ChemComm

FEATURE ARTICLE

View Article Online
View Journal



Cite this: DOI: 10.1039/d3cc01455j

# Quantitative NMR spectroscopy of complex mixtures

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Complex mixtures are ubiquitous in many branches of chemistry, be it a complex pharmaceutical formulation, a collection of biofluids analysed in a metabolomics workflow, or a flowing mixture in a reaction monitoring setting. The accurate quantitative determination of mixture components is one of the toughest challenges posed to analytical chemists, requiring the determination of often heavily overlapped signals from compounds in very diverse concentrations. NMR spectroscopists have developed an impressive variety of approaches to deal with such challenges, including the development of innovative pulse sequences, hyperpolarization methods and processing tools. We describe the most recent advances in the field of quantitative NMR, and the many subsequent application perspectives in fields where the sample complexity is a daily challenge, such as pharmaceutical science, metabolomics, isotopic analysis, and monitoring.

Received 24th March 2023,
Accepted 26th April 2023

DOI: 10.1039/d3cc01455j

rsc.li/chemcomm



## 1. Introduction

Complex mixtures are essential in the daily life of chemists. This is particularly the case in the field of analytical chemistry, where a great variety of mixtures are encountered, for applications in a broad diversity of areas: pharmaceutical or medical sciences, food or environmental chemistry, microbiology, *etc.* Mixtures of biological interest (extracts, biofluids, *etc.*) are particularly complex, as they contain a wide variety of chemical structures in very diverse concentrations, from small molecules (amino-or organic acids, sugars, *etc.*) to bigger molecular structures such as lipids and proteins. This is also the case for environmental or food samples or mixtures of natural products. In synthetic chemistry, complexity has a different meaning. Indeed, while the number of mixture components is more limited, the mixture complexity arises from the very similar molecular structure of reactants, products and intermediates. Similarly, in pharmaceutical sciences, an apparently simple purified drug sample can turn out to be very complex due to the presence of impurities with structures close to the one of the main compounds.

In all the above-mentioned scientific domains, analytical chemists are called in to determine the composition of these complex samples. They are asked to determine the structure of the species present, but also their quantity. Indeed, the accurate quantitative determination of mixture compounds is often crucial to ensure the relevance and validity of the answer provided to the initial question. In the study of biological mixtures, the concentration of metabolites is for example a very important data to model and understand the metabolism, or to provide reliable diagnostic data. In the pharmaceutical industry, the purity of marketed products is a key data for the health of the consumer. The same is true for the analysis of contaminants in the environment. In the chemical industry, the determination of concentrations and yields is essential for process optimization.

While many complementary analytical methods are used for the analysis of complex mixtures, nuclear magnetic resonance (NMR) spectroscopy has unique advantages.[1] First, it is a non-destructive method that can provide both structural and

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quantitative information at the same time. Second, the level of accuracy that can be achieved with NMR gives a very high degree of confidence in the quantitative results obtained. Third, NMR offers an impressive variety of data acquisition and processing modalities among which the analytical chemist can choose to tackle a specific scientific question. This point is particularly interesting because NMR has the unique ability of offering an infinite reservoir of methodological developments based on quantum mechanics and spin physics. The role of analytical chemistry is then crucial to transform these innovative developments into real-life applications.

Over the last decade, the development of new NMR methods for the quantitative analysis of complex mixtures has been particularly dynamic, both driven by challenging applicative questions and by the inventiveness of NMR spectroscopists.[2] The present review aims at describing how such developments have enabled -or could enable in the near future- new applications in very diverse fields. In this context, one should pay particular attention to the meaning of the term ''quantitative'', often overused in the literature. Therefore, we will first discuss the different types of ''quantitative analysis'' that NMR can address, based on the quantification needs (*e.g.* absolute or relative) and on the associated target in terms of trueness and precision. Then, we will highlight the most recent literature in the four application domains where quantitative analysis by NMR is mostly used: quality control and pharmaceuticals, metabolomics, isotopic analysis at natural abundance, and reaction monitoring. For each of these fields, we will show how recent developments have led to a diversity of quantitative applications, and provide perspective discussions on the potential of the most recent methodological advances.

## 2. Quantitative NMR spectroscopy

The aim of this section is to provide an accurate description of the quantitative NMR framework, in order to shed analytical light on the developments and applications described in the following sections. We will not discuss in details the principles of quantitative NMR, which have been described in excellent reviews,[3–5] but rather highlight the most important points.

### 2.1. Analytical considerations

The general equation describing the quantitative nature of the NMR signal is $S = k \times N$. In this expression, $S$ is the signal intensity (area or volume), $N$ is the number of spins giving rise to this signal, and $k$ is a proportionality constant that depends on numerous factors (spectrometer, probe, NMR pulse sequence, temperature and the sample itself).[6] Since $k$ is generally not known, NMR is always a primary ratio method providing *relative* quantification results.

However, *absolute* quantification results can also be achieved, and this is undoubtedly one of the major strengths of NMR spectroscopy as an analytical technique. In a first case, often known as ''qNMR'', a reference method and specific experimental conditions ensure that $k$ is the same for all the peaks in the spectrum. This characteristic makes it possible to determine simultaneously the concentration of multiple analytes in complex mixtures, with a single reference signal. In a second case -typically for most multi-pulse NMR experiments- $k$ is different for each peak, and achieving absolute quantification requires suitable calibration methods that will be mentioned later in this article.[7]

A review of recent literature on quantitative NMR clearly reveals how broadly the term ''quantitative NMR'' is used. As an illustration, a search limited to the 2022 literature with this exact term yields results as eclectic as (i) the monitoring of enzyme kinetics where absolute concentrations are monitored in the course of time,[8] (ii) the relative variation of lipoprotein profiles in a cohort of Alzheimer's disease patient serum samples,[9] or (iii) the quality assessment of medicinal plants by NMR and chemometric techniques.[10] Not only do these three examples-taken among many-illustrate a diversity of application areas, but they also cover a very different meaning of the word ''quantitative'', in terms of information being sought and of analytical target.

It is important to recall the existence of appropriate analytical terms to define the quantitative character of a method. Throughout this manuscript, we will rely on the terms *accuracy, trueness and precision*. Trueness is defined as ''the closeness of agreement between the average value obtained from a large series of test results and an accepted value''.[11] Precision is ''the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions''.[12] Precision can be defined at different levels: repeatability, intermediate precision and reproducibility. Finally, accuracy is a term that generally includes both precision and trueness.

