The integration of LC-MS and NMR for the analysis of low molecular weight trace analytes in complex matrices

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Abstract

This review discusses the integration of liquid chromatography (LC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) in the comprehensive analysis of small molecules from complex matrices. We first discuss the steps taken toward making the three technologies compatible, so as to create an efficient analytical platform. The development of online LC-MS-NMR, highlighted by successful applications in the profiling of highly concentrated analytes (LODs 10 µg) is discussed next. This is followed by a detailed overview of the alternative approaches that have been developed to overcome the challenges associated with online LC-MS-NMR that primarily stem from the inherently low sensitivity of NMR. These alternative approaches include the use of stop-flow LC-MS-NMR, loop collection of LC peaks, LC-MS-SPE-NMR, and offline NMR. The potential and limitations of all these approaches is discussed in the context of applications in various fields, including metabolomics and natural product discovery.

Keywords

characterization; LC-MS; NMR

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[†]We dedicate this review to the memory of Dr. Roger Kautz who contributed significantly to writing of this work. He passed away on May 27, 2017. He is dearly missed.

CONFLICTS OF INTEREST

BSK is the inventor on general metabolomics-related IP that has been licensed to Metabolon via Weill Medical College of Cornell University and for which he receives royalty payments via Weill Medical College of Cornell University. He also consults for and has a small equity interest in the company. Metabolon offers biochemical profiling services and is developing molecular diagnostic assays detecting and monitoring disease. Metabolon has no rights or proprietary access to the research results presented and/or new IP generated under these grants/studies. BSK's interests were reviewed by the Brigham and Women's Hospital and Partners Healthcare in accordance with their institutional policy. Accordingly, upon review, the institution determined that BSK's financial interest in Metabolon does not create a significant financial conflict of interest (FCOI) with this research. The addition of this statement where appropriate was explicitly requested and approved by BWH.

1 | INTRODUCTION

The unambiguous identification of known—and more importantly unknown—analytes in complex mixture often requires the use of chromatographic separation coupled to detectors that give high structural information. Mass spectrometry (MS) and Nuclear Magnetic Resonance (NMR) are the two most widely used techniques for structural identification of compounds. In the analysis of unknowns, MS and NMR provide complementary data and both are often required for full characterization. MS can provide the atomic formula of an analyte while NMR indicates the structural moieties those atoms are organized into. For example, NMR, but not MS, can distinguish isobaric compounds and positional isomers. Conversely, MS, but not NMR, can identify certain functional groups such as sulfate and nitro groups, which are NMR silent. These complementary capabilities of MS and NMR have led to their coupling in the analysis of compounds that cannot be characterized with either detector alone in fields such as natural product discovery, metabolomics, and drug metabolite identification. The coupling of LC-MS and NMR is not trivial and requires compromises both in instrumentation and method development. For example, a fairly thorough MS analysis (including fragmentation) can be completed in under a second with a nanogram of analyte. NMR on the other hand, requires minutes to hours at the microgram level for the simplest 1D spectrum. In this review, an overview of the different ways LC-MS and NMR have been coupled, and the compromises that enable each technique to be used at its maximal sensitivity are discussed.

2 | LC-MS AND NMR

The section below provides a brief introduction of LC-MS and NMR. The analytical advantages, in terms of the structural information gained from each detector, and some of their contrasting features are presented (Table 1).

2.1 | LC-MS

Since the development of electrospray ionization, MS combined with liquid chromatography (LC-MS) has become the method of choice for structural identification of analytes found in complex mixtures, due to its high sensitivity and selectivity. The limits of detection of MS are comfortably in the femtomole range for analytes with high ionization efficiency. The LC separation greatly reduces the complexity of samples in MS, which, in turn, reduces ion suppression because reducing the number of charged analytes simultaneously entering the mass spectrometer reduces competition for the amount of charge available at any one time. MS provides molecular weight information, and from exact mass measurements the elemental composition of compounds can usually be deduced. Furthermore, tandem mass spectrometry (MS/MS) provides structural information of compounds based on their fragmentation patterns. Spectral scan rates for MS are in the nanosecond to microsecond range depending on the mass analyzer, making it ideal for high-throughput analysis. Combining MS to LC, makes it ideal for analysis of complex samples. A limitation of MS, however, is that it does not provide definitive structural identification of compounds in the absence of authentic standards. Definitive structural identification by LC-MS is usually

based on comparison of the retention time and the MS/MS spectral pattern of the analyte of interest with those of an authentic standard.”

2.2 | NMR (and LC-NMR)

NMR (and LC-NMR) is generally the analytical method of choice when more definitive structural characterization is needed. The NMR phenomenon is based on the interaction of magnetically-active nuclei with an applied external magnetic field. In principle, any magnetically active nucleus is detectable by NMR; however, due to the low sensitivity of most nuclei, those primarily studied in NMR are ^1H , ^{13}C , ^{19}F , and ^{31}P . Some nuclei like ^1H and ^{19}F , are found at ~100% abundance and are detected with better sensitivity than nuclei like ^{13}C , which is found at 1.1% abundance. ^{15}N is also frequently studied but it is detected indirectly through attached ^1H . Structural information is deduced from (i) the chemical shift, which is dependent on how shielded a nucleus is by surrounding electrons; (ii) the splitting patterns, which provides information on the number of neighboring nuclei; and (iii) multi-dimensional experiments which indicate atomic connectivity. Also, unlike MS, the NMR signal is not affected by matrix effects, so NMR is intrinsically quantitative. Moreover, NMR analysis is non-destructive, so after analysis, a sample can be recovered and analyzed further using other techniques. Another advantage of NMR over MS, is that NMR data are constant and reproducible across different NMR instruments (regardless of vendor or field strength). This is not the case in MS analysis where data is dependent on the ionization or even the mass analyzer and software used. In comparison to MS, however, NMR requires relatively large concentrations of material for analysis.

