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Review

Online hyphenated liquid chromatography–nuclear magnetic resonance spectroscopy–mass spectrometry for drug metabolite and nature product analysis

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Abstract

Screening analysis that aims at rapidly distinguishing new molecules in the presence of a large number of known compounds becomes increasingly important in the fields of drug metabolite profiling and nature product investigation. In the past decade, online-coupled liquid chromatography–nuclear magnetic resonance spectroscopy–mass spectrometry (LC–NMR–MS) has emerged as a powerful tool for the detection and identification of known and, more important, emerging compounds in complex clinical, pharmaceutical samples and nature product extracts, due to the complementary information provided by the two detectors for unambiguous structure elucidation. This review discusses the practical conditions under which LC–NMR–MS is suitable as a routine tool for unknown analysis, as well as the fundamental concepts and their advantage aspects. Particular attention is paid to its major operating parameters that include the instrumental configurations, working modes, NMR probe improvement and LC mobile phase selection. Finally, the recent applications of LC–NMR–MS to clinical metabolite and nature product analysis are summarized which have shown the benefit of this promising hyphenated technique.
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Keywords: LC–NMR–MS; Liquid chromatography; Mass spectrometry; Nuclear magnetic resonance spectroscopy; Hyphenation; Metabolite; Nature product; Review

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1. Introduction

A well-established protocol for drug metabolite and nature product analysis is the utilization of rapid assays to screen maximum number of active components in small quantity of biological mixtures within shortest possible time. The most common challenge faced by analysts in these investigations is how to rapidly and efficiently identify unknown components emerging from the compound pipeline, which contains a large number of known entities. Without unequivocal identification of these unknowns, valuable information leading to new biomarkers or new drug candidates might be neglected or inaccurately interpreted. Identification of unknowns in these samples is a difficult task since they display very complex mixtures of compounds varying greatly in structure and in concentration. Among a wide array of analytical techniques and methods, high performance liquid chromatography–mass spectrometry (LC–MS) is without question the pre-eminent technique for the analysis of expected analytes in complex mixtures without prior component isolation. It is also widely used for the determination of unknowns, due to the complementary structure information generated by multiple stage MS experiments [1]. However, LC–MS on its own is insufficient to unequivocally identify some unknowns because LC–MS is unable to distinguish between co-eluting geometrical or optical stereoisomers [2,3], and is blind to compounds that can undergo severe thermal degradation or cannot be effectively ionized in the ionization interface [4]. Therefore, in many cases, nuclear magnetic resonance spectroscopy (NMR) is needed for unambiguous structure determination, especially for the stereospecific identification of unknown bioactive compounds that may be of interest for the development of pharmaceuticals and functional foods.

NMR is the most powerful spectroscopic tool for obtaining structural details of complex organic compounds. The structure assignment of all isomers is possible by considering chemical shifts, coupling constant, and integration ratios of the NMR data. Unlike MS, NMR response is compound independent and all the compounds having NMR-measurable nuclei can be detected including ^{1}H and ^{13}C, which are major structural elements for organic compounds. Conventionally, NMR spectroscopic analysis has required time-consuming isolation and purification steps in order to acquire NMR spectra on individual component. The direct linking of high resolution NMR and LC [5] eliminates the need of extensive sample purification, and increases the NMR capability of solving structural problems of complex mixtures. During the past decade, LC–NMR has been successfully applied to the analysis of mixtures of drug related and biological origins [6], drug metabolites [7], plant metabolites [8], interconverting labile natural products [9] and in the identification of *cis*/*trans* isomers or other possible co-eluting stereoisomers [10]. However, an inherent drawback of NMR is its low sensitivity and its inability to detect some NMR "silent" function groups having poor or non-existent magnetic properties (e.g. SO_4, NO_2) [11]. The lack of sensitivity makes the on-flow LC–NMR measurement of minor constituents impossible, and hinders the direct access of ^{13}C signal that provides fundamental information for structure elucidation. Furthermore, NMR has difficulty in observing analyte signals in the presence of the large resonance of the LC mobile phases, typically when reverse phase LC separations with multiple protonated solvents are employed, and when solvent gradients are used. Thereby, the capability of the determination of an unknown structure by LC–NMR alone is limited. Even though LC–NMR has been investigated since late 1970s [12,13], commercially available instruments were not launched until last decade. This long development time of LC–NMR may be attributed to the intrinsic low sensitivity of NMR spectroscopy. With recent technical advances in high resolution NMR and NMR probe development [14], online LC–NMR hyphenation becomes technically applicable.

Using LC–MS and LC–NMR in a synergistic way for rapid and unequivocal identification of unknowns has shown great promise. MS rapid screening and preliminary structure investigation, followed by supplementary NMR structure determination, has become a typical structure elucidation protocol in clinical, biological and natural product research. However, data correlation based on independent LC–MS and LC–NMR results of the same sample is sometimes difficult, due to possible different chromatograms obtained by the two systems. To avoid this ambiguity, MS and NMR are combined with one LC to operate as LC–NMR–MS, and this combination has attracted considerable investigation interest.

LC–NMR–MS allows for the acquisition of MS and NMR data simultaneously in a single LC run, which provides inclusive analysis of a complex matrix through the real-time comparison and complementation of NMR and MS data. Because MS analysis can provide the number of exchangeable protons of a compound by comparing the MS data in deuterated and non-deuterated solvents [15], it helps the determination of, for example, the number of hydroxyls in an unknown. On the other hand, the acidic proton exchange also complicates MS data interpretation. The first online-coupled LC–NMR–MS system [16] appeared in middle 1990s, right after the introduction of commercially available LC–NMR instruments. This first "in-house" built LC–NMR–MS system consisted of an LC, NMR and a particle beam chemical ionization MS, and was used to analyze a complex mixture of pharmaceutical interest [16]. Only about 1 year later a second application, based on an iron trap MS, was developed for rapid identification of some expected acetaminophen glucuronide and sulfate containing metabolites in human urine [17]. Acetaminophen provides a useful example to illustrate the value of LC–NMR–MS because of its sulfate metabolite. NMR clearly detected this acetaminophen related compound but unequivocal structure determination is impossible because sulfate has no NMR signal. MS, on the other hand, easily resolved the sulfate moiety based on the molecular weigh and diagnostic fragmentations that are consistent with sulfate. In addition, a number of unknown endogenous metabolites that are not normally observed by other techniques were detected by this hyphenated system. This study clearly demonstrated the superior value of LC–NMR–MS in the comprehensive analysis of drug metabolites. Over the past years, LC–NMR–MS has been applied to speed up the process of screening active fractions in biomedical and pharmaceutical materials [18], drug

metabolites in biological specimen [19,20] and bioactive constituents in nature products [21].