In quantitative NMR, the method and experimental conditions that should be used highly depend on whether the targeted application requires focusing on precision, trueness, or both. In addition, the required level of trueness and precision strongly depends on the application. These considerations should be central in the development of any new quantitative NMR method. Table 1 describes the main applications of quantitative NMR together with the corresponding required analytical performance. For instance, many applications of

Table 1 Analytical targets (in terms of trueness and precision) for major applications of quantitative NMR. Typical values encountered in the literature are given

| Application | | Trueness | Precision |
|---|---|---|---|
| Purity determination | | <1% | <1% |
| Metabolomics | Untargeted | — | <5% |
| | Targeted | <5% | <5% |
| | Fluxomics | <1% | <1% |
| Isotopic analysis | Profiling | — | 0.1% |
| | irm-NMR[a] | 0.1% | 0.1% |
| Reaction monitoring | | ~1%[b] | ~1% |

[a] IRM: isotope ratio monitoring. [b] Required only for some applications.

 

quantitative NMR only require relative quantification: this is the case for profiling approaches where the evolution of analyte concentrations is monitored across a large set of samples. In such case (typically for untargeted metabolomics), trueness is not crucial since absolute concentrations are not necessarily sought, but a good precision is required to ensure that the variations observed arise from biological differences between samples and not from an analytical bias.[13] This is also the case a number of reaction monitoring applications, where the relative evolution of reactant and product concentrations is sufficient to obtain kinetic data.[14] On the other hand, some applications require both a high trueness and precision, such purity determination[15] or targeted metabolomics.[13] The most demanding analytical targets for quantitative NMR are found in the field of isotopic analysis at natural abundance,[16] that will be described later in this review.

### 2.2. Quantitative NMR specificities

The above-mentioned analytical targets also drive the optimization of the numerous experimental parameters involved in quantitative NMR procedures, from sample preparation to data acquisition and processing. The choice of these parameters should be carefully considered when developing a new quantitative NMR method. Many parameters in the quantitative NMR workflow can impact the accuracy of quantitative analysis, and this concerns sample preparation as well as data acquisition and processing. However, not all parameters have the same importance depending on whether the application requires good trueness, good precision, or both.

Sample preparation is of course crucial, as for many quantitative analytical methods. Special care should be taken on the material used for sample preparation (scale, glassware, NMR tubes) depending on the targeted analytical performance, and parameters such as pH, ionic strength, stability, solubility, and potential chemical or physical interactions should be considered.[2]

The choice of acquisition conditions strongly depends on the required precision and trueness. While basic precautions (probe tuning and matching, shimming, careful pulse calibration) are always indispensable to ensure even a basic level of repeatability, extra care should be taken depending on the application. In metabolomics or reaction monitoring, specific pulse sequences are often required to suppress the solvent signal(s) and address dynamic range issues.[17] In quantitative $^{13}$C NMR, special conditions are sometimes needed to avoid the impact of nuclear Overhauser effect (nOe) on NMR signals, or to ensure homogeneous $^{1}$H decoupling over a broad range.[18] Moreover, the targeted precision dictates the number of transients, since there is an inverse relationship between these two parameters.[19] These are just examples among the many parameters that can impact the precision, but additional parameters should be considered when absolute concentration needs to be determined. In qNMR conditions, such determination involves, in particular, an accurate determination of longitudinal relaxation times ($T_1$) for the NMR peaks of interest, to avoid partial saturation of the corresponding magnetizations.[3]

Processing parameters also have a strong impact on the quantitative nature of NMR experiments, and again depend on the targeted application. Phasing and baseline correction deserve special attention since they may be largely operator-dependent and can have a strong impact on precision and trueness. The determination of peak areas, by integration or deconvolution, is also a critical step of the data processing workflow, which has been the focus of numerous developments.[20–23]

In addition to these specific acquisition and processing parameters, quantitative measurements generally involve normalization or reference methods.[24] Data normalization is generally used for relative quantification only, and consists in correcting the NMR spectra to account for differences between samples such as dilution, extraction yield, *etc.* Reference methods rely on an additional signal which generally arises from a chemical substance solubilized within the sample (internal reference) or placed in an external or co-axial NMR tube (external reference). Electronic reference methods are also available, where an artificial peak in the spectrum is generate from the spectrometer hardware or software. The choice of an appropriate reference method is highly dependent on the nature and number of samples, and on the targeted analytical strategy and expected trueness and precision.

As we will see in the next sections, while the conventional 1D quantitative NMR approach has been well described, the NMR community has recently developed a large ensemble of quantitative NMR methods based on complex, multi-pulse and/or multi-dimensional experiments to address the limitations of 1D qNMR. However, most of them differ from quantitative 1D NMR in that the coefficient of proportionality between the concentration and the signal is different for each analyte (and possibly for each signal). In that case, relative measurements are still straightforward (for instance when a peak is monitored across a set of samples, or in the course of time), but absolute concentration determination generally requires signal calibration, like for many other non-quantitative analytical methods.[7] Still, we will show that many of these new approaches can provide excellent levels of trueness and precision, addressing applications in complex mixtures which are out of reach of 1D NMR.

It is important to stress that although the basics of quantitative NMR have been largely described and reviewed, numerous papers still claim to achieve quantitative NMR measurements while not reporting in details -and sometimes not correctly setting- the corresponding parameters. Therefore, it seems important that readers are carefully aware of the analytical considerations associated with quantitative NMR before developing or applying quantitative NMR methods. The recent developments and applications in quantitative NMR, described in the following sections, will be discussed in light of these reflections.

## 3. Pharmaceuticals and quality control

### 3.1. Complex problems in a constrained environment

In chemical industry, NMR is often a central tool in the quality control of final products or synthesis intermediates. This is

particularly the case in the pharmaceutical industry, where quality control plays a pivotal role in drug development.[25] Quantitative NMR is used for a broad variety of purposes:[26]

•Determination of the purity of an active pharmaceutical ingredient (or determination of impurity levels),

•Determination of isomeric compositions, such as the ratio of diastereoisomers or enantiomers in the case of chiral compounds,

•Quantification of residual solvent levels,

•Determination of the content of excipients,

•Quantification of complex multicomponent drugs such as heparin, polysaccharides, *etc.*

The pharmaceutical industry has been traditionally relying on liquid chromatography (LC) methods for such quantitative determination. However, each quantification issue requires developing and fully validating an ad-hoc LC method, which can be costly and time-consuming. As a consequence, the industry is increasingly relying on quantitative NMR in early drug development stages.