2.3 | NMR sensitivity

In the hyphenation of LC-MS and NMR, the limitations are derived largely from the low sensitivity of the NMR experiment. The low sensitivity of NMR derives from the very small energy difference between the spin states (low energy and high energy states) of the nucleus. The detectable signal strength is proportional to the population difference, which is about 0.01% for ^1H at room temperature making the sample requirement for a simple ^1H experiment at least 100-fold higher than that for MS. Moreover, NMR experimental acquisition timeframes are significantly slower than the standard MS experiment. To produce an NMR signal, radio frequency energy is applied and it stimulates the spins in the lower energy state to the higher energy state. After each perturbation of the sample (ie, measurement), 1-2 sec are required for the population difference to relax back to equilibrium (return to ground state), which further limits the scan rate. Thus, NMR requires long observation times to increase the signal to noise ratio (S/N). The observation times range from minutes to hours for a simple ^1H spectrum, and hours to days for 2D experiments at the low microgram level and for the analysis of low sensitivity nuclei like ^{13}C which are particularly useful in the structural analysis of unknowns. In light of these limitations, several strategies have been developed to allow the routine use of NMR in the analysis of low concentration analytes. One approach is the development of higher field spectrometers; an increase in spectrometer frequency by a factor of three leads to a 5.2-fold increase in S/N (eg, from 300-900 MHz). Moving to higher fields primarily improves resolution, which is useful in highly crowded spectra, high field spectrometers are, however, very expensive. A second approach is the development of more sensitive NMR probes of which the principal

approaches involve the use of small probes (microcoil probes), cryogenically cooled probes (cryoprobes), or high temperature superconducting probes (HTS). Cryoprobes improve sensitivity by reducing the noise from the electronic components. The electronics in a cryoprobe are kept at about 20°K while the sample is at room temperature. This leads to a fourfold improvement in S/N for organic solvents and twofold for aqueous solvents, when compared to a room temperature probe of the same dimensions. Microcoil probes have reduced coil dimensions, which lead to a reduction in noise thereby leading to an increase in signal-to-noise ratio. In addition, a feature of microcoil probes is their small observe/active volumes, that is, the portion of the sample that is in the coil that contributes to signal. This volume may be as low as 1.5 μ L, and dissolving analytes in such low volumes of solvent increases their concentration and hence the NMR signal.

3 | CHALLENGES IN INTEGRATING LC, MS, AND NMR

Online coupling of LC-MS and NMR into a single integrated system would provide essentially complete spectroscopic characterization of a sample in a single injection, while minimizing sample preparation and reducing the opportunity for sample degradation during drying or offline handling. Hyphenating two analytical methods requires accommodating the features that can make them incompatible, which in turn defines the complexity of hyphenating them. Bearing this in mind, we discuss next the technical issues that need to be considered in the integration of MS and NMR. This includes the solvent limitations of each (and of the LC mobile phase), and the different chromatographic requirements of MS and NMR detection.

3.1 | Mobile phase for LC-MS-NMR

The mobile phases commonly used in reversed-phase HPLC are acetonitrile or methanol as the organic mobile phase, and water as the aqueous mobile phase. All of these solvents have protons at concentrations of 30-100 M; although NMR has a wide dynamic range of 10^5 such strong solvent signals can overwhelm the NMR signal of low concentration analytes and add to the background noise. To reduce interference from solvent resonances, deuterated solvents are preferably used for separation but, due to cost, often only water is substituted with D₂O (relatively inexpensive, below \$0.50/mL) while the organic phase (> \$1/mL) is often reported in its non-deuterated form. Although many papers report the cost of using deuterated organic phase as prohibitive, our experience has been the \$100 cost of switching to deuterated acetonitrile for a critical run is modest (unpublished results).

The use of deuterated water in NMR brings a few minor concerns in LC-MS-NMR. In the LC dimension, a slight shift in the retention time of the analytes relative to the protonated form (due to the deuterium isotope effect) may be observed. In the NMR, when H₂O is substituted with D₂O, exchangeable hydrogen in, for example, -SH, -NH, and -OH, are not observed due to their rapid exchange with the solvent. Deuteration also shifts the mass of analytes that contain exchangeable hydrogens. Deuterium incorporation should be considered when interpreting mass spectra, especially when an analyte is partially deuterated, which leads to observation of several masses for the same analyte. For example, Figure 1 shows the mass spectrum of propranolol obtained from a LC-MS-NMR experiment

that utilized deuterated water and acetonitrile (non-deuterated) for separation. Two masses at m/z 262 and m/z 263 are observed due to partial H/D exchange. To account for the changes observed in both LC and in MS, an initial LC-MS separation may be first conducted with non-deuterated mobile phases, and then compared with a second separation in the deuterated solvent. This is useful when differences between data with and without deuterated solvents can assign the number of exchangeable protons in structural characterization. An alternative technique to avoid deuteration in MS, is to introduce a “make-up” flow—to mix the eluent with an excess of non-deuterated solvent between LC and MS analysis—so that deuterated sites backexchange to protons.

When non-deuterated solvents are used for LC-MS-NMR, interference from the solvent resonance can be reduced through solvent suppression techniques, such as pre-saturating the solvent proton resonances. A water resonance is generally observed in all NMR solvents, due to adsorption from air and this may also warrant suppression. For trace analytes, the residual protons of deuterated solvents may warrant suppression as well. Figure 2 shows NMR spectra of a 10 mM sample of sucrose before and after solvent suppression. Suppression of the residual water resonance at 4.5 ppm and the acetonitrile resonance at 2 ppm increases the analyte signal 100-fold. Solvent signal suppression, however, tends to also suppress resonances in close proximity to that of the solvent. Suppression of the water signal can also diminish the intensity of exchangeable protons that are in rapid exchange with water.

Modifiers and buffers are often added to the mobile phase to improve the peak shape of ionic compounds, to ensure reproducible retention times in LC, or to aid ionization of analytes in MS. Most additives will produce high background in online LC-MS-NMR. For NMR, additives that do not have protons are preferred, while for MS, additives that will not interfere with the ionization process are recommended. Buffers that are NMR-friendly (eg, sodium phosphates) are generally salts incompatible with ESI; most are non-volatile and can contaminate the ion source. Trifluoroacetic acid (TFA) is NMR-friendly, is volatile and, is a great ion pairing reagent for LC, but TFA causes ion suppression in ESI-MS. It is particularly problematic in the analyses of acidic analytes, which are neutralized by ion pairing with TFA, and become undetectable in MS. Formic acid has been found to be the best compromise for both MS and NMR. Although it has a sharp proton resonance at 8.5 ppm, this single sharp signal is easily suppressed.