Despite the promising performance of this hyphenated technique, there is only a small number of publications on LC–NMR–MS every year. Besides the high initial cost of capital instruments, this phenomenon may reflect the suspicions against the applicability and effectiveness of coupling all of the three instruments together, especially the two very different detectors in regards to sensitivity, acquisition time and solvent compatibility. Practically, relatively few chromatographic and spectroscopic compromises are necessary to modify an LC–MS or LC–NMR system into an LC–NMR–MS system. Since many publications have focused on the benefit of the technique without detailed discussion of its practical aspects, this paper intends to offer an overview of the current instrumental development of LC–NMR–MS, with a deep insight into its technical features. Its applications to metabolite and nature product analysis in the past 10 years are also reviewed. Studies without online LC, NMR and MS hyphenation are not included in the review; among those are, for example, studies performed on separated LC–MS and LC–NMR runs [22], or LC–MS followed by offline NMR determination [23]. The term LC–NMR–MS rather than LC–MS–NMR is preferred to designate this hyphenation in this article, because the analysis takes place in that order if the system is configured in series, and the limitations and improvements are always in the LC–NMR side. It needs to mention here that some researchers prefer to use LC–MS–NMR because they think MS data should be obtained initially since some NMR experiments take hours or days to complete [24].

2. Instrumentation

2.1. LC–NMR–MS configuration

Generally, there are two ways to hyphenate MS and NMR to an LC system, either in parallel or in series (inline) [25]. Because NMR is a relatively low sensitive and mass dependent technique, large volume and high concentrate analytes (pure sample in the order of several milligrams) are required to achieve enough detection sensitivity. Practically, a flow rate of the order of 1 mL/min on a large LC column is necessary to provide enough sample mass for NMR measurement. Though it can be accommodated by modern mass spectrometers, such high flow rate will affect the performance of MS, in particular electrospray ionization (ESI) MS. Therefore, the LC flow needs to be split before entering mass spectrometer. The most common way of interfacing LC to the two detectors is to place them in parallel by splitting the LC eluent at a ratio of 20:1 or similar, directing the major faction to the NMR flow probe and the minor portion to the mass spectrometer ionization interface. This configuration allows the MS detector to be operated at optimal condition without compromising the NMR sensitivity. In addition, parallel configuration avoids the concern of backpressure produced by LC–MS interface that could damage the NMR flow probe. The split flows can also be adjusted easily, depending on the type of experiments being conducted. An analyte can be detected in both systems simultaneously, or alternatively, at different time. If NMR and MS analyses of the same peak are essentially simultaneous, there is much less risk of the peak identification being different, which may result from sample degradation and peak dispersion during the time delay [26]. If LC–NMR–MS is configured in such a way that a peak reaches UV or MS earlier than NMR by a certain period of time, the UV or MS data, which is acquired rapidly, can be used to monitor more sensitive stop-flow NMR experiments for the particular peak. Because of this advantage, this layout is the most widely adapted parallel configuration in published literature. As noted before, sample degradation and peak distortion during the time delay may occur.

Coupling NMR and MS in serial is to place mass spectrometer inline right after NMR by connecting the inlet tubing of MS to the outlet of NMR flow probe. The sample is first analyzed by NMR and then is directed to mass spectrometer. A flow-splitting device/interface is employed in front of mass spectrometer to control the sample amount introduced to the MS ionization interface, and to release the backpressure generated by the interface that could lead to NMR probe leakage [27]. Serial coupling sacrifices the benefit of fast peak identification ability of MS, and introduces the possibility of peak dispersion and retention time drifting when peaks reach MS. However, serial arrangement is a simpler and more robust setup. It can be easily disassembled for standalone mass spectrometric studies [26] and is free of data synchronization problems. Additional advantage brought by serial operation is the possibility to re-protonate exchangeable protons that have been deuterated through the use of deuterated solvents [28]. Usually, serial configuration only applies to on-flow NMR measurement. Typical online LC–NMR–MS configurations are outlined in Figs. 1 and 2.

All the detectors are best located outside the 5 Gauss line (about 3 m radius of a 500 MHz NMR) of the stray field generated by NMR spectrometer, because the high magnetic field greatly influences the performances of other detectors, especially mass spectrometer [29]. The mass spectrometer performance was about 100-fold less sensitive than normal, when it was turned within the NMR's magnetic field [15].

2.2. LC–NMR working modes

Because LC–MS is already a highly sophisticated technique, the overall performance of LC–NMR–MS is determined by the performance of LC–NMR. The working mode of LC–MS is the condition under which the NMR spectra are acquired. There are two general working modes for LC–NMR analysis: on-flow (continuous-flow) and stop-flow [25,29]. On-flow LC–NMR operation is to directly direct LC eluent to the NMR flow probe, where the NMR spectra are continuously being acquired when the LC eluent is moving through at a constant speed. Stop-flow is a mode that allows NMR spectra of peak of interest acquired under static condition in the flow probe. Unlike conventional NMR measurement, the peak of interest is delivered online to a NMR flow probe instead of a conventional static probe. According to how the peak is sat in the flow probe, stop-flow splits into two submodes: direct stop-low (time-slicing) and loop storage (loop collection/peak picking). Direct stop-flow provides static sample condition for NMR data acquisition by stopping the flow

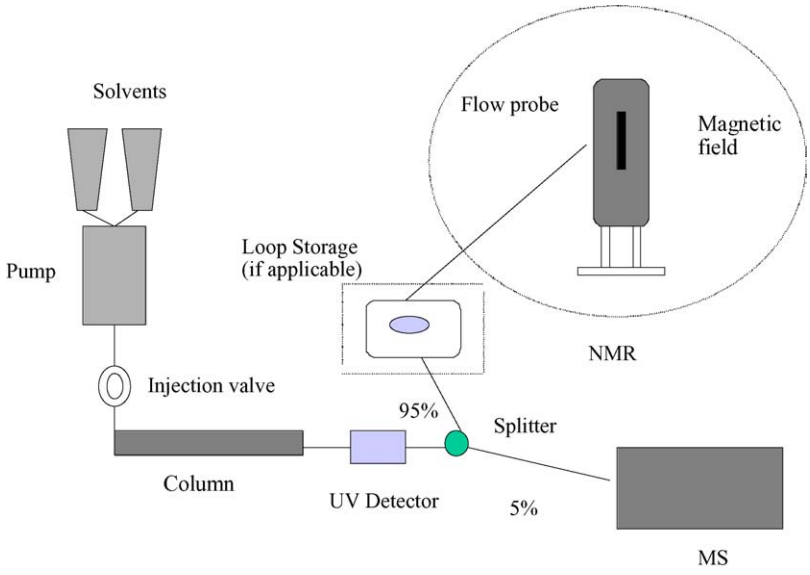

Fig. 1. Typical in parallel hyphenated LC–NMR–MS system.

for a short interval when a peak of interest enters NMR probe, and the flow resumes after the data acquisition completes. The same procedure repeats for next peak of interest till the end. In loop storage method, the LC peaks are stored in a capillary loop and the static NMR measurements begin after the LC separation completes. Because loop storage is similar to an off-line NMR study except using a flow probe, the stored peaks can be analyzed in different order from the LC separation. In both stop-flow modes, methods are required to trigger flow stop or to select the peaks for loop storage.