The pharmaceutical industry is probably one of the fields where analytical requirements are best controlled, due to strong regulations that drive the different stages of pharmaceutical drug development. For instance, the United States Pharmacopeia (USP) has recently decided to revise its NMR chapters to include strong and detailed recommendations on quantitative NMR.[27] Beyond pharmaceutical industry, other fields where quality control is central have similar requirements in terms of quantitative NMR, such as chemical industry where quantitative NMR is used as a tool to determine the purity of organic reference materials.[28] The field of forensics is also concerned by such determination, since assessing the purity of drugs is essential to trace their origin and production pathways. The ENSFI drug working group has edited an official guideline for qNMR analysis.[29]

While the level of targeted analytical performance is different for each application, most of the above-mentioned applications require a precision and trueness of *ca.* 1% (or lower for purity determination). However, quantifying the above-mentioned samples by NMR with such accuracy is challenging, since they often form complex mixtures. Even though the number of compounds can be limited, spectral overlap can be very difficult to address since mixture components often have very similar structures and spectral signatures. Fig. 1 illustrates typical cases of sample complexity that can be encountered in pharmaceutical analysis. Fig. 1A shows how $^{1}$H NMR provides an opportunity to identify and quantify an impurity (kaempferol) present in a sample of quercetin (a flavonoid used as a food supplement).[30] While the mixture contains only two compounds, it can already be considered as "complex" because the two molecules have very similar structures. Fig. 1B, C and D show $^{1}$H NMR spectra of heparin, an anticoagulant polysaccharide drug whose adulteration is a worldwide problem.[31] This is a much more challenging example of sample complexity since the signals from heparin residues are heavily overlapped together and with those of potential contaminants. Fig. 1E shows the $^{1}$H NMR spectrum

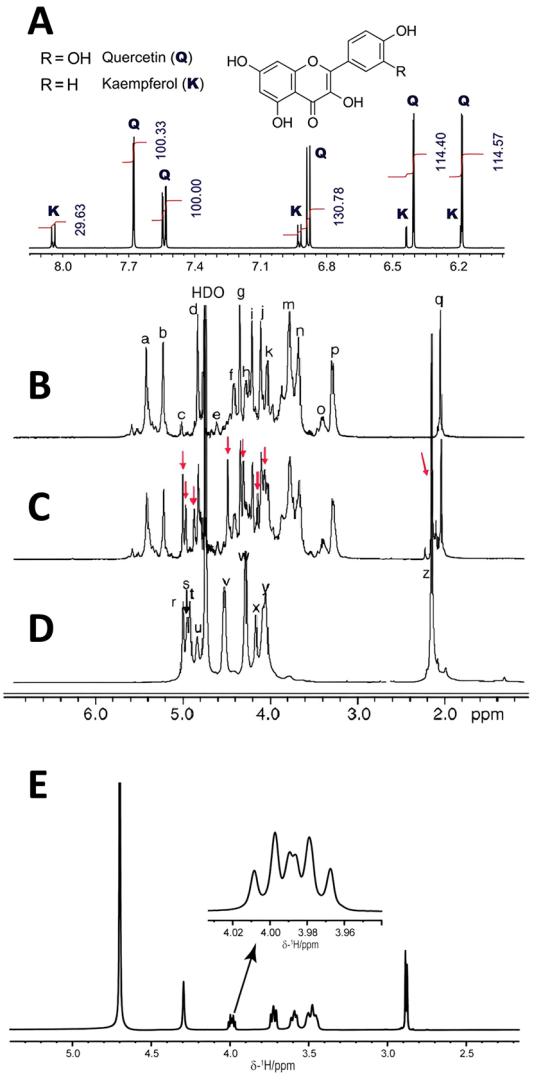


Fig. 1 $^{1}$H NMR spectroscopy examples illustrating the complexity of mixtures and of their quantification in pharmaceutical industry. (A) Application of the relative (100%) qHNMR. A commercial sample of quercetin (Q; declared purity >99%; 24.67 mg mL$^{-1}$ [not required for purity calculation] in DMSO-$d_6$, 600 MHz) was analyzed. A structurally related compound, kaempferol (K), was identified as an impurity. On the basis of the relative integral ratios, the content of quercetin and kaempferol in the sample was determined as 87.8% and 12.2% w/w, respectively. (B, C and D) $^{1}$H-NMR spectra acquired at 800 MHz and assignment of heparin, contaminated heparin and OSCS contaminant. (B) $^{1}$H-NMR spectrum of heparin. (a) H1 GlcNS, GlcNS6S; (b) H1 IdoA2S; (c) H1 IdoA; (d) H5 IdoA2S; (e) H1 GlcA; (f) H6 GlcNS6S; (g) H2 IdoA2S; (h) H6′ GlcNS6S; (i) H3 IdoA2S; (j) H4 IdoA2S; (k) H5 GlcNS5S; (l) H6 GlcNS; (m) H4 GlcNS6S; (n) H3 GlcNS, GlcNS6S; (o) H2 GlcA; (p) H2 GlcNS6S; (q) acetyl $CH_3$. (C) $^{1}$H-NMR spectrum of contaminated heparin; peaks with red arrows show peaks not observed in heparin. (D) $^{1}$H-NMR spectrum of OSCS contaminant. (r) H4 GalNAc2S4S6S; (s) H3 GlcA2S3S; (t) H1 GlcA2S3S; (u) H1 GalNAc2S4S6S; (v) H2 and 4 of GlcA2S3S; (w) H6 GalNAc2S4S6S; (x) H5 GlcA2S3S; (y) H2,3 and 5 GalNAc2S4S6S; and (z) acetyl $CH_3$ of GalNAc2S4S6S. (E) Conventional 1D–$^{1}$H NMR spectrum of aspartic acid (1 eq.) containing CSA (−)-(18-Crown-6)- 2,3,11,12-tetracarboxylic acid (0.25 eq.) in solvent $D_2O$. (A) Reproduced with permission from ref. 30. (B, C and D) Reproduced from ref. 31. (E) Reproduced with permission from ref. 32.

of a racemic mixture of aspartic acid (an essential amino acid) in the presence of a chiral solvating agent (CSA), highlighting the signal overlap between diastereoisomeric pairs formed between the analyte and the CSA.[32]

In spite of this sample complexity, the vast majority of quality control applications in industry rely on 1D $^{1}$H quantitative NMR, probably because the quantification method has to meet the traditional validation requirements that regulated industries impose to liquid chromatography methods. Limitations associated to sample complexity are generally addressed by adapting the sample preparation conditions.[26] Indeed, signals can be shifted by adjusting the sample pH, concentration and temperature, by using a different solvent (or mixture of solvents), or by adding shift reagents (CSA in the case of enantiomer quantification). Recording NMR spectra on other nuclei is also sometimes an option to solve the spectral crowding issue associated with $^{1}$H NMR. Quantitative $^{19}$F NMR has gained popularity in drug discovery and biological applications,[33] owing to its high sensitivity and 100% natural abundance. Recent examples include the characterization of a conformational ensemble of the human $A_{2A}$ adenosine receptor,[34] or the quantification of amphetamine regioisomers with quantitative $^{19}$F benchtop NMR.[35] Precautions should be taken due to the large chemical shift range of fluorine[36] but the use of tailored broadband pulses can significantly increase the quantitative bandwidth of $^{19}$F NMR measurements.[37] $^{13}$C quantitative NMR is also an option, although its low sensitivity limits its use to highly concentrated samples.