3.2 | LC parameters and NMR sample efficiency

Signal in NMR is proportional to the concentration of analyte present in the NMR coil's active volume. Flow probes used in LC-MS-NMR have observed volumes of 60-300 μ L. At an LC flow rate of 1 mL/min, a 60 μ L volume is equivalent to 8 sec of flow. LC peaks are typically 10-90 sec wide, which means that only a fraction of the available analyte produces NMR signal (Figure 3) which further reduces the overall sensitivity. The term “sample efficiency” refers to the percent of the available sample which is in the observed volume, and is responsible for the produced signal. The NMR flow-probe volume of an LC-NMR system is chosen when purchasing an LC-NMR system to balance the higher sample efficiency (better sensitivity) of larger probes, against the better chromatographic resolution of smaller

probes. An LC probe's flowcell volume is typically twice its observed volume, so even sharp peaks have sample efficiency below 50%.

Although the probe volume of a given system is fixed, the residence time of the analyte can be increased by reducing the LC flow rate, or by stopping the LC pump to acquire more scans. Either of these actions can compromise the LC performance, leading to broader peaks through diffusion. Although capillary LC typically shows sharper peaks, the capillary LC columns have a 10-fold lower loading capacity than analytical columns which limited their applicability for LC-NMR analysis, especially of low concentration analytes.

The column dimensions, that is, column internal diameter and the column length, play an important role in LC-MS-NMR. These two dimensions determine the amount of sample that can be injected (thus detected) and the separation of different analytes in a complex sample (ie, analyte resolution), respectively. The column length chosen is dependent on the complexity of the sample. For highly complex samples, longer column lengths provide better analyte resolution. The use of long columns, however, leads to long analysis times, and requires a longer time for column equilibration between injections. For LC-MS-NMR, the most commonly used columns are 15-25 cm long. The column internal diameter determines the amount (in mass) of material that can be injected on column, thus larger diameter columns allow for the injection of more material on column. For LC-MS-NMR, because of the high sample mass requirements for NMR, the most commonly used columns are 2.1 and 4.6 mm ID columns, with loading capacities of 50 and 100 µg, respectively. Because different analytes in a complex sample are at different concentrations, to maximize the concentration of analytes at lower levels the column should be loaded to capacity. Unfortunately, overloading of column also leads to peak broadening and in-turn, peak-to-peak contamination of closely eluting analytes.

The above discussion illustrates the disparate requirements of these three analytical techniques and the challenges involved in their incorporation into an integrated platform. Challenges in the hyphenation of LC-MS and NMR are largely derived from NMR. The approaches taken to overcome these disparities are discussed next.

4 | ONLINE LC-MS-NMR

HPLC can be interfaced to MS and NMR either in series or in parallel, as shown in Figure 4. When connected in series (Figure 4A), LC eluent passes first through the NMR detector (which is non-destructive) and then MS analysis. Due to the differences in sample requirements between MS and NMR, a small fraction of the flow is split off for MS analysis, with the majority directed to waste or to a sample collector for further analysis. This configuration has not been used extensively because it is difficult to correlate the MS and NMR data as the analyses occur at significantly different times in each detector (and dispersion in the large NMR flowcell degrades chromatographic resolution of the LC-MS data).

A parallel configuration of MS and NMR detectors is the more extensively used approach of hyphenating LC-MS and NMR (Figure 4B). In this mode, the LC eluent is split at typical

ratios of 1:5 or 1:20 between the MS and the NMR, with most of the flow directed toward the NMR and the smaller fraction to the MS system. In online LC-MS-NMR, data can either be acquired simultaneously and continuously on both detectors (onflow), or the NMR experiment can be temporarily decoupled (at-line) from the LC-MS using stopped-flow or loop storage, to allow for longer NMR acquisition time to enhance the signal-to-noise ratio in NMR.

4.1 | On-flow (Continuous) LC-MS-NMR

On-flow or “continuous mode” LC-MS-NMR is characterized by simultaneous and continuous acquisition of MS and NMR data. A UV detector is often included immediately post-column, to monitor the transfer of the analyte simultaneously to both the MS and the NMR. The delays in the transfer lines from the LC to the MS and the NMR are calibrated, to correlate the data from the three detectors. Even when stopped-flow analysis is anticipated, a preliminary on-flow run is generally performed with a small amount of analyte to check for changes in the chromatogram caused by deuterated solvents, before committing the bulk of a limited sample to stopped flow.

On-flow LC-NMR data are generally displayed as a contour plot of stacked ^1H or ^{19}F spectra. As shown in Figure 5A, the LC retention time is displayed vertically and the NMR chemical shift horizontally. The MS data are recorded concurrently but are displayed separately as insets for selected time points. For data interpretation, the ^1H NMR spectra corresponding to each component based on the retention time are extracted from the stack. In the example on Figure 5, continuous flow LC-MS-NMR has been used to distinguish between different carbohydrates in beer. The carbohydrates in beer give it its “mouth feel,” thus, monitoring their composition during fermentation can be used to optimize or confirm the quality of the product before release. These carbohydrates in beer are mainly composed of linear and/or branched dextrans. MS data alone cannot unambiguously distinguish branched or linear carbohydrates, whereas by NMR the two configurations have distinct chemical shifts with branched dextrans exhibiting resonances between 5 and 6 ppm while linear dextrans are found between 4 and 5 ppm. The results shown in Figures 5B and 5C, indicate the carbohydrates found in an ale.

Gradient elution, which aids the separation of compounds with very different polarities in a complex sample in the LC dimension, can be problematic in NMR. Steep gradients—changes of more than a few percent per minute—can create solvent gradients in the NMR flowcell, and thus a gradient of magnetic field (magnetic inhomogeneity), which causes resonances to broaden, split, and weaken. Moreover, even when a gradient is slow enough to avoid broadening, the chemical shifts of analytes are affected. The chemical shift in NMR is solvent dependent, thus when the solvent composition changes, (eg, during gradient elution), the chemical shift of analytes also changes. A 1% change in solvent composition, for example, can shift the solvent resonance by up to 15 Hz. This shift is problematic for solvent suppression, which requires the solvent frequency to be known to within about 5 Hz. An approach developed by Jayawickrama et al counters the minor effect of gradient elution on analyte chemical by mixing the LC eluate with a reverse gradient between the LC and the NMR probe, keeping the solvent composition in NMR constant.

On-flow LC-MS-NMR is also affected by the flow rate used for elution. Analytical columns with a loading capacity of >100 µg are preferably used to ensure enough material for NMR. The optimal flow rate for these columns is in the mL/min range, which can be detrimental in both MS and NMR detection, although the flow rate can be reduced in MS by splitting at higher ratios. In NMR however, higher flow rates reduce the residence time of the analytes in the NMR probe—fewer scans are acquired before the analyte leaves the detector—thus compromising the advantage of isolating more material on higher-capacity column. Reduced flow rates can increase the residence time for NMR analysis but may lead to broadening of the LC peaks (loss of LC resolution). Flow rates on the order of 0.1–0.4 mL/min have been found to be a good compromise between longer residence time in the NMR probe with acceptable broadening of LC peaks.”