The on-flow mode, in practical, is limited to only acquire high abundance ^{1}H or ^{19}F NMR spectra of major components in a sample due to the limited NMR data acquisition time (about 3.6 s in a conventional NMR flow probe having an active volume of 60 μL at 1 mL/min LC flow rate). Decreasing the flow rate to increase measurement time is a way to increase the NMR sensitivity, but still not enough for low abundance nuclei such as ^{13}C and for minor constituents in the samples. Because no interruption of chromatographic eluent occurs, the instrumentation is simple and allows for the detection of all NMR active compounds under identical conditions. And therefore it provides a straightforward and real-time NMR and MS comparison of individual compounds [17]. Stop-flow mode offers unlimited time (minutes to hours depending on the concentration of analytes) for static NMR measurement, which enables the acquisition of high resolution NMR spectra (e.g. 2D NMR data) for most of the components that are not concentrated enough for quality on-flow NMR measurement. The amount of sample required for the analysis can be reduced as well. Stop-flow mode is the method of choice if more detailed structural analysis is desired, or the sample amount is not enough for on-flow NMR measurement, or the analytes of interest are not major components of a sample. Fully automated analysis is also practicable in this mode when system is able to automatically monitor flow stop or loop storage.

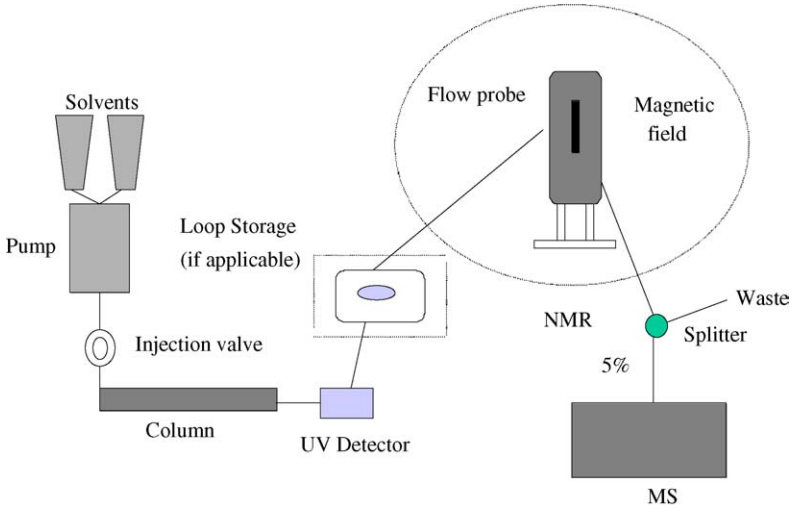

Fig. 2. Typical in series hyphenated LC–NMR–MS system.

However, direct stop-flow suffers from the possibility of peak dispersion and resolution impairing for peaks trapped between the NMR and MS systems when the flow is stopped, and possible carry over (memory) effects if a small peak is preceded by a large one. As well as the carry over effect, loop storage suffers from the possibility that some compounds may undergo degradation or isomerization during a long-time storage. Because stop-flow mode requires additional device for peak selection control and is time-consuming, the possibility of system and personal errors increases [30]. In addition, some important compounds may be neglected because they are not selected for NMR detection for some reasons. An important consideration for stop-flow mode is the backpressure in the closed system when the flow is stopped. The backpressure may push peak shifting in the NMR flow probe even when the pump stops, thus the whole system needs to be carefully configured, usually with a dedicate interface, to solve this problem. So far, the direct stop-flow measurement is the most widely applied technique in LC–NMR or LC–NMR–MS applications.

2.3. Stop-flow monitor

As noted above, both direct stop-flow and loop storage modes require additional device to trigger the temporary LC flow stop or the transfer of an LC fraction to the temporary sample storage unit. In independent LC–NMR systems, this job is done by UV or fluorescence spectrometer, either by manual peak selection or automatic control. However, using UV spectrum to select peaks for consequent stop-flow NMR analysis is insufficient because a large number of organic compounds lack UV absorbance. Fluorescence detector suffers from the similar drawback.

Coupling a MS to an LC–NMR system, MS can be used as a first step for the chemical profiling of clinical and nature product samples. Most compounds can be tentatively identified based on the molecular weight and fragmentation information. With the advent of searchable mass spectra database and compound library, a rapid search of obtained MS spectra against the database will help the selection of peaks of interest or the detection of emerging unknown compounds. Upon peak recognition, with a signal from the mass spectrometer manually or automatically to the central control system, the LC pump stops to reside the peak in the NMR probe or the fraction is directed to a loop storage unit for further static NMR analysis. Undoubtedly, the well-designed protocol results in significant reduction in time and effort in metabolite profiling and nature product analysis that always look for new discovery leads. In order to take advantage of this approach, LC–NMR–MS has to be configured in parallel and the length of the capillary tubing connecting MS interface needs to be adjusted such that an LC peak passes through MS a certain period before it enters the NMR flow probe. The certain period of time should be long enough for manual, or more recently automatic, mass spectra search in various “in-house” or online spectral libraries. To achieve this goal successfully, dedicated software is needed to export an obtained mass spectrum of an LC peak for library search, and then trigger the pump stop or loop transfer upon being directed to do so.

The benefit of using MS data to trigger stop-flow NMR measurement was not fully recognized until early this century [5]. In this investigation, MS/MS data were used to monitor the NMR stop-flow measurement for selected peaks or the transfer of the LC fractions to a temporary sample storage unit, in comparison with the performance of UV data as triggers. Both methods were believed to be selective (specific) regarding to the distinction between quercetin- and phloretin-derived glycosides. However, the author concluded that the MS/MS might be more suitable for the selection of the fractions of interest.

2.4. NMR probe improvement

A flow probe is the only prerequisite to modify a static NMR instrument to an online LC detector. Most commercial NMR flow probes have active volumes between 60 and 240 μL using 3–5 mm radio frequency (rf) coils around the detection cell. Modern high field NMR, 500 MHz or higher, can detect proton signal well into the high nanogram range. With the introduction of new probe techniques, the sensitivity of NMR has been improved significantly. Recently, many developments made to increase LC–NMR sensitivity have focused on the dedication of the flow probe, since the overall system performance largely depends on the performance of the probe. A probe is a sensor placed in the centre of the magnet containing the coil that is used to send rf pulses to the sample and to detect the NMR signals returning from the nuclei. The sensitivity of NMR is primarily limited by the thermal noise of the detection components.