### 3.2. NMR methodological developments

There are, however, a few cases of quality control where the sample complexity has been recently tackled by novel methodological developments. A first one is the analysis of enantiomers, where peak overlap can remain significant even with a careful choice of CSA structure and concentration (Fig. 1E), leading to small values of chemical shift difference between diastereoisomeric complexes ($\Delta\Delta\delta$).[32] Several recent studies have shown the potential of ''pure-shift'' NMR methods to address this limitation, by providing homonuclear decoupling of $^{1}$H multiplets, yielding single lines for each $^{1}$H chemical shift. While pure-shift NMR is limited by a relatively low sensitivity and potential artefacts, Pérez-Trujillo and co-workers showed that pure-shift 1D and 2D HSQC spectra could form efficient tools for the detection and accurate quantification of enantiomers exhibiting very small $\Delta\Delta\delta$ values.[38] More recently, selective rather than broadband decoupling methods have been suggested for enantiodiscrimination, offering a more sensitive -although less general-analysis than pure-shift NMR.[32,39]

Another field where several methodological advances have enabled new applications is the quality control of complex drug mixtures, with heparin as a common example (Fig. 1B). These drugs, often produced by biotechnological means or extracted from natural sources, are complex mixtures of polymers for which classical techniques fail to establish the conformity of the product with the expected standards.[40] The ability of NMR to quantify multiple species in a single experiment is particularly interesting for such analysis, but the multi-component nature of such drugs makes $^{1}$H NMR poorly suited to resolve and integrate signals from the individual components of complex polymers. Chemometric techniques based on $^{1}$H NMR profiling can be used to tackle such complexity, and several papers demonstrated the utility of such methods to detect contaminated heparin samples.[41] It was recently shown that such chemometric methods can also be applied to diffusion-ordered spectroscopy (DOSY) datasets, allowing to predict the molecular weight of heparin samples.[42] But the most efficient separation of overlapped heparin signals for quantitative purposes has been obtained with quantitative 2D NMR methods. Guerrini and co-workers applied a quantitative 2D NMR experiment to determine the percentage of mono- and di-saccharides in heparin samples.[43] Fig. 2 clearly illustrates the potential of their method to separate signals from individual entities, which are heavily overlapped in 1D $^{1}$H spectra. The authors relied on a quantitative HSQC method where an appropriate selection of magnetically comparable signals made it possible to minimize the peak-specific response arising from different $J_{C-H}$ couplings and relaxation times. Their approach led to a precision between 2% and 10%, depending on the concentration of the different fragments. A method validation was carried out, highlighting the suitability of the method for pharmaceutical quality assessment purposes.

Quantitative 2D NMR has also been applied to other challenging pharmaceutical examples. Dufour *et al.* developed a calibration-based method relying on 2D $^{1}$H COSY to quantify cyclodextrins in plasma.[44] Similarly, Martineau *et al.* evaluated the potential of several quantitative 2D NMR methods for the quantification of a polysaccharide of pharmaceutical interest.[45] Although they observed a surprising non-linearity of quantitative 2D NMR, which was most likely specific to the studied molecule, the authors showed that quantitative results could be obtained with a trueness of a few percent, relying on an external calibration method.

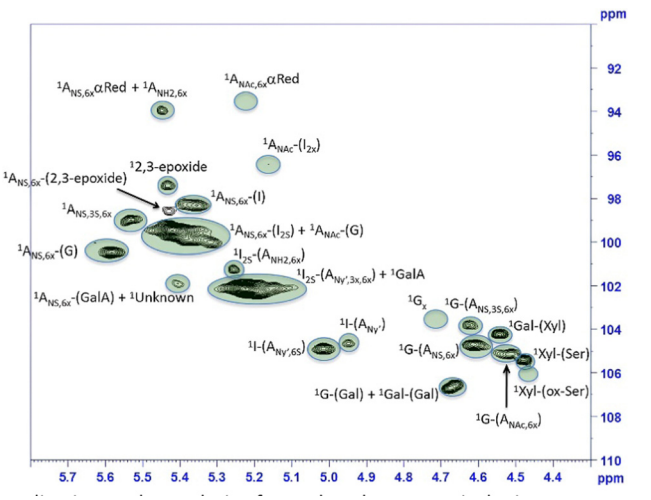


Fig. 2 Heparin HSQC spectrum with signals assignments: region showing anomeric signals. Reproduced with permission from ref. 43.

While the use of advanced NMR methods has enabled new applications in targeted areas of chemical and pharmaceutical industries, elaborated multi-pulse sequences have not yet been widely adopted in these fields. This will require numerous method validation steps to meet regulatory requirements, as well as inclusion of these approaches in pharmacopeia. Moreover, the need for quantitative NMR methods that can handle highly overlapping spectra may be further enhanced by the advent of compact ''benchtop'' spectrometers, operating at magnetic fields of 1 to 2 *T*.[46] In fact, these fields are of the same order of magnitude as those used in the very first quantitative applications of NMR.[47] Benchtop NMR spectroscopy is a promising perspective for quality control in the industry, thanks to its accessibility, low cost and portability. However, the limited spectral range leads to ubiquitous peak overlap that is detrimental to accurate quantification. Therefore, recent development of multi-pulse, gradient-based methods on benchtop NMR spectrometers could be key to their application to the analysis of complex pharmaceutical mixtures.[48]