Although the limits of detection (LOD) in the on-flow mode are relatively high (LODs are on the order of 10 µg for 1D proton spectra), it is useful as a screening tool to give an overview of compounds eluting from an LC column. In addition, since NMR provides absolute quantitation, on-flow LC-MS-NMR can also provide concentrations of the major constituents of a complex sample. On-flow LC-MS-NMR has been particularly useful in identifying drug metabolites from fluorine-containing compounds, which are not uncommon in drug design. For instance, ^{19}F NMR has good inherent sensitivity, and ^{19}F NMR spectra of biological materials are nearly devoid of other signals. On-flow LC-MS-NMR has also been a valuable step in natural product discovery to provide an overview of a plant extract. Recognizing extracts with known compounds (dereplication) can avoid wasting effort on re-discovery. Other applications of on-flow LC-MS-NMR include: quality control in the beverage industry, as a screening tool in metabolomics, and in the characterization of degradation products in the pharmaceutical industry. In all these applications, however, on-flow LC-MS-NMR does not generally provide the type or quality of NMR data required for the structural elucidation of unknowns.

Nearly synchronous with the introduction of continuous-flow LC-MS-NMR, variations were introduced that temporarily decouple the MS and NMR experiments, (also called at-line methods). These methods increase NMR sensitivity by either allowing longer NMR data acquisition time, or by increasing analyte concentration. The goals were to obtain (i) better NMR data of low concentration analytes; (ii) spectra of low-sensitivity nuclei (ie, ^{13}C or ^{15}N); and (iii) acquisition of the 2D NMR experiments which are often required for complete structural characterization of unknowns. The alternatives to on-flow LC-MS-NMR were stop-flow mode, LC peak collection into loops and pre-concentration by collection onto solid phase extraction (SPE) cartridges.

4.2 | Stop-flow LC-MS-NMR

Stop-flow LC-MS-NMR was developed to improve S/N in NMR analysis. In the stop-flow mode, the LC pump is stopped when an analyte of interest is in the NMR probe, and therefore can increase its residence time in contrast to on-flow LC-MS-NMR. In NMR, the signal increases linearly with the number of scans and the noise (which is random) increases as the square root of the number of scans. Thus, the S/N increases with the square root of time. For example, doubling the S/N of an NMR spectrum requires a four-time increase in

time (ie, for a twofold increase of the signal of a 1 min spectrum, the spectrum should be acquired over 4 min). Stop-flow LC-MS-NMR also enables the acquisition of more time-consuming ^{13}C spectra and 2D NMR spectra. An additional advantage realized through stop-flow NMR is that conditions are static, thus solvent peaks remain constant for suppression.””

Stopping the LC pump for NMR analysis can be triggered by either UV peak detection or by the MS detector. Delays between peak UV and peak NMR intensity need to be calibrated, and prior knowledge of the elution patterns of analytes and interferences is valuable. Triggering stop-flow from MS has an advantage over UV in detecting analytes that do not contain a chromophore or for compounds with a low UV absorbance. Increased specificity on peak selection can be achieved by use of tandem MS (MS/MS), whereby a particular MS/MS transition can be used to trigger the stop of the LC pump.” An illustrative example was shown by Lommen et al, in the identification of O-glycoside-modified flavonoids in apple peels. Some flavonoids have been shown to have antioxidant properties and to inhibit tumor growth in animal studies, thus their identification in foods is important. Figure 6 shows four chromatograms, UV chromatograms at 370 and 280 nm, and MS/MS chromatograms at m/z 301 and 274, which monitor the two classes of O-glycoside flavonoids (Quercetins and Phloretins, respectively) found in apple peels. The fragment ion monitored was produced by the loss of the sugar moiety. Although the two classes of flavonoids have some differences in their UV absorbance, as shown in Figures 6A and B (right panels), the use of an MS/MS transition to detect the phloretin glycosides is much more selective because multiple compounds with different masses can have the same UV absorbance. In this work, MS provided the molecular weights of the O-glycoside flavonoids while NMR showed the configuration and position of the sugar moieties.

The introduction of cryoflow NMR probes provided a strong boost in sensitivity of on-line NMR, in both on-flow and stop-flow modes. The increased sensitivity of cryo-flow LC-MS-NMR was demonstrated in the analysis of metabolites from acetaminophen. The analysis of the metabolites using a cryoflow probe were compared with previously published LC-MS-NMR results that had utilized a conventional flow probe. The experiments with the cryoflow probe utilized 40% less material, and detected new metabolites. In addition, the use of a cryoflow probe led to a 10-fold reduction in the NMR analysis time and also facilitated the detection of labile analytes.

A challenge of stop-flow LC-MS-NMR relative to the onflow mode is that diffusion mediated peak broadening can lead to peak-to-peak contamination of closely eluting compounds. The diffusion-mediated peak broadening can be circumvented using gradient elution for LC separations rather than isocratic elution. A second limitation is the contamination of the NMR probe, especially when a low concentration analyte is analyzed immediately after a high concentration one. The problem of contamination of the NMR probe was addressed by the loop-storage method discussed next.

4.3 | Loop-storage LC-MS-NMR

Another early enhancement to LC-MS-NMR involved the collection of analytes of interest during continuous LC, for subsequent NMR analysis.” Loop-storage avoids the need to stop

the LC pumps for NMR analysis by directing the LC flow to capillary loops, which are matched to the volume of the NMR probe. This improves performance over stop-flow analysis in two main ways: (i) avoids diffusion-mediated broadening of the LC peak, but still allows for the improvement of NMR S/N through signal averaging or for 2D experiments and (ii) it can mitigate carryover in the flowcell, as discussed below. As in the stop-flow mode, peak selection for NMR may be done either by MS or by UV. The NMR experiments are usually performed after completion of LC-MS analysis. Although we distinguish “at-line” methods in which NMR data collection is done immediately after chromatography from offline methods, the loops can also be stored indefinitely before NMR analysis if the samples are stable and do not degrade. An example of a loop collection application was the discovery of a novel glutathione adduct of diclofenac. Yu and O’Connell improved the usual MS assay for GSH adducts (a neutral-loss scan for m/z 129) by using an offline nanoelectrospray ion source to improve MS sensitivity. When a novel adduct was detected (after incubating the parent drug with microsomes), straightforward LC-NMR using a loop collector provided NMR spectra.