The most significant advance in NMR probe development is the introduction of CryoProbes. In these NMR probes, the electronic components are cryogenically cooled to −20 K. By operating the electronic components at this low temperature, while the sample remains at ambient temperature, the electronic noise is greatly reduced [31]. On an average, CryoProbes enable a three- to four-fold enhancement of the sensitivity in high resolution NMR compared to conventional probes. A novel cryoflow probe, the flow configuration of CryoProbes, was built and evaluated for its potential to advance the NMR sensitivity for LC–NMR–MS in the determination of acetaminophen urinary metabolites in 2003 [32]. The signal-to-noise ratio (S/N) advantage of the novel cryoflow probe over conventional flow probes used for LC–NMR–MS studies was demonstrated by manual injection of 2 mM sucrose in 90% D_2O solution. As a result, the ^1H spectra obtained in one scan at 500 MHz with a line broadening factor of 1 Hz gave a S/N of 215:1 for the anomeric sugar proton using the cryoflow, in contrast with those of 80:1 for a typical 120 μL LC selective inverse ^1H–^{13}C non-cryoflow probe and 20:1 for a typical non-cryo 30 μL LC probe. Since there is at least three times S/N improvement over conventional NMR flow probes, the cryoflow probe enables the NMR analysis of lower concentrations of metabolites than was previously possible for untreated samples. Another advanced NMR probe developed for capillary LC–NMR system is wounding a solenoid rf coil directly on the separation capillary [33,34], which is called solenoid probe. Atypical solenoid probe has a detection volume between 5 and 5000 nL, and is capable of detecting sample down to 160 pmol. The major drawback is that a new coil has to be

prepared for every separation capillary. An alternative design consists of a double-saddle Helmholtz if coil directly affixed to a 60–180 μL glass tube, into which the detection capillary is inserted [35,36]. This design leads to optimal NMR resolution values allowing the determination of coupling constant in on-flow NMR spectra, which is not applicable by using conventional NMR probes. However, the close proximity of the rf coil and sample may result in increased line-width and loss of resolution.

For LC–NMR or LC–NMR–MS analysis, ^{1}H and ^{19}F are the commonly recorded nuclei because their nature abundances are close or equal to 100%. ^{13}C NMR is also an important structural elucidation technique, and in many cases it is a prerequisite for unambiguous assignment of an unknown structure. However, ^{13}C NMR is seldom used in LC–NMR–MS or LC–NMR applications because of its low signal intensity resulting from low nature abundance (1.1%). The difficulty of accessing ^{13}C information, which represents an important structural element, is a major hindrance to a wider use of LC–NMR–MS. Indirect access of ^{13}C information can be achieved by acquiring the 2D ^{1}H and ^{13}C correlation spectra in stop-flow NMR modes. Recently, on-flow ^{13}C NMR spectra acquisition becomes possible by using a specially designed ^{13}C flow probe. A 15 mm long ^{13}C rf coil is directly attached to the glass tubing, and an outer rf coil, mounted co-axially to the first one, is turned to decouple $^{1}H/^{1}H$ coupling and stabilize (lock) the field frequency [37].

As mentioned before, memory effect is another possible problem when highly concentrated analytes were encountered in the analysis. Though memory effect of LC–NMR–MS has not been intensively evaluated, the phenomena is common in all kinds of flow through instruments such as instruments containing nebulizer and spray chamber, flow through (online) infrared spectrometer (IR), and post-column derivation device. Using special material to make NMR probe and using low dispersion tubing to connect the instruments will be beneficial.

2.5. Mobile phase compatibility and solvent suppression

Mobile phase constituent of LC is another important consideration for LC–NMR–MS experiments. Solvent and additive selection has to be a compromise between the ideal conditions of individual instruments. Though the use of inorganic buffers is ideal for NMR because no additional proton-carrying species are introduced, they are incompatible with ESI, a principal ionization technique for LC–MS. Deuterated organic buffers are the best choice; however, they are extremely expensive and not always readily available. In real LC–NMR or LC–NMR–MS applications, non-deuterated formic acid [38], acetic acid [27], trifluoroacetic acid (TFA) [17] and their corresponding ammonium salts are widely used, although they give typical NMR signals. In some studies, TFA was found to suppress the ESI and mask the analyte ions, and thus is not recommended for LC–NMR–MS [39].

Most of the LC separations for LC–NMR–MS are performed on reverse phase columns with binary solvent mixture mobile phase: acetonitrile/water or methanol/water. The intensity of the solvent protons is higher than sample NMR signal in the order of 10,000 times in a routine LC–NMR run with a 10 μL sample injection. Any characteristic NMR signal buried under the solvent resonance cannot be detected; as a consequence, structure information cannot be fully elucidated. If solvent gradients are used, the change in solvent resonance during the chromatographic run further complicates the NMR analysis. In the early stages of LC–NMR development, LC separation with fully deuterated solvents was mandatory to eliminate the interfering solvent signal. However, deuterated solvents are prohibitively expensive for a routine reverse phase LC run operated at a typical flow rate of 1 mL/min, and sometimes cause problems in MS data interpretation as informative proton change between analyte and solvent disappears [15]. A breakthrough technological achievement that overcomes the solvent signal interfering problems is the development of solvent suppression techniques [40], which has transformed LC–NMR and LC–NMR–MS from research tools to the stage where routine applications are possible. In summary, there are three ways to perform solvent signal suppression: pre-saturation (NOSEY pre-saturation), soft-pulse multiple irradiation and WET (water suppression enhanced through T1 effects) pre-saturation employing a *z*-gradient. Detailed information on those techniques can be found elsewhere [40]. All the three techniques can be applied to both on-flow and stop-flow NMR acquisitions. Pre-saturation works very well in the stop-flow modes, whereas WET seems to be superior in the on-flow mode. WET solvent suppression technique is considered the standard for LC–NMR because it has the capability of suppressing several solvent lines with minimum baseline distortions, compared to other techniques. These solvent suppression techniques lead to great gain in NMR sensitivity by eliminating or reducing strong solvent signals, however, they also result in the loss of valuable analyte signals that reside near or under the solvent peak and are suppressed along with the solvent signal. For example, crossing signals in a 2D experiment (i.e. COSY and TOCSY) will not appear if they coupled to a signal in the suppression area. For unknown constituent analysis, this is a major drawback. Multiple-solvent suppression for unknown analysis is not suggested because it eliminates too much useful NMR information. Therefore, from a practical point of view, it is beneficial and economically acceptable to use part of deuterated solvents, such as acetonitrile/D_2O, to simplify signal suppression by suppressing only the part of non-deuterated solvents, and to prevent the loss of valuable information. A better, but more complicate, approach is to perform the LC–NMR experiments with two independent solvent systems, such as acetonitrile/D_2O and methanol/D_2O. Because the suppression regions for acetonitrile and methanol are different, full range of ^{1}H signal is obtainable after combining both spectra. Such an approach was demonstrated to analyze complex alkaloids in the crude extract of *Senecio* species [41].