# 4. Metabolomics and fluxomics

## 4.1. Incredibly complex mixtures

Metabolomics is certainly the area of analytical chemistry where one of the highest mixture complexities is encountered. Indeed, metabolomics aims at providing the broadest view of the metabolites present in complex biological samples such as biofluids, extracts, biopsies, *etc.*[49] The analysis of such samples is generally included in a complete analytical workflow that includes the design of a biological study to answer a specific question, followed by careful sample preparation, data acquisition and processing steps, then by statistical analysis and data interpretation. Metabolomics includes different sub-approaches.[50] Untargeted methods aim at capturing the broadest possible snapshot of metabolites in order to identify potential biomarkers of a particular phenotype. They do not require *a priori* knowledge on the detected metabolites. Targeted approaches focus on the quantification of known metabolites, for instance to validate a biomarker or to obtain further insight into metabolic pathways. A third approach, fluxomics, aims at providing a quantitative measurement of metabolic fluxes focusing on labelled atoms (generally $^{13}C$).[51]

Applications of metabolomics cover a broad range of scientific fields including personalized medicine,[52] clinical studies,[53] plant sciences,[54] biotechnologies,[55] among others. Metabolomics strategies are also applied in other fields that do not necessarily involve metabolites but also deal with highly complex (bio)chemical mixtures such as the authentication of food products (foodomics)[56] or the analysis of environmental samples.[57] A particularly high complexity is encountered in the analysis of dissolved organic matter, an emerging field where NMR shows great promises and where considerations related to metabolomics are also applicable.[58] However, in this section, we do not focus on a specific application field, but we rather describe the challenges associated with the quantitative analysis of complex biological samples, focusing on the innovative NMR methods which have been developed to tackle them.

NMR spectroscopy is a central tool in metabolomics and in related approaches, owing to its robust and non-destructive character, associated with its ability to provide both structural and quantitative information.[59] Focusing on the latter, one should highlight that the analytical targets in terms of trueness and precision are much less demanding than for other applications (Table 1). In untargeted metabolomics, where relative measurements are performed across large sets of samples, trueness is not necessarily a goal, while precision must be sufficient to reflect biological variations between sample groups. In targeted metabolomics, absolute concentrations are determined, but again with a target of a few percent in trueness and precision. The case of fluxomics is more challenging, since isotopic enrichments must be determined with a precision of *ca.* 1%, in order to provide reliable data that can be used to model metabolic fluxes. However, while the analytical objectives may seem undemanding, the extreme sample complexity also raises additional challenges that make quantification very difficult. A great diversity of molecular structures is present in biological samples (from small amino and organic acids to sugars, lipids and proteins) with a broad range of concentrations. This raises important challenges in terms of sensitivity, resolution and dynamic range, which are discussed in the next paragraph. An additional difficulty arises from the need to suppress the signal of non-deuterated solvents (most often $H_2O$) without impacting the quantitative nature of NMR.[17]

## 4.2. Improving peak separation

In routine untargeted NMR metabolomics, where relative signal variations are studied across large sample cohorts, a limited set of 1D NMR pulse sequences are generally used, which provide a spectral fingerprint of all the detectable analytes in the sample, or of a selected subset of those analytes by relying on relaxation- or diffusion-edited experiments. When large sample cohorts are analysed, which is often the case in metabolomics, the acquisition duration is limited to a few tens of minutes per sample at most. While these methods are generally efficient to provide an overview of major concentration variations across samples, the identification of metabolites that contribute the most to the discrimination between sample groups can be made difficult by the strong and numerous peak overlaps that occur on 1D spectra. Such overlap becomes even more critical when absolute quantification is sought, be it for the determination of absolute concentrations in targeted metabolomics, or for the determination of position-specific isotope enrichments in fluxomics where the spectra are further complicated by the $^{13}C$ isotopic patterns. The next paragraphs describe how, over the last decade, NMR spectroscopists have developed advanced methods to solve the overlap problem in NMR metabolomics.

As for many other spectroscopic techniques, spectral decomposition methods have been developed to quantify individual metabolite signals from complex overlapping patterns, both in the case of metabolomics and fluxomics.[23,61–63] However, these

 

methods-sometimes commercial and expensive-rely on the use of databases which are specific to certain matrices and which must be used with specific data acquisition conditions to provide accurate results. A more general strategy is offered by numerical lineshape fitting methods, most often relying on non-linear least-square minimization methods between a model and the experimental spectrum.[21,64,65] However, these methods require expert human eyes to process highly overlapped spectral regions, making the approach relatively low-throughput and prone to errors. Very recently, a strategy to automate spectral decomposition based on machine-learning has been proposed by Brüschweiler and co-workers, showing excellent performance for the analysis of heavily overlapped spectral regions. The model was trained on a large number of synthetic spectra of known composition with variable complexity.[60] Fig. 3 shows the application of this method to mouse urine 1D and 2D spectra. While the quantitative performance of the approach will need to be fully evaluated, it certainly provides a promising strategy for quantitative metabolomics. Another promising approach consists in a decomposition of the analyte signals in the time domain.[20] This approach has recently been applied to the lipidomics profiling of mice tissue extracts.[66]

Alternatives to these processing methods include a number of developments to simplify NMR data at the acquisition stage. A first possibility arises from sample preparation methods that rely on the addition of nanoparticles into the NMR sample.[67,68] Depending on the specificities of the nanoparticles (charge, coating with ligands, *etc.*), some metabolites bind to them and corresponding signals are suppressed from the NMR spectra, while signals from metabolites who do not interact to the nanoparticles remain visible. These "chemosensing" methods simplify the spectra in a way that can be tuned by the experimentalist, depending on the nanoparticle design. The application of these methods to quantitative metabolomics has not been demonstrated yet, but they could provide an interesting tool for targeted analysis of complex biological samples.

An alternative to simplify the 1D NMR spectra of complex metabolite mixtures relies on pure-shift NMR methods[69,70] that remove homonuclear couplings, as described in the previous section. While the potential of pure-shift methods has been illustrated on a variety of complex mixtures, its application to metabolomics is much more recent. This is probably because pure-shift methods-that rely on complex NMR pulse sequences-have only recently reached a sufficient level of robustness to meet the analytical requirements of metabolomics. In particular the initial pure-shift methods were hampered by artefacts due to the data chunking mode applied during data acquisition. However, this limitation was recently circumvented by methods capable of delivering clean 1D pure-shift $^{1}$H spectra with a repeatability that enabled their application into metabolomics workflows. Several studies were subsequently published, including the differentiation of fruit extracts from different Andean ecosystems,[71] or the detection of adulteration of food samples.[72] The combination of pure-shift methods with water suppression techniques was demonstrated by Bertho *et al.*, yielding high quality metabolomics data on extracellular media samples.[73] Very recently, Chen *et al.* investigated the ability of pure-shift NMR to provide absolute quantitative data (Fig. 4).[74] As for any multi-pulse sequence in NMR, the signal response in pure-shift experiments exhibits a site-specific coefficient, however the authors introduced a library-based calibration approach to fit the pure-shift spectral fingerprint of individual metabolites. They evaluated the method performance on model mixtures, then on cell culture media. A trueness better than 10% was obtained, with coefficients of variations of less than 15%. The potential of pure-shift NMR for metabolomics remains limited by its low sensitivity (typically a few percent of conventional $^{1}$H NMR), but it provides an appealing solution for applications which are not sensitivity-limited.