The loop-storage mode of LC-MS-NMR can eliminate carry-over of high-concentration analytes that can contaminate low concentration compounds that directly follow chromatographically. This contrasts with the stop-flow mode, which, due to the large dead volume of NMR flowcell, causes significant tailing of peaks. Figure 7 shows an example of two such peaks, with retention times 0.4 min apart, contrasting the spectra acquired using loop-storage mode against stop-flow. Carryover of Peaks 1-2 is observed in stopped flow, but not in the loop collection mode. Carryover can be eliminated with loop collection because the probe can be flushed with solvent between loading the different loops.

Loop collection has several limitations. Some additional broadening is inevitable. Moreover, deuterated solvents are still preferred for separation, as with both on-flow and stopped-flow LC-MS-NMR. Loop collection also requires more complicated fluidics than stop-flow analysis. Typically, a breadbox-sized accessory with multiple valves is needed, to switch between a dozen or so collection loops and to reroute collected fractions through a UV detector for positioning them in the NMR probe, along with software to control loading and data collection (preferably unattended). A loop collection interface is, however, available with most commercial LC-NMR systems.

4.4 | LC-MS-SPE-NMR

An alternative to LC peak collection into loops, is collection onto small columns or solid phase extraction (SPE) cartridges. LC-MS-SPE-NMR has several strong advantages which include: (i) the possibility to capture a broad LC peak (capturing all analyte from a larger volume than an LC-NMR probe) and (ii) eluting analytes in a small volume thereby achieving much higher sample efficiency. This translates into the presence of more analyte in the observed volume to gain higher NMR sensitivity. In fact, several repeated LC injections can be captured onto the same SPE cartridge before elution, doubling, tripling, or more the effective sensitivity. In this final section of at-line methods, we will discuss some of the advantages and considerations in using LC-MS-SPE-NMR.

Post-column trapping of analytes for NMR was first demonstrated by Griffiths et al, using a guard column for analyte enrichment. This method has since evolved and analyte trapping is now widely done using an SPE fraction collector, although good results are still obtained on home-built systems.

Post-column trapping with SPE can be compared to 2D LC whereby a complex sample is first separated on one column and, as each analyte elutes, it is routed to a second column. The second LC column (here an SPE or guard column) is smaller, so after being loaded, the analyte can be eluted with a small volume of solvent for subsequent NMR analysis. The instrumental set-up for LC-SPE-MS-NMR is shown on Figure 8. First, separation of a complex mixture (eg, plant extract, serum, etc.) is done on a large column with a high loading capacity (a 4-mm analytical column, or SPE enables use of semi-prep columns). The LC flow is split between MS and the SPE fraction collector with MS and/or MS/MS data are acquired continuously. Detection of LC peaks to trigger SPE fraction collection is preferably based on MS spectra (mass-guided fractionation). Because the compounds eluting from the LC are in high organic solvent (thus would not bind to the SPE cartridge), the eluate is mixed with aqueous solvent to reduce the elutropic strength of the mobile phase prior to being passed over the SPE column). A ratio of 3:1 of the make-up flow to the strength of the mobile phase is typically used. After analyte trapping, cartridges containing analytes of interest are then dried with nitrogen to remove residual mobile phase, and finally eluted using deuterated solvents into the NMR probe.

LC-MS-SPE-NMR hyphenation has several advantages over onflow, stop-flow, and loop-storage methods. One advantage, is that the LC separations may use non-deuterated solvents, which greatly reduces expense and avoids adjusting chromatography for differences in elution. Importantly for MS, the interpretation of mass spectral information is more straightforward without deuterium exchange. Moreover, since SPE cartridges for NMR analysis can be eluted with aprotic deuterated solvents, exchangeable hydrogens may be observed.

Another advantage of LC-MS-SPE-NMR is that because multiple trappings of an analyte of interest can be made, the NMR analysis of low concentration analytes is possible. The concentration of these analytes is increased via multiple injections. Moreover, multiple trappings can also be used to reduce NMR analysis time as it is often faster or more efficient to make multiple injections than to acquire NMR spectra overnight. As shown on Figure 9, multiple trappings of an analyte of interest reduces the NMR experimental time exponentially with linear increase in analyte concentration. In this specific case, by making three repeat trappings, the experimental time is reduced 10-fold and all proton resonances are still observable. Multiple trappings can even isolate sufficient quantities of minor components for acquisition of 2D NMR spectra or for ^{13}C analysis, which are necessary for de-novo structural elucidation.

LC-MS-SPE-NMR has some limitations and requires some method developments. First, one must ensure efficient trapping of analytes onto the SPE cartridges, and then subsequently ensure efficient elution and transfer of trapped analytes into the NMR probe. Efficiency in trapping and elution are highly dependent on the analyte of interest and often knowledge of

the physicochemical properties of compounds is required.” Analyte trapping is influenced by the choice of sorbent material, which is a compromise in the analysis of complex mixtures that have different analytes—a single sorbent might not work for all analytes in a mixture. For example, Clarkson et al investigated the trapping efficiency of eight SPE phases for 25 model natural products with a variety of polarities. Overall, the authors found that hydrophobic compounds were retained on most phases but that hydrophilic and charged compounds were best retained on polymeric phases made from poly(divinylbenzene) as compared to silica-bonded phases (eg, -C18). Another concern associated with analyte trapping on SPE cartridges is that overloading the cartridges can lead to sample loss. Moreover, analytes can inadvertently elute from the cartridges due to the high flow rate of the make-up flow. The flow rate of the makeup flow is up to three times that of the LC flow rate (2.5-5 mL/min) which leads to high pressure on the trapping cartridge.

Some final concerns in LC-MS-SPE-NMR are the choice of solvent used to elute and transfer the analyte into the NMR probe, which is also highly dependent on the analyte of interest. Deuterated methanol and acetonitrile are frequently used because they are common organic mobile phases for reversed-phase LC and so expected to elute the analyte from the cartridge. As with the choice of sorbent material, however, the different analytes within a mixture being analyzed may behave differently. Additionally, because it is desirable to elute the trapping cartridges with a small volume of solvent for NMR analysis, elution from the cartridge may be incomplete leading to analyte loss.