Several experiments have been conducted to evaluate the usage of superheated D_2O as the reverse phase LC eluent for LC–^{1}H NMR and LC–MS–^{1}H NMR systems [42,43]. In these investigations, superheated D_2O was found to be an effective eluent for reverse phase LC–NMR–MS. Using superheated D_2O as LC mobile phase, no any buffer systems or organic modifiers are necessary. Compared with other deuterated organic

solvents, D_2O is comparatively cheap and is available in a high state of purity with no organic impurities. In addition, the absence of large NMR signals coming from LC eluent considerably simplifies the NMR spectra interpretation. NMR signals originating from the principal impurity of D_2O, non-deuterated water (0.1%), can be suppressed using conventional NMR pulse techniques. Though it has not been widely applied in LC–NMR–MS, superheated D_2O provides a promising alternative to other expensive deuterated organic solvents.

One of the latest technological developments to reduce the consumption of expensive deuterated solvents is the introduction of post-column solid phase extraction (SPE) into LC–NMR system, termed as LC–SPE–NMR [44]. SPE is a well-recognized approach for concentrating and purifying samples either online or offline before chromatographic separation. In this LC–SPE–NMR setup, however, SPE column (cartridge) is placed online between the LC column and the NMR flow probe. Analytes separated from the LC are trapped on SPE column and are then eluted to NMR flow probe for measurement at a later time. LC–SPE–NMR is similar to loop storage LC–NMR setup, but has several advantages over loop storage and other approaches. Besides peak concentration, this online LC–SPE–NMR approach allows operating LC separation with non-deuterated mobile phase and then using deuterated solvent to transfer the analytes from SPE to NMR flow probe. Only a small amount of deuterated solvent (several hundred microliters or less) that matches the detection volume of the flow probe is needed for the elution. As a result, the expense of high purity deuterated solvents is no longer an issue and the use of solvent suppression techniques, which can lead to analyte signal loss, is strongly reduced or no longer necessary. To take advantage of the solvent-changing benefit for LC–NMR–MS, NMR and MS need to be configured in parallel, and the online SPE column be positioned between the solvent splitter and NMR flow probe. Often, a second pump is required to deliver the SPE eluting solvents.

2.6. Miniaturized techniques

The trend toward instrumental miniaturization has had significant impact in the field of LC–NMR–MS. Several goals are the primary drives behind the miniaturization: reduced usage of expensive deuterated solvents, shortened analysis time and increasing demand to analyze quantity-limited biological samples. Over the past years, there has been a trend of hyphenating miniaturized separation techniques, such as microscale or capillary LC (capLC), to high field NMR equipped with miniaturized micro/nanoliter NMR probes in order to analyze mass/volume-limited samples. This miniaturization requires so little solvent that fully deuterated solvents can be employed in the capLC separation without significantly increasing the overall cost. Because capLC can provide higher analyte concentration in the elution bands due to high separation efficiency, the improved sensitivity and reduced sample/solvent requirement make this technique more applicable than standard LC–NMR in the analysis of quantity-limited biological samples [45,46]. In order to interface with commercial microscale LC systems using columns between 0.3 and 1 mm in diameter, an optimized NMR probe operated at 500 MHz was designed [45]. The custom probe consists of an rf coil with an active volume of 1.1 μL and a 1.4 Hz static line-width. Using sample as small as 70 ng on column, two-dimensional COSY spectra in stopped-flow mode were obtained in shorter acquisition periods than previously reported studies using larger amounts of samples.

Recent advance in actively shield NMR magnets greatly reduces the stray magnetic fields and, therefore, shortens the necessary distance between NMR, MS and LC, which results in great reduction in the consumption of solvents as well as the dead volume in between the instruments that could cause peak distortion. For example, with shield cryomagnets the LC can be as close as 30–50 cm to the magnet versus 1.5–2 m for conventional magnets [29].

3. Applications of LC–NMR–MS

The published LC–NMR–MS applications cover a variety of sample types but mostly focus on drug metabolite identification and nature product extract analysis [24,29]. In majority of those publications, LC–NMR–MS was perceived as a new analytical entry, and often known compounds were used as targeted models to evaluate this technique. Though a search for real unknowns was not the primary goal, some unknown or previously unreported compounds were detected and identified occasionally.

3.1. Drug metabolite analysis

Drug metabolite analysis in biological specimen plays a vital role in drug discovery and therapeutic drug monitoring. Predicting possible metabolites of a target drug is applicable, since it is subject to a set of biotransformation rules. Online LC–NMR has been proved useful for the metabolite profiling of some drugs via structural characterization of their metabolites, but NMR itself is not sufficient for structure elucidation of metabolites containing NMR non-detectable nuclei such as sulfate. Upon realizing this drawback of LC–NMR, several groups investigated the benefit of using LC–NMR–MS to identify sulfate-containing compounds. The first LC–NMR–MS system separated and identified a mixture of fluconazole and two related triazole configurations [16], which were used as test models. An isocratic separation was performed on a C18 column using acetonitrile/D_2O (25:75) at a flow rate of 1 mL/min. Sixty percent of the eluent was split into the flow probe of a 500 MHz NMR for on-flow ^1H data acquisition, while the remainder was sent to a particle beam mass spectrometer operated in chemical ionization mode. Gradient reversed phase LC–NMR–MS was developed to determine the metabolites of acetaminophen in human urine [17]. The gradient D_2O/acetonitrile-d_3 LC separation was operated at 1 mL/min. After going through the UV detector, the eluent was split by a ratio of 95:5, with major proportion traveling 29 s to a 500 MHz NMR and minor part traveling 45 s to a positive ion ESI ion trap MS. The residue water resonance was suppressed using a selective sine pulse. Both NMR and MS spectra were acquired in real time. In addition, tandem experiments

utilizing MS/MS studies to identify fragment ions were also carried out in positive ion mode. Besides confirming the expected metabolites, LC–NMR–MS also identified a number of endogenous metabolites that are not normally observed in human urine. The same drug model was further analyzed by a similar gradient LC–NMR–MS system equipped with a single qudarupole mass spectrometer operated in both positive and negative ion modes [47]. Still, the ^{1}H NMR spectra were obtained on-flow using WET solvent suppression technique. As mentioned before, standalone LC–NMR and LC–NMR may have some data synchronization problems. In order to solve these problems, NMR and MS data acquisitions were combined in a single LC system. The combined LC–NMR–MS was then applied to identify the metabolites of 2,3-benzofuran (BF), including a sulfate-containing compound, in the urine of rate given BF intraperitoneal [48]. The eluent was immediately split after a gradient reverse phase LC separation, with 5% of the flow to the ESI ion trap mass spectrometer and 95% of the flow to the NMR flow probe via the UV detection cell. The system was arranged in such a way that the eluent reached the mass spectrometer earlier than it reached UV detector and NMR flow probe, which allowed the use of rapid MS detection in search of particular molecular ion fragmentation to direct stop-flow NMR measurements. Stop-flow ^{1}H NMR spectra were acquired at 600 MHz using a pre-saturation pulse sequence, with double pre-saturation for suppression of the signals of acetonitrile and residue water in D_2O. The LC–NMR–MS approach with mass-directed NMR detection was believed to greatly facilitate the structural determination of compounds containing “NMR silent” moieties as exemplified in this study.