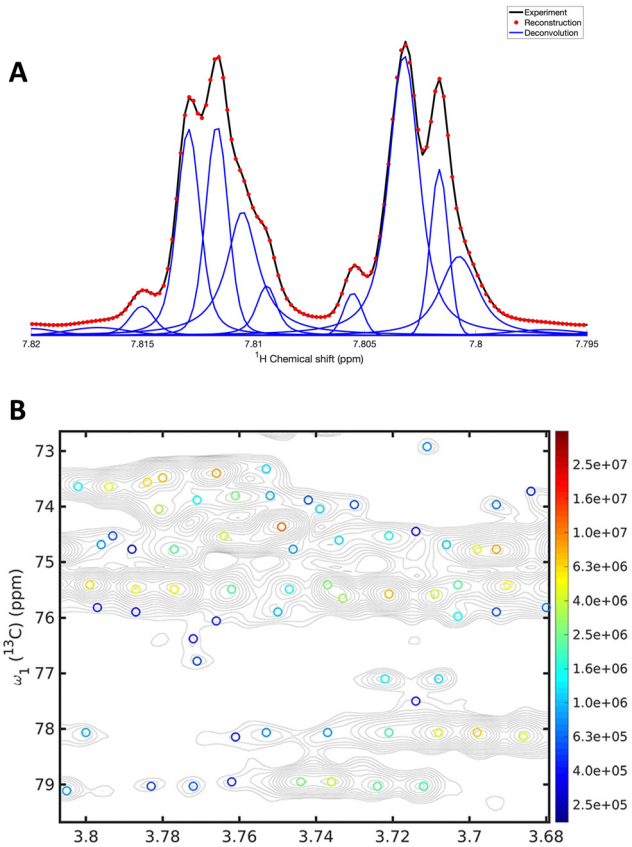


Fig. 3 Potential of machine-learning methods for the decomposition of complex overlapped spectra in 1D NMR metabolomics. (A) Application of DEEP Picker1D and Voigt Fitter1D to a spectral region of the 1D $^{1}$H spectrum of mouse urine. Experimental and reconstructed spectra are depicted as black lines and red dots, respectively. Deconvoluted individual peaks are depicted as blue lines. The deconvolution by DEEP Picker1D was performed with model 2 with a PPP number of 8. (B) Illustration of performance of DEEP Picker for 2D $^{13}$C–$^{1}$H HSQC of mouse urine. DEEP Picker is able to identify and distinguish between cross-peaks that strongly overlap, which poses a significant challenge for their analysis by traditional peak pickers. Picked cross-peaks are indicated as circles and color-coded according to their amplitude on a logarithmic scale with logarithmic contour line spacings. (A) reproduced from ref. 60; (B) reproduced from ref. 22.

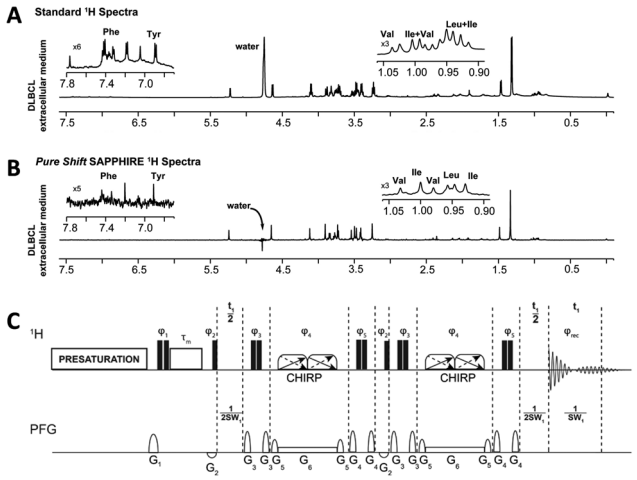


Fig. 4 Potential of pure-shift $^{1}$H NMR for metabolomics. Standard (A) and pure-shift SAPPHIRE (B) $^{1}$H NMR spectra recorded on DLBCL cancer extracellular medium. Regions of interest are magnified to show the evolution of proton lineshape. (C) Pulse sequence used to record pure-shift SAPPHIRE $^{1}$H NMR spectra. Reproduced with permission from ref. 74.

Rather than simplifying spectral patterns, one could also think of relying on multi-dimensional NMR to spread them along orthogonal dimensions. In particular, 2D NMR has several advantages for metabolomics (Fig. 5).[75] Since peaks are spread over two orthogonal dimensions, the determination of individual metabolite contributions in overcrowded areas is expected to be facilitated. As a consequence, 2D NMR has the potential to improve classification performance in untargeted metabolomics, but also to facilitate the identification of potential biomarkers. Moreover, the determination of absolute metabolite concentrations should be made easier by the separation of overlapped peaks. Finally, the great diversity of 2D NMR pulse sequences available offers a full toolbox of experiments with complementary characteristics in terms of sensitivity and resolution.[76]

For untargeted analysis, the systematic use of 2D NMR experiments in metabolomics workflows was first suggested by Van *et al.* who showed an improved performance for 2D TOCSY compared to 1D $^{1}$H NMR for the metabolic profiling of mice urine samples.[77] However, this study also highlighted the main limitation associated with the use of 2D NMR in

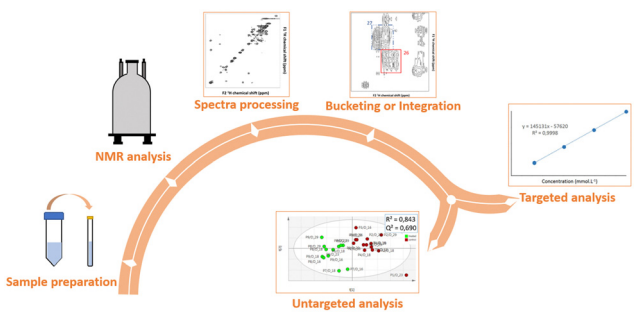


Fig. 5 Typical workflow for untargeted or targeted metabolomics using 2D NMR. Reproduced from ref. 81.