5 | OFFLINE LC-MS/NMR

This section will cover examples where the NMR experiment is offline and completely decoupled from the LC-MS experiment (LC-MS/NMR). This mode offers more advantages to the methods previously discussed and they include: (i) the LC-MS and NMR methods can be developed independently and each performed at its optimal conditions (without compromise); (ii) LC-MS data can be reviewed to select which peaks require NMR analysis, and to plan what specific structural information is needed from the NMR analysis; (iii) The MS and NMR do not need to be in the same room, operational, and manned and waiting for each other; and (iv) Analytes can be concentrated by dissolving them in the smallest volume possible, then analyzed using the most sensitive NMR probes available including microcoil probes

A typical LC-MS/NMR workflow is summarized in Figure 10. First, a complex mixture (eg, plasma, plant extract) is separated by LC on normal bore analytical columns (ie, 2.1 or 4.6 mm columns) so that a large amount of the complex mixture can be loaded on column. The LC eluent is then split post-column and a portion of the flow directed to the MS (5%) and the rest, to a fraction collector. Multiple LC-MS injections to collect the same peak(s) can be used to increase the analyte concentration for subsequent NMR analysis. The LC solvent is then dried down and after review of the LC-MS data, select analytes can be analyzed by NMR using the most sensitive NMR probe available. Each step of the offline LC-MS/NMR experiment is discussed in detail in the context of selected examples.

5.1 | Analyte collection and concentration for offline NMR

In offline LC-MS/NMR, analytes can be collected for NMR either by post-column trapping on SPE cartridges, or by fraction collection on 96-well plates, or tubes. The advantages and limitations of using SPE for analyte trapping are the same as discussed in the LC-MS-SPE-NMR section. The difference is that, in the offline mode, the LC-MS experiment that includes analyte isolation is completely decoupled from the NMR experiment. Fraction collection on plates or in tubes, is a less expensive alternative to the use of SPE cartridges and does not require expensive specialized equipment. Additionally, unlike when SPE cartridges are used, fraction collection does not require extensive method development and optimization to ensure optimal analyte trapping, since it does not add an additional analytical step. Moreover, unlike SPE, which has mainly been limited to hydrophobic compounds (the solid-phases currently available for SPE trapping), concentration by drying allows for analysis of hydrophilic compounds, because any validated LC method can be used, including separation using HILIC-LC which is mainly for hydrophilic compounds.”

A succinct demonstration of LC-MS with offline NMR using a fraction collector was demonstrated by Willman et al in the structural elucidation of sphingomyelins from bovine brain. Sphingomyelins are a class of biologically relevant lipids that have been found to be altered in some diseases, for example, heart disease. The structure of sphingomyelins is comprised of a phosphocholine head group, and two long carbon chains. The structural elucidation of SMs, is hindered by (i) the complexity of the samples they are found in; (ii) their wide range of concentrations (spans three orders of magnitude); and (iii) their structural diversity, specifically their different isomers of SMs. Isomeric SMs differ according to position and configuration (cis/trans) of double bond on the fatty acyl chains, the orientation of the fatty acyl chains along the backbone, and by the length of each of the fatty acyl chains (eg, SM[d16:1/18:0] and SM[d18:1/16:0] are isomeric). Typically, LC- MS/MS (with exact mass measurements) is used to elucidate the number of carbon and double bonds of the SMs, and the constitution of each fatty acyl chain. NMR may however be required to establish the exact position and orientation of the double bond along the carbon chains. The example from Willman et al discussed here, used reversed phase LC-MS/MS with fraction collection, followed by NMR analysis on a 3 mm cryoprobe of the dried fractions. Figure 11A shows the baseline separation of a wide range of concentrations of 17 SMs in bovine brain. In addition to reducing the complexity of the bovine brain, LC separation reduced the risk of ion suppression of low concentration SMs by the higher concentration ones. For structural determination, the empirical formula of the 17 SMs was determined by measuring their exact mass on a time of flight mass spectrometer, while MS/MS was used to determine the number of carbons and double bonds on the two carbon chains that is, the spingoid base and the fatty acyl. Both 1D and 2D NMR were used for complete structural elucidation of the SMs including determination of the exact position of the double bond and the connectivity between the different carbons. Figure 11B shows a 2D heteronuclear single *quantum* correlation (HSQC) spectrum of the chromatographic peak at 51.3 min (structure on in-set). The HSQC spectrum correlates the chemical shifts of the different hydrogen and carbons on the sphingomyelin.

5.2 | Use of LC-MS results for NMR follow-up

In offline LC-MS/NMR, the LC-MS results are commonly used as a first step in the analysis, while NMR analysis, which is more time consuming and requires much more material, can be done only when necessary. The high sensitivity of MS-methods enables the rapid detection of trace levels of compounds without the need to scale-up. Tentative identification of these compounds is then based on their exact mass and fragmentation data which can then be searched in publicly available databases. Novel compounds, or compounds that cannot be readily identified by MS/MS alone especially if authentic standards are not available, can then be isolated for more comprehensive analysis using NMR.

The rapid MS-based ID of analytes followed up by NMR has been especially useful in the search of new pharmaceutical agents via natural product discovery. The two goals of natural product studies are to (i) isolate and purify compounds found in crude extracts and (ii) unambiguously characterize the isolated compounds. Since compounds of interest can be at very low concentrations, it is important to establish whether a compound that shows bioactivity is novel. This process known as dereplication, ensures that already known compounds are not re-identified and avoids time consuming structural elucidation steps, and expensive scale-up. The dereplication process, which has been reviewed extensively elsewhere, combines MS data with database searches (and in some cases 1D ^1H NMR) to quickly identify already known compounds. Unknown or difficult to identify compounds are then isolated by scaling up for extensive 2D NMR.,,

The emerging field of metabolomics has also benefited from LC-MS/NMR. The metabolome is the set of low molecular weight compounds present in a biological system (eg, in cells, blood, tissue etc.). Metabolomics experiments (especially untargeted experiments) have two major components (i) Metabolite profiling, the process of determining one or more metabolites/sets of metabolites that can be used to characterize a biological state, or distinguish two biological states of interest (eg, disease vs non-disease), based on the presence or concentration of the metabolites and (ii) the structural characterization of the metabolites identified in the profiling. A majority of metabolites from the profiling experiments, are structurally unknown thus the identification of these unknown metabolites is the primary challenge in metabolomics. Metabolite identification is challenging because the metabolome is comprised of small molecules with different physicochemical properties and with a wide range of concentrations, thus a single analytical platform is not sufficient for its analysis and because many platforms do not provide sufficient information to characterize markers of interest.