Stimulated by the promising potential of LC–NMR–MS, one group of researchers has successfully used LC–NMR–MS for a variety of applications. The targeted analytes of interest include ibuprofen metabolites in an extract of human urine [49], urinary metabolic fate of 2-chloro-4-trifluoromethylaniline [38] and 2-bromo-4-trifluoromethylaniline [50] in rat, metabolites of the HIV-1 reverse-transcriptase inhibitor BW935U83 [51], GW420867 and GI265080 [52], and fate of 5-trifluoromethyl pyridone in hydroponically grown maize [53]. Their prototype LC–NMR–MS system always consists of a UV detector, which can be used alone or along with MS to monitor stop-flow NMR measurements.

Taking the coupled technique one step further, the same group evaluated the suitability of a multiple hyphenated LC–FT/IR-time of flight (TOF)/MS–NMR system using mixtures of non-steroidal anti-inflammatory drugs as examples [54]. There was no technical difficulty to link additional spectroscopic detectors, such as FT-IR, to a parallel LC–NMR–MS system. And it was satisfactorily demonstrated in this study that quality UV, IR, MS and ^{1}H NMR data could be obtained on-flow, and in a single LC run, for quantities of materials on the order of 50–100 μg. The major difficulty encountered in this multiple hyphenation was how to maintain the optimal operating conditions for each detector according to their intrinsic differences in the sensitivity and in the sample quantity requirement. As a result, multiple splitting of eluent had to be employed to avoid the signal overloading of UV and MS, which could induce possible band broadening. Despite incomplete LC resolution and the presence of co-eluting interferences, the combination of detectors provided a wealth of information on the identity of the major components and impurities. The same LC–FT/IR-TOF/MS–NMR system was also applied to the identification and characterization of ecdysteriods in plant extracts [55].

A milestone in the improvement of NMR sensitivity for LC–NMR–MS is the development of cryoflow NMR probe [32]. The performance of this novel probe for LC–NMR–MS was demonstrated using acetaminophen [32], a well-documented test compound whose urinary metabolic profile has been previously studied by LC–NMR–MS equipped with conventional flow probe [17]. The superior sensitivity of this probe allowed the use of 100 μL of untreated urine (40% less material than previous studies that required pre-concentration). Besides the known sulfate and glucuronide metabolites, previously undetected metabolites of acetaminophen were directly observed in a 15 min on-flow experiment at 500 MHz. In addition, stop-flow NMR experiments were conducted for greater S/N on minor metabolites. This strategy could also be applicable for samples containing mass-limited analytes, such as those from drug metabolism studies, biomarker and toxicity profiling, impurity analysis and natural product analysis.

Several methods for online concentrating the analytes of interest have been developed to increase NMR sensitivity, and therefore to shorten the acquisition time of some important minor constituents in a sample. Incorporating an online post-column SPE to an LC–NMR–MS system was reported to provide an additional at least two-fold NMR sensitivity enhancement over that of five-fold cryoflow NMR microprobe advantage at 500 MHz [56]. The significant sensitivity gain enabled the running of 2D NMR experiments for structural elucidation of the unknown metabolite. In the case described here, SPE was used online after the analytical column to trap LC peaks for pre-concentration as LC–SPE–NMR. The peaks of interest were detected by UV and mass responses to trigger the SPE trapping. And then the SPE trapped peaks were flushed directly into NMR flow probe for stop-flow measurements. The LC–SPE–NMR–MS system was applied to the structural elucidation of a low concentrated paracetamol metabolite present in human urine. In this study solvents for SPE elution were non-deuterated, and the solvent signals were suppressed with time-shared double pre-saturation of the water and the acetonitrile ^{1}H frequencies. An online column trapping approach was developed for concentrating LC peaks before transferring them to NMR probe [57]. Like online LC–SPE–NMR approach, the trapping column was placed after the analytical column to trap separated analytes. After trapping, a high organic content solvent was used to back flush the analyte to the NMR microflow probe, where NMR spectra were obtained at 600 MHz with WET pulse sequence for solvent signal suppression. A flow splitter was placed after the trap column, delivering 5% of the LC eluent to the mass spectrometer. This approach showed over three-fold increase in peak intensity on target compounds, which resulted in dramatic saving in NMR acquisition time as demonstrated in the determination of tacrine metabolites in microsomal incubate (Fig. 3). The column trapping-NMR system was believed

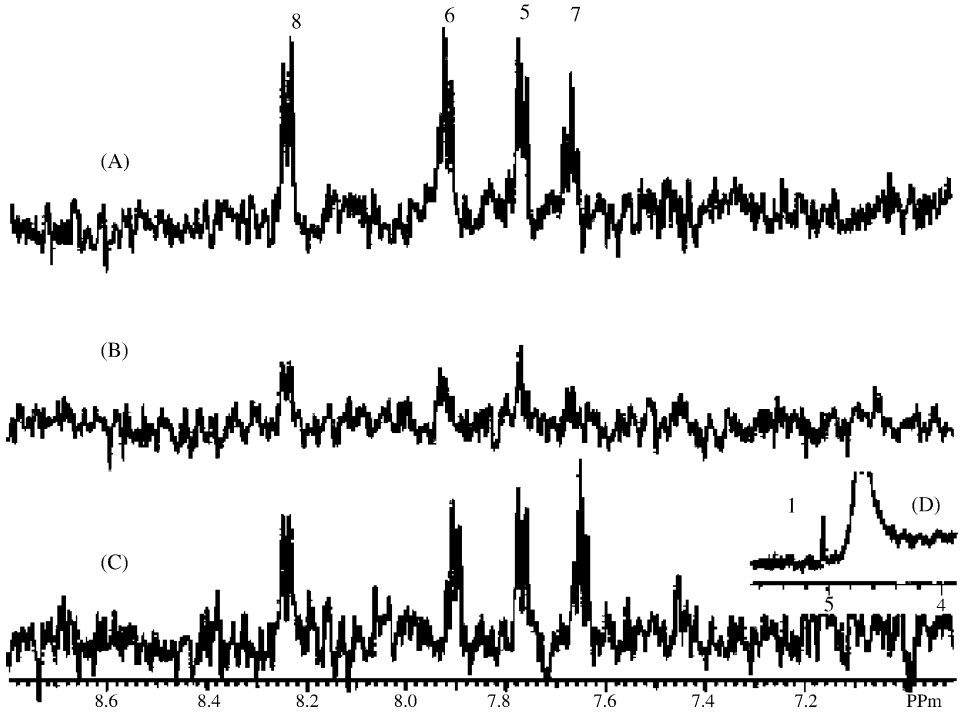

Fig. 3. Aromatic region of the ^{1}H NMR spectra (600 MHz) of 1-OH-tacrine from dog microsomal incubate (Ref. [57]). LC–NMR in stop-flow mode: (A) 512 scans acquired, (B) 64 scans acquired and (C) with column trapping, 64 scans acquired. (D) Expansion of the region near water of the bottom spectrum, showing the methine resonance of 1-OH-tacrine.

to be most useful where the amount of analyte is limited and chromatography peaks are well separated.

