10,41,45] naphthylisoquinoline alcaloids, [7,46,47] lignanes, [48] triterpene saponins, [49] fasciculol triterpenes, [50] taxanes, [51] tocopherols and tocotrienols, [52] phenylphenalenones, [53] polyhydroxy steroids, [54] carotenoid isomers, [55] flavonoids [56,57] as well as hop bitter acids [53,58]. Mostly, applications of LC–NMR published so far have been the characterization of plant-derived natural products and their metabolites, while applications to micro-organisms or marine based natural products are still rare [50,59–61].

A heteronuclear (heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC)) on-flow mode of LC–NMR experiments has also been reported by Garo and his coworkers [9]; however, this was found to be practical only for a highly enriched fraction of a natural product, showing the limitation of this approach. In natural product analysis, the stop-flow mode is more popular and employed to acquire ^{1}H spectra, or if further structural information is required to perform two-dimensional ^{1}H NMR spectra, such as correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), nuclear Overhauser experiment spectroscopy (NOESY) or rotating frame nuclear Overhauser experiment spectroscopy (ROESY). In many cases, an on-flow NMR chromatogram (usually at flow rates 0.3–1 mL min^{-1}) is recorded earlier for screening of particular groups of compounds or to gain a general overview on the sample composition. Moreover, time-sliced stop-flow [7,46,47] and a modified on-flow approaches at low flow rates [60,61] have been also applied to natural product extracts in order to combine the advantages of both on-flow (dynamic condition) and stop-flow (static condition which allows the samples to achieve a sufficient acquisition time for trace compounds) modes. According to our literature review, LC–SPE–NMR, which is the latest hyphenated method and seems to be a promising technique as compared to the other modes when considering the cost issues, sensitivity as well as compatibility with 2D NMR techniques. Table 2 describes the early starting breakthrough real practical applications of LC–NMR which could be considered as an illustrative example in the natural product analysis field where in all LC–NMR operational modes all solvents used are deuterated solvents for both isolation (LC) and detection (NMR), except in LC–SPE–NMR where deuterated solvents to elute the components in the SPE are used for detection.

Table 2. Different LC–NMR and/or capillary LC (capLC)–NMR modes of operation and their application in natural product.

Mode of Operation	Plant Species	Plant Parts	Class of Compounds Analyzed	Reference
On-flow	*Orophea enneandra*	Leaf	Lignans, Tocopherol, Polyacetylene	[62]
	Iris domestica	Root	Isoflavonoids	[63]
	Ancistrocladus guineensis	Leaf	Alkaloids	[47]
	E. vacciniifolium	Bark	Crude alkaloid	[31]
	Urtica dioica	Root	Phytosterols	[64]
	Vitis vinifera, Vitis amurensis, Vitis cinerea and Vitis X champinii	Grape barry skin	Anthocyanin composition	[65]
	Bobgunnia madagascariensis	Root bark	new antifungal constituents	[66]
Stop-flow (including loop storage)	*Monotes engleri*	Leaf	Prenylated flavanones	[9]
	Schizanthus grahamii	Stem-bark	Tropane alkaloids	[67]
	Swietenia macrophylla	Seed	Limonoids	[68]
	E. vacciniifolium	Bark	Crude alkaloid	[31]
	Isoplexis species	Seed	glycosides	[69]
	Ravensara crassifolia	Aerial parts	6-alkylated a-pyrones	[70]
	Heliotropiu ovalifolium	Aerial parts	tetrahydrophenanthrene	[71]
	Schizanthus grahamii	Stem-bark	Isomeric tropane alkaloids	[67]
	Nandina domestic	Stem part	Alkaloids, crude extracts and cultured cells	[72]
	Vitis species	Grape barry skin	Anthocyanin composition	[65]
HPLC–SPE–NMR	*Taraxacum officinale*	Root	4-hydroxyphenylacetic acid derivatives of inositol	[73]
	Hypericum perforatum	Aerial parts	Naphtodianthrones, Phloroglucinols, Flavonoids, Phenolic acids	[74]
	Warburgia salutaris	Bark	Sequisterpenes	[75]
	Schizanthus grahamii	Stem-bark	Isomeric tropane alkaloids	[67]
	Smirnowia iranica	Root	Isoflavonoids	[21]
	Neolitsea sericea	Leaf	Flavonoid glycosides	[76]
	Neolitsea sericea	Leaf	Isoquinoline alkaloids	[77]
	Strychnos usambarensis G.	Fruit	Akagerine, Palicoside	[78]
	Steganotaenia araliacea	Stem-bark	Cytotoxic stem bark extract	[79]
	Ormocarpum kirki	Root	Minor natural products	[80]

5. Conclusions and Future Outlooks

The technological improvements in NMR achieved over the past three decades have rendered it a practical, versatile and reliable LC detector in different application areas such as natural product analysis. NMR offers advantages over other detectors as it enables the acquisition of better structural information including complex unstable natural stereoisomers. Major overall performance improvements have been achieved through technological advances such as (a) selective solvent signal suppression, (b) cryogenic probes, (c) miniaturization of probes (microprobes) and (d) automation of the different working modes. In addition, the ability to accumulate multiple analytes using storage loops (peak parking)

and SPE cartridges (peak trapping) has improved the sensitivity to the nanogram level. The combination of these technical developments has taken LC–NMR from an academic curiosity to a practically useful analytical tool. Based on the current and future needs of the end-user, a literature survey and our opinion, LC–SPE–NMR and capLC–NMR modes will still have strong emphasis placed upon them due to their cost effectiveness which is achieved by avoiding consumption of an excess amount of expensive deuterated solvents throughout the experiment. Since the plant kingdom produces an abundance of potential natural products, further developments in (a) finding compatible solvents for better sensitivity, (b) simplicity and cost issues as well as (c) overall miniaturization are still required to produce indispensable technologies throughout analytical laboratories in both industry and academia. Additionally, technological advancements in both hardware and software aspects of the isolation (LC) and detection (NMR) parts are also needed.

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Abbreviations

1D: one dimension; 2D: two dimension; COSY: correlated spectroscopy; HMBC: heteronuclear multiple bond correlation; HMQC: heteronuclear multiple quantum correlation; HPLC: high-performance liquid chromatography; HSQC: heteronuclear single quantum correlation; IR: Infrared-spectroscopy; LC: liquid chromatography; LC–NMR: liquid chromatography–nuclear resonance spectroscopy; LC–SPE–NMR: liquid chromatography–solid phase extraction–nuclear magnetic resonance; MS: Mass Spectroscopy; NMR: nuclear magnetic resonance; NOE: nuclear Overhauser experiment; NOESY: nuclear Overhauser experiment spectroscopy; RF/rf: radio frequency; ROESY: rotating frame nuclear Overhauser experiment spectroscopy; SPE: solid phase extraction; TOCSY: total correlated spectroscopy; UV: Ultra-Violet; WET: Watergate excitation technique.

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