metabolomics, *i.e.* the long experiment time associated with the acquisition of 2D datasets. The development of several fast 2D acquisition methods, such as the popular non-uniform sampling (NUS), spectral aliasing or ultrafast 2D NMR methods, has changed this paradigm and enabled a larger use of 2D NMR in metabolomics.[75] In addition to their high-throughput nature, fast 2D NMR methods can also be more repeatable than their conventional counterparts, which confers them an additional advantage for metabolomics. Recent studies include the analysis of human urine samples, where 2D COSY NUS spectra provided a higher level of clustering after statistical analysis,[78] or the application of several NUS and UF methods to the analysis of pig serum lipid extracts in a food chemical safety study.[79] Note that although less used in metabolomics, DOSY methods have also shown potential in this field, in particular in a very recent study that highlighted their potential for the profiling of biofluids.[80]

The application of 2D NMR to targeted metabolomics has also been extensively explored in the last decade, with the development of quantitative 2D NMR methods to determine the concentration of metabolites in complex biological mixtures. Different directions have been explored to address the peak-specific response factor problem posed by 2D NMR, and all of them have been applied to targeted metabolomics studies.[7] A first approach consists in calibrating the response factor for each targeted metabolite signal, either by external calibration or by relying on standard additions. This method, which can be combined with fast 2D data acquisition methods, has been applied in various contexts, such as the quantification of metabolites in plant or cell extracts,[82–84] showing an excellent trueness and precision (a few percent) provided that the mixtures of standards used for signal calibration are carefully designed and prepared. However, calibration methods require commercial availability of targeted analytes, which may be problematic for the quantification of specialized metabolites. In that case, alternatives have been proposed based on "intrinsically quantitative" 2D NMR methods based on $^{1}$H–$^{13}$C HSQC. These include approaches that compensate for the impact of *J*-couplings on peak volumes, making it possible to quantify multiple metabolites in a single 2D experiment with an internal or external ref. 85,86. This strategy has been successfully applied to determine the concentration of natural products in herbal supplements[87] or the concentration of sugars in plants.[88] While their accuracy (*ca.* 5–10%) is not as good as the one of calibration methods, these methods are useful when metabolites which are not available as standards need to be quantified. An alternative method is the $HSQC_0$ approach, where time-zero HSQC peak volumes are extrapolated from a series of three HSQC experiments recorded on each sample, yielding response factors that are independent of the chosen peak.[89,90]

2D NMR methods -and their fast version- are also increasingly applied in the field of $^{13}$C NMR fluxomics, where the use of a second -and sometimes a third- dimension makes it possible to extract the peak-specific isotopic enrichments more accurately than from 1D NMR data.[91] Several strategies have

 

been developed based on UF 2D NMR, NUS or on fast-repetition 2D NMR methods.[92–95]

Overall, fast 2D NMR methods are increasingly applied in metabolomics and fluxomics thanks to their strong signal separation capability. Their systematic incorporation into high-throughput metabolomics workflows will however require the development of automated and high-throughput processing tools for the integration of 2D NMR peaks, which are currently not widespread in the metabolomics community. Recent developments based on machine-learning (Fig. 3B) are very encouraging from this point of view.[22]

The previous paragraphs show the interest of the methods allowing to limit peak overlap in NMR metabolomics. It is interesting to note that these developments could become even more topical with the development of compact NMR spectrometers, whose interest for metabolomics has been demonstrated in recent years.[96] While benchtop spectrometers have many advantages for metabolomics, such as cost or point-of-care capabilities, spectral overlap is even more problematic.

### 4.3. Enhancing sensitivity

Another major limitation of quantitative NMR in metabolomics is, as for many other applications of NMR, its low sensitivity. This drawback is the main reason why many metabolomics studies rather rely on mass spectroscopy, although the latter is less reproducible and informative than NMR. Addressing the sensitivity limitation of NMR has been a longstanding goal in all application fields of NMR, but recent developments in the field of hyperpolarization have opened particularly promising perspectives for metabolomics.[97] Hyperpolarization methods -which are not described in details here- can increase the nuclear polarization by several orders of magnitude, and among the many hyperpolarization strategies available, two have recently shown great promises for the analysis of metabolite mixtures. The first one is dissolution dynamic nuclear polarization (d-DNP), which relies on polarization transfer from unpaired electrons to nuclei.[98] The DNP process takes place in the solid state, by freezing the sample in a glassy state at low temperatures and irradiating it by microwaves. Still, it can be used to increase the sensitivity of liquid-state NMR by dissolving the hyperpolarized sample with a hot solvent and transferring it to a liquid-state spectrometer. Due to the short lifetime of hyperpolarization, d-DNP has been mainly applied to $^{13}$C NMR, opening the way to sensitive $^{13}$C NMR detection in complex mixtures of metabolites. In spite of its apparent complexity, the d-DNP experiment has recently shown to be surprisingly repeatable (*ca.* 5% on complex metabolite mixtures),[99] and a thorough optimization of the experimental workflow has been carried out to improve its sensitivity and resolution, making it compatible with metabolomics.[100] A first application has been reported to an untargeted metabolomics study on model plant extracts at natural abundance (Fig. 6).[101] Applications to labelled samples have also been described, highlighting the potential of d-DNP for stable isotope resolved metabolomics.[102,103] Several recent developments could contribute to strengthen the role of this methodology for quantitative metabolomics, such as the developments

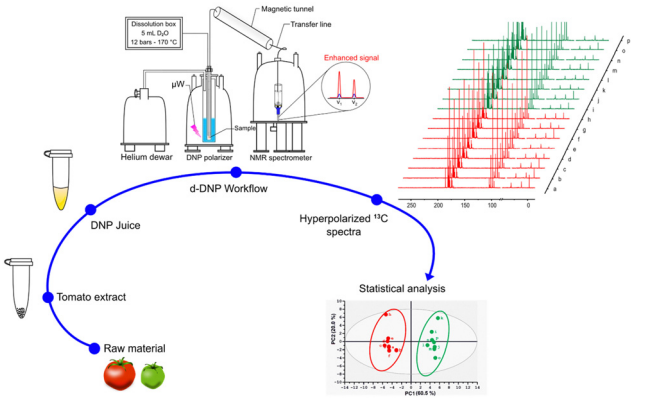


Fig. 6 Illustration of untargeted metabolomics workflow on tomato extracts using $^{13}$C d-DNP. Tomato extracts were prepared from a single pooled sample of tomato powder and mixed in a DNP juice ($d_8$-gly: $D_2O$: $H_2O$, 6 : 3:1, v/v). 200 μL of solution were polarized at 1.2 K and 7.5 T using cross-polarization. Sample were dissolved with 5 mL of hot $D_2O$, spectra were acquired after a total transfer time of 12.8 s. In spectra, letters a to h refer to red-ripe tomatoes, letters i to p refer to green tomatoes. Adapted from ref. 100.