Superheated D_2O is considered an ideal eluent for on line LC–NMR or LC–NMR–MS, it can be used for both on-flow and stop-flow NMR measurements. To demonstrate the suitability of superheated D_2O as an eluent for LC–NMR–MS, several experiments were performed using salicylamide [42] or a series of sulfonamides [43] as model compounds. The LC–NMR–MS systems were configured in parallel, and stop-flow ^{1}H NMR measurements were performed at 500 MHz when the sample reached the NMR flow probe. The residual water resonance was suppressed using pre-irradiation during the relaxation delay of 2.0 s and mixing period of 0.10 s. The systems were dedicatedly designed to ensure the eluent cooling down to room temperature from 180 °C when it reached the NMR flow probe. It was found that use of superheated D_2O as an eluent has a number of advantages for LC–NMR or LC–NMR–MS as it is relatively inexpensive compared to deuterated organic solvents, is pure, and produces a minimal background signal. Moreover, unexpected but specific deuterium exchange reactions can occur in superheated D_2O, which may provide a selective and specific method for the preparation of deuterium-labeled analytes.

3.2. Nature product analysis

Nature products such as medical plants account for a big portions of prescribed and over-the-counter medicines, and functional foods in the world. Because the vast variety of compounds in nature still exceeds the number of that included in the largest combinatorial library, screening nature products for emerging compounds that could become new drugs or functional foods has been one of the most prominent driving forces for analytical method development in nature product analysis. LC–NMR–MS allows for the rapid detection and identification of a broad range of nature compounds, and thus markedly speeds up the analytical process and stereo-specific identification of unknowns. It is also of particular interest to nature product researchers because a systemic analysis of the compound network promises to provide deep insight into the nature process and biological response to changing environments.

The first application of LC–NMR–MS to nature product analyzed the complex extract of *Silene otites* for ecdysteriods identification [58]. Additional MS information helped in the identification of those ecdysteriods that could not be identified by LC–NMR alone [59]. In this study, a 500 MHz NMR equipped with a ^{1}H–^{13}C flow probe and an ion trap MS operated at ESI positive ion mode was hyphenated in parallel, allowing same peaks arriving at both detectors simultaneously. NMR data were acquired on-flow for all peaks and direct stop-flow mode for selected peaks of interest. Peak selection and temporary flow stop were monitored by a UV detector, and the water signal in deuterated acetonitrile/water mobile phase was suppressed by WET. This hyphenated system was further modified for the identification of constituents in an extract of *Hypericum perforatum* L. [27]. In the modified system, both UV and MS were used to trigger the stop-flow NMR measurements in a ^{1}H–^{19}F flow probe. The solvent signals were suppressed using a ID NOESY sequence for double solvent suppression. All the major known constituents in the extract were identified by LC–NMR–MS, along with the arabinoside and galacturnoide of quercetin that

had not been previously reported as constituents in the extract. Food samples are rich sources of nature products, many of which already known to have therapeutic properties. A rapid LC–NMR–MS method was developed to the direct analysis of the aromatic components in beer, grape juice and wine extract [60]. ^{1}H NMR spectra were acquired at 500 MHz in a ^{1}H–^{13}C inverse-detection flow probe using on-flow mode for grape juice and wine extract, direct stop-flow and loop storage modes for beer samples. The results presented in study showed that, in all cases studied, LC–NMR–MS has enabled the assignment of many aromatic compounds, which were difficult or not possible to be identified by NMR alone typically because of their relative low intensity and overlap with other signals in the region.

Flavone glycosides in apple peel were determined by a loop storage LC–NMR–MS system [5]. Novelty in this setup is the use of MS/MS data obtained by an ion trap MS to trigger the transfer of LC fractions of interest to a temporary sample storage unit (BPSU-36), prior to over night ID TOCSY NMR experiments. This study clearly demonstrated that the results obtained from MS and NMR complemented each other, because only with additional NMR data was it possible to discover the co-eluting stereoisomers: glucopyranosid and galactopyranosid. A similar loop storage LC–NMR–MS system was employed to isolate and identify steroid oligoglycosides from the extract of starfish *Asterias rubens*, the assignment of the signals attributed to the various sugar units was achieved by the application of 2D NMR techniques at 600 MHz [18]. Stereo-specific identification of bioactive compounds from natural sources is considered as a prerequisite for the physiological and toxicological evaluation of compounds that may be of interest for the development of pharmaceuticals and functional foods. LC–MS alone is impossible to completely characterize the chemical composition of complex natural products due to the occurrence of complex mixtures of conjugates (glycosides) and aglycones present in natural extracts. Because of the inability of LC–MS, parallel LC–NMR–MS was employed for the structure elucidation of two secoisolariciresinol diglucoside (SDG) diastereoisomers extracted from flaxseed [61]. The purified flaxseed extract was separated on a C18 column using a linear gradient mixture of D_2O and acetonitrile at a flow-rate of 1 mL/min. After splitting the LC eluent, a portion of H_2O and acetonitrile mixture was added to the flow heading to the ESI interface, which enabled the comparison of deuterated and non-deuterated molecular masses. Stop-flow ^{1}H NMR spectra were acquired at 600 MHz with WET solvent suppression sequence. The two optical isomers were clearly identified by comparing the MS and NMR data, which was further confirmed by circular dichroism (CD) analysis. The sample preparation in this study was a time-consuming procedure, which involved one alkaline hydrolysis and two separated chromatographic purification processes before submitted to LC–NMR–MS. Readers interested in online LC–MS/MS–NMR–CD "triad" for absolute stereostructure determination of nature products from crude extracts are encouraged to consult a recent review in this topic [62].