Work conducted in our laboratory demonstrated the use of multiple analytical strategies applied to the identification of candidate metabolites in a metabolomics study. The candidate markers had been identified using LC with electrochemical detection (LC-EC) which provided limited structural information. In LC-EC, compounds are detected based on their oxidation or reduction potential. Thus, LC-EC analysis is limited to compounds that are electrochemically active (within a specified range for example, between 0 and 900 mV), and it provides no structural information of the detected compounds. We therefore developed a platform that allowed us to move between LC-EC, LC for concentration, and fractionation of

metabolites, MS, MS/MS, and NMR for complete structural elucidation. Figure 12 shows a proof-of-concept application of this platform to characterize endogenous tryptophan in human plasma. The chemical formula was determined from high resolution MS data, while MS/MS experiments provided initial structural information. The identity of the isolated compound was then confirmed by ^1H NMR data and by comparison of all analytical results using an authentic standard. The platform was then applied to the characterization of other electrochemically active metabolites in human plasma including the low concentration metabolite (2 nmol in plasma) indole-3-propionic acid.

5.3 | Use of highly sensitive probes

Success in analysis of low concentration, mass-limited samples by offline NMR can mainly be attributed to the development of highly sensitive NMR probes. In all the examples presented above, offline NMR was conducted either using room temperature microcoil probes, or using micro-cryoprobes. The small diameter probes are ideal for purified LC fractions that can be dissolved in lower volumes of solvent for NMR, thus increasing their concentration. In our own work, we use room temperature 1 mm NMR probes, which, when compared to 5 mm room temperature probes, show a 10 $\times$ increase in sensitivity. Microcoil probes are flow probes, which means that they have a large dead volume from the probe's inlet to the probe's observed volume. Dead volumes from the probe's inlet can range from 6 μL (when using a syringe) to 40 μL (when using a sample loader). In most cases, the solvent used to dissolve the sample is used to push the sample through the large dead volume leading to sample dilution (Figure 13A). Segmented flow sample loading (Figure 13B), whereby a sample is carried as a droplet in an immiscible fluid (like a droplet of oil in water), facilitates the loading of a concentrated sample into a microcoil probe through large dead volumes. This avoids dispersion and confines all sample into the microcoil probe observe volume, in-turn increasing sensitivity for NMR analysis."

Most applications using microcryoprobes have been conducted using 3 mm microcryoprobes, however, a recent example from Johansen et al used a 1.7 mm microcryoprobe. They used SPE to collect NMR analytes from LC-MS separations of a safflower and a *Penicillium* fungus. The SPE cartridges were then eluted in 25 μL of solvent directly into 1.7 mm NMR tubes. The transfer of samples from the SPE to the NMR tube was all automated. Although limits of detection were not explicitly discussed, the data appear to show at least partial NMR spectra from compounds at the 1% level of a single 50 μg LC injection, in NMR acquisition times just under 10min, consistent with expectations of the 1.7 mm micro-cryoprobe.

An alternative method for restricting samples into the coil region of a standard size NMR probe (5 or 10 mm probe) uses a microscale assembly known as a Shigemi tube. When using a regular 5 NMR tube in a 5 mm probe, samples are usually dissolved in ~ 700 μL of deuterated solvent to cover $\sim 2\times$ the coil diameter to avoid loss of resolution due to magnetic inhomogeneity. The use of Shigemi tubes reduces the volume needed to dissolve a sample by half, in-turn, increasing the mass sensitivity of the sample in the NMR probe. Shigemi tubes have a region (the region outside the coil area) that is matched to the magnetic

susceptibility of specific deuterated solvent (eg, chloroform, D₂O) to avoid magnetic inhomogeneity.

LC-MS and NMR are not always comprehensive and sometimes require follow-up with other techniques (eg, GC, IR).⁷ One such case is an example from our own metabolomics work whereby we included GC-MS to an LC-MS offline NMR platform. The LC-MS/NMR platform had not been sufficient for complete metabolite identification and the addition of GC-MS increased confidence in our structural assignments. An example of the identification of indole-3-acetic acid (I3AA) using four detectors (LC-EC, LC-MS, GC-MS, and ¹H-NMR), is shown on Figure 14. The exact mass of the metabolite (Figure 14A), was not sufficient for its complete identification as it yields two possibilities. Fragmentation data eliminated 5-hydroxyindole acetaldehyde (5-HIA) based on the loss of CO₂ (*m/z* 130.0646). ¹H NMR and GC-MS were used to further support the annotation of the metabolite as I3AA (Figures 14B and 14C). In addition to complementing LC-MS and ¹H NMR analysis, for some analytes, we found that even after fractionating a plasma sample for LC-MS analysis, some metabolites were not detected due to ion suppression and others were at too low a concentration for NMR detection. The addition of GC-MS, which is ideal for analysis of volatile and semi-volatile compounds, provided orthogonality to LC-MS and NMR and enabled the identification of metabolites that either did not ionize well by ESI-MS due to ion suppression or were at too low a concentration for NMR analysis.

We note that there have been some successful attempts to hyphenate GC to NMR online.⁸ The hyphenation of GC to NMR is however not trivial and it is complicated by the need to condense gas samples into the NMR probe. The difficulty in obtaining spectra of a flowing gas, reduces the sensitivity of GC-NMR, and the technique requires concentrated samples (LODs at ~2 mg per analyte). Due to these limitations, the technique has not found extensive application.⁹ The addition of IR (online) to an LC-MS-NMR platform was discussed in detail by Wilson and Brinkman in their review “hype and hypernation.”

6 | SUMMARY AND CONCLUSIONS

The platforms that have been discussed throughout this review show that there are different options for LC-MS-NMR in the analysis of analytes in complex matrices. Analysis can be done either online (continuous flow or at-line), or offline; the platform used is dependent on the application, sample availability, and instrument availability.

There are different modes of the online LC-MS-NMR methods. Continuous flow and loop storage LC-MS-NMR methods, are best suited for high concentration analytes. The advantage of these two techniques is that separation is done only once and in continuous flow analysis, all the required data are acquired concurrently. They are, however, limited by the low sensitivity of NMR—the sensitivity of any hyphenated technique is defined by its least sensitive detector. An additional disadvantage of continuous flow and loop storage analysis, is that they both require extensive method development to ensure the compatibility of all detectors.

It is increasingly clear that, in the analysis of low concentration analytes, NMR is best done offline and after exhaustive LC-MS analysis. The use of offline LC-MS/NMR allows for each detector to be optimized independently so it can be used at its most optimal sensitivity. Samples can be fractionated and concentrated for NMR analysis either by using SPE cartridges, or by collection on plates or tubes, all of which allow for multiple injections to increase analyte concentration. The use of SPE sample enrichment can be either partially or completely decoupled from the NMR experiment; it requires specialized equipment and extensive method development to ensure efficient sample trapping. The use of NMR for analysis of low concentration analytes has improved in recent years due to advances in NMR technologies including probe technologies (microcoils and cryoprobes) and higher field strengths).

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Abbreviations:

5-HIA	5-hydroxyindole acetaldehyde
I3AA	indole-3-acetic acid
LOD	limits of detection
MS	mass spectrometry
NMR	nuclear magnetic resonance
S/N	signal to noise ratio
SPE	solid phase extraction
TFA	trifluoroacetic acid
TRP	tryptophan.

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FIGURE 1.

The mass spectrum of propranolol (molecular weight 259) obtained during an LC-MS-NMR (mobile phase D_2O and protonated acetonitrile), shows masses at m/z 262 and m/z 263 from different states of deuteration. Adapted from *Rapid Commun Mass Spectrom.* 1998;12:1732-1736 with permission from John Wiley and Sons, copyright 1998

FIGURE 2.

A 10 mM of sucrose in 50:50 D₂O: acetonitrile, before suppression of solvent resonance (Top Spectrum). Selective suppression of the solvent resonances at 2 ppm (acetonitrile) and 4.3 ppm (residual water) lead to the enhancement of the analyte signal (bottom spectrum). Adapted from *J Mag Reson, Series A*. 1995;117:295-303 with permission from Elsevier Limited, copyright 1995

FIGURE 3.

Wider LC peaks may have only a small percentage of the analyte in the NMR probe's observed volume. Peaks as sharp as the NMR observed volume may be diluted twofold in the dead volume of the NMR flow cell. Adapted from *Mag Reson Chem.* 2003;41:448-454 with permission from John Wiley and Sons, copyright 2003

FIGURE 4.

Online coupling of LC-MS-NMR, in series (A) or in parallel (B). In series, after LC separation, the LC eluent passes through the NMR, then to the MS after NMR detection. In parallel, the LC eluent is split between the MS and NMR with most of the flow directed toward the NMR due to the low sensitivity of NMR

FIGURE 5.

On-flow LC-MS-NMR analyses of Carbohydrates in Beer. A, Contour plot representation of the LC-NMR results. B, Extracted ¹H NMR spectra (RT 1-17 min) of the carbohydrates in the ale under study. C, Positive ion MS results recorded of the carbohydrates in (B). The multiplicity of the MS signals is due to the partial deuteration of hydroxyl groups in sugars. Adapted from *J Agric Food Chem.* 2003;51:4847-4852 with permission from American Chemical Society, copyright 2003

FIGURE 6.

A, LC-UV analysis of apple peels monitored at 370 nm. LC-MS targeted analysis (MS/MS) of m/z 301 (the quercetin moiety) was used to determine which of the peaks contain quercetin glycosides (Peaks Q1-Q6). B, LC-UV (280 nm) analysis of apple peel extracts. Targeted MS/MS monitoring of the phloretin moiety (m/z 274) selectively indicates peaks D1 and D2 are phloretin glycosides. Adapted from *Anal Chem.* 2000;72:1793-1797 with permission from American Chemical Society, copyright 2000

FIGURE 7.

NMR spectra of closely eluting LC peaks (retention time difference 0.4 min) under two different working modes, stopped-flow, and loop transfer. Adapted from *Online LC-NMR and Related Techniques*. With permission from Chichester, UK: John Wiley & Sons, Ltd; 2002

FIGURE 8.

Schematic representation of LC-MS-SPE-NMR instrumentation set-up. Adapted from *Magn Reson Chem.* 2009;47:S157-S162 with permission from John Wiley and Sons, copyright 2009

FIGURE 9.

A, LC separation of paracetamol metabolites. B, NMR spectra of paracetamol (compound 3): Single trapping of paracetamol on an SPE cartridge required 1024 scans (NS) while with triple trapping the NMR time is reduced 10-fold (NS = 128). Adapted from *J Chromatogr A*. 2004;1058:191-196 with permission from Elsevier Limited, copyright 2004

FIGURE 10.
A typical LC-MS/Offline NMR platform

FIGURE 11.

A, Baseline LC separation a bovine brain extract that contains 17 sphingomyelins in bovine brain. B, HSQC-NMR spectrum of the chromatographic peak at 51.3 min (SM(d18:1/24:1)) Inset- structure with carbon assignments. Adapted from *Anal Chem.* 2007;79:4188-4191 with permission from American Chemical Society, copyright 2007

FIGURE 12.

LC-MS, LC-EC, and microcoil NMR analysis of an endogenous plasma metabolite isolated using the fractionation and concentration scheme developed for structural analysis of dietary metabolites. The MS spectrum provides evidence that the compound is tryptophan (TRP), while the NMR confirms this characterization. Adapted from *Anal Chem.*

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FIGURE 13.

Comparison of flow injection sample loading and segmented flow sample loading. The sample dye is dissolved in DMSO. A, The dye is pushed with a miscible solvent (DMSO) which leads to dispersion, diffusion, and sample dilution. B, The dye sample is pushed using immiscible fluorocarbon and remains as a discrete plug.

FIGURE 14.

A, The high-resolution MS spectrum in the negative ion mode of a metabolite identified as Indole-3-acetic acid. B, The aromatic region (6.5-11 ppm) of the ¹H NMR analysis of the metabolite. C, The GC-MS spectrum of the metabolite after TMS derivatization. Adapted from *Anal Biochem.* 2014;454:23-32 with permission from Elsevier Limited, copyright 2014

TABLE 1

Points of comparison of MS and NMR

Advantages	Limitations
MS LC-MS	
• Low LODs 10^{-13} mol • Specific (MS/MS)	• Suffers from matrix effects • Difficulty distinguishing isomers • Structural identification requires comparison with authentic standards
NMR (LC-NMR)	
• Detailed structural information (multidimensional experiments) • Non-destructive • Inherently quantitative	• High LOD 10^{-9}mol • Long acquisitions for low conc. And multi dimensional experiments.