of faster and more automated systems,[104] and the combination of d-DNP with UF 2D NMR acquisition methods.[105]

A second family of hyperpolarization methods that can increase the sensitivity of quantitative NMR metabolomics rely on non-hydrogenative *para*-hydrogen induced polarization (nh-PHIP). In this approach, the reversible binding of $p$-$H_2$ and of the analytes to a metal complex is exploited to transfer, in a reversible way, hyperpolarization from $p$-$H_2$ to compounds of interest.[106] Combined with 2D NMR acquisition strategies, this method can detect selected metabolites at sub-micromolar concentrations.[107] Reimets *et al.* showed that this method could be applied to targeted quantitative analysis of nicotine and its derivatives in urine, relying on a standard addition workflow.[108] Although the nh-PHIP method only allows the detection of specific metabolites -those that can bind to the metal catalyst- it forms an extremely sensitive chemosensing approach for targeted quantitative metabolomics.

While these hyperpolarization approaches aim at improving the molar limit of detection of NMR metabolomics, there are also cases where the limitation comes from the available sample size. This includes precious samples such as medical or environmental samples, or small biological systems such as small organisms or cell spheroids. In such cases, a goal is to improve the mass limit of detection, *i.e.*, to be able to detect metabolites on samples of very small volume (a few μL). Utz and co-workers have shown the potential of lab-on-a-chip microfluidic devices to reach this goal, and demonstrated that it could be used to monitor metabolite concentrations from a single spheroid in the course of time.[109] Interestingly, recent developments have shown that the sensitivity of NMR microfluidic devices could be improved even further by combining them with hyperpolarization methods.[110]

It is quite impressive to see how the detection and quantification of metabolites has motivated an impressive number of

developments in NMR over the last decade. A number of these developments are still at the proof-of-concept stage, and their adoption by a large community of users will require many steps of standardization and automation. Nevertheless, they form an excellent illustration of the synergies offered by the combination of spin physics with analytical chemistry, and could also inspire developments in other areas where sensitive and resolved quantitative NMR experiments are required.

## 5. Isotopic analysis at natural abundance

The measurement of very small variations in the natural abundance of stable isotopes is certainly one of the toughest challenges for quantitative analytical methods. The phenomenon of isotopic fractionation induces very small but highly informative variations in the isotope composition of molecules, and the quantitative determination of these variations is key to track the origin of molecules.[111] Isotopic analysis has found applications in many fields such as food science, pharmaceutical or environmental sciences, geochemistry, archaeology or metabolic studies. Measuring the variation of the natural abundance of stable isotopes is commonly done by isotope ratio mass spectrometry (IRMS), but this very accurate technique only provides access to the average isotope composition of each analyte.[112] There are, however, many cases where the isotopic abundance variations need to be measured for each atomic position of the analytes. Although recent MS-based developments have carried out to access such information,[113] NMR is currently the only general technique for position-specific isotope analysis (PSIA).[16] The corresponding approach has been named irm-NMR (isotope ratio monitoring by NMR).

In the late 1980s, Prof. Martin and Martin developed irm-$^{2}$H NMR for authentication purposes, and it has since then become an official method for several applications such as the detection of sugar addition in wine and fruit juices, or the origin determination of vanillin and acetic acid.[114] A decade later, irm-$^{13}$C NMR was developed as an alternative, allowing applications that were out of reach irm-$^{2}$H NMR, such as the detection of counterfeiting in active pharmaceutical ingredients, the authentication of caffeine samples from different sources or the characterization of photosynthetic pathways in plants.[115]

From the quantitative point of view, irm-NMR poses very strong requirements, since the natural abundance variations are often very small.[115] When absolute isotopic abundance values are sought, a precision and trueness of one per mil (0.1%) are required, since the isotopic abundance variations in nature are spread over a limited range (a few percent). For some applications such as authentication, isotopic profiling (recently dubbed as ''isotopomics'') does not require the determination of true values, but a precision of 0.1% is still required. Such performance has been reached by the development of a very well controlled acquisition and processing workflow and associated parameters. In irm-$^{13}$C NMR, key developments included the implementation of highly homogeneous and repeatable adiabatic decoupling,[116] as well as the use of acquisition conditions ensuring a high SNR, including polarization transfer methods.[117]

In most applications of irm-NMR, a key feature in the achievement of a 0.1% precision was to work on pure and concentrated compounds. But recently, this approach has found an increasing interest for the analysis of complex mixtures. Indeed, Akoka and co-workers developed a methodology called ''metabisotopomics'' that combines metabolomics and isotopomics profiling approaches, that was applied to the study of triglycerides in food samples.[118] In this relative quantification method, the targeted precision of 0.1% cannot be reached for most of the $^{13}$C peaks, therefore peak area variations between samples reflect compositional changes of the fatty acids, in a similar way to metabolomics profiling. But some $^{13}$C NMR signals (glycerol moiety, a part of the fatty acid signals and the terminal methyl groups) are common to all triglyceride molecules and can be used for isotopic profiling when the signal-to-noise ratio is sufficient. Based on this original methodology, Merchak *et al.* showed that olive oils could be classified according to their geographical origin.[119] High-precision $^{13}$C NMR spectra were recorded with an adiabatic INEPT pulse sequence, ensuring sufficient SNR and repeatability for metabisotopomics. The same approach was applied by Hajjar *et al.* for the profiling of triacylglycerols from animal origin.[120]

As for metabolomics, isotopic analysis of complex mixtures has to face the problem of peak overlap, which can be very critical even in the case of a slight overlap, since it impacts the precision on peak area determination. Therefore, the use of highly repeatable 2D NMR methods has been investigated, and Martineau *et al.* first showed that a precision of 0.2% could be reached in a few tens of minutes on concentrated samples of pure ibuprofen, relying on fast methods such as NUS and spectral aliasing.[122] This approach was then applied to mixtures by Merchak *