Compared to clinical specimen, nature products are more complex matrices. As illustrated before [61], extraction followed by purification is always needed for preparing LC friendly samples. Simplifying the sample preparation process for nature product will result in great saving in the turnaround time. An automated online LC–UV–SPE–NMR–MS was developed and showed great promise in concentrating the peaks prior to NMR analysis [63]. The sample subjected to the hyphenated system was the acetone extract of Greek oregano, which was selected because of its previously reported anti-oxidant activity and its chemical complexity. After sample was separated on a C18 column using gradient acetonitrile/H_2O mobile phase, 95% eluent was directed to an online SPE column placed before NMR flow probe, while the other 5% was split to the ESI interface. The separated peaks were trapped on the SPE column and then flushed with deuterated acetonitrile to the dual inverse ^{13}C–^{1}H cryoflow probe, where ^{1}H NMR spectra were acquired at 600 MHz with double pre-saturation suppressing any residual water and acetonitrile signals. Online SPE in conjunction with advanced cryoflow probe resulted in large sensitivity improvement, and rendered the setup a valuable tool for the investigation of new compounds or for a rapid identification of known or undesirable compounds from natural products. Matrix solid phase dispersion (MSPD) is a special type of solid phase extraction. By combining both sample extraction and purification in one single step starting from the intact sample material [64], MSPD significantly reduces the time and steps needed for preparing nature product that usually consist one extraction and at least one purification process. The use of MSPD in one simple step to provide nature product extracts which are suitable for direct LC–NMR–MS unknown analysis was demonstrated in the determination of a class of closely related glycosidic compounds, asterosaponins, from *A. rubens* (starfish) [65]. The MSPD treated extract was subjected to separation on an ODS column using 20 mM ammonium formate in D_2O/acetronitrile as eluent. H_2O was added to the MS flow after splitter for D–H back-exchange experiments. Both on-flow and MS triggered stop-flow ^{1}H NMR spectra for major peaks were obtained in a 4 mm *z*-gradient flow probe at 600 MHz, with solvent suppression achieved by the WET sequence with ^{13}C decoupling during the WET pulse train. Within 2 days of work, an overview of the composition of the asterosaponin fraction was obtained starting from the intact animal material, compared to an initial procedure required for 4 days.

The authors concluded that the combination of MSPD with LC–NMR–MS is a powerful approach for the rapid chemical screening of natural products, as shown for a class of closely related glycosidic compounds that cannot be distinguished by LC–MS alone. Furthermore, the LC–NMR–MS information could be used to guide further preparative work, and facilitate the identification of unknowns in the presence of known compounds without necessary re-isolation. By correlating on-flow LC–^{19}F NMR, MS/MS, with stop-flow LC–^{1}H NMR data, it was possible to identify the peaks of interest from a complicated chromatogram, after minimal sample preparation and cleanup [53]. The proposed LC–NMR–MS/MS strategy was applied to the study of the metabolism of a trifluoromethylpyridone in hydroponically grown maize plants. MS/MS data were used to identify peaks of interest for stop-flow ^{1}H NMR measurements in this study. The benefit of combined use of NMR and MS

data derived from the same experiment was clearly demonstrated in distinguishing between the glucoside and malonylglucoside conjugates.

Using superheated D_2O as the mobile phase, LC combined with online diode array UV, FT-IR, NMR and MS was employed for the analysis of a standard of 20-hydroxyecdysone- and ecdysteroid-containing plant extract. This combination of spectrometers enabled the on-flow collection of UV, ^{1}H NMR, IR and mass spectra not only for pure 20-hydroxyecdysone (100–400 g on column) but also for the major ecdysteroids present in crude extracts of *Silene otites*, *Silene nutans* and *Silene frivaldiskyana*. The ecdysteroids unequivocally identified in these extracts included 20-hydroxyecdysone, polypodine B and integristerone A [66]. This set of model compounds was chosen because there is considerable structure diversity within some 300 identified ecdysteroids. The system could obtain full UV, IR, atmospheric pressure chemical ionization MS and on-flow pseudo-2D ^{1}H NMR data on these compounds in quantities in the region of 100 μg on column, providing a typical application in the area of nature product chemistry. Gradient LC coupled to UV, MS/MS and NMR was applied to the rapid structure determination of three new isomeric divanilloylquinic acids from *Fagara zanthoxyloides* collected in Burkina Faso [67]. The first piece of information about structure similarity of these three compounds was partially identified by LC–MS/MS, because the positive and negative ESI MS spectra of them were almost identical. The locations of the vanilloyl groups on the quinic ring of the divanilloylquinic acid isomers could not be solved by reference to MS alone, but were clearly assigned with the aid of stop-flow ^{1}H NMR spectra obtained in a 60 μL LC–NMR probe at 500 MHz.

The structures of the minor constituents of active crude extracts were elucidated thanks to this powerful hyphenated technique, and so without any need for offline sample purification. The hyphenated LC–DAD–MS/SPE–NMR technique allowed the characterization of isobaric iridoid glycoside regioisomers (Fig. 4) present only as minor constituents in a *Harpagophytum procumbens* DC. (Pedaliaceae) root extract [68]. Like previously discussed LC–SPE–NMR approach, the LC separations were performed with regular solvents, and the SPE trapped analytes were eluted to NMR flow probe using 225 μL pure deuterated organic solvent. ^{1}H NMR data were obtained in stop-flow mode using multiple-solvent suppression with time-shared double pre-saturation for residual water signal. The ^{1}H NMR spectra with sufficient S/N ratio were obtained within a short time, even the analyte present in the lowest amount (~10 nM). Consequently, data mining and structure based literature search led to the rapid identification of the four analytes including one new natural product, two metabolites previously not described from *H. procumbens*, and a prominent chemotaxonomical discriminator in the genus *Harpagophytum*. This study provides a good example for rapid LC–NMR–MS analysis of minor secondary plant metabolites.

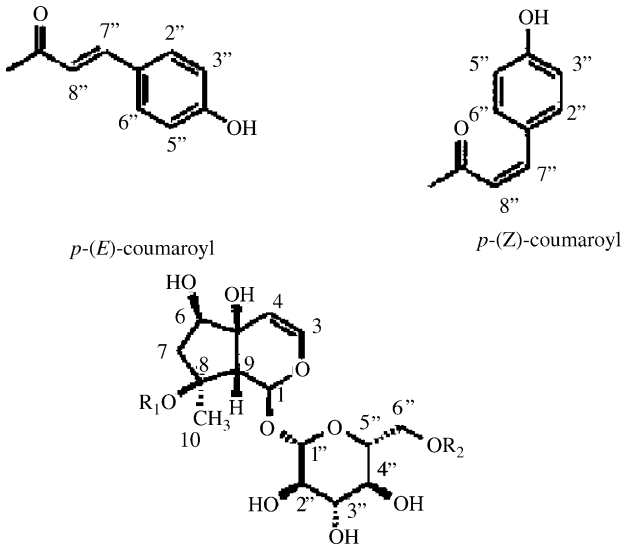

Fig. 4. Basic structure (below) and substituents (above) of the elucidated isobaric iridoid regioisomers. R_1 and R_2 can be H or *p*-(*E*)-courmaroyl or *p*-(*Z*)-courmaroyl (Ref. [68]).

4. Conclusions and expectations

Online LC–NMR–MS has been shown in many studies to be a powerful tool for solving identification and structure related problems that a LC–MS or LC–NMR alone cannot handle, without the necessity of laborious fractionation and purification. Numerous obstacles to coupling both MS and NMR to LC have been overcome during its 15 years of development, especially the improvement of NMR on-flow detection sensitivity.

The high level analytical excellence achieved may trigger the development of dedicated instruments with dedicated software to further improve the performance of this technique for routine use in clinical metabolite and nature product analysis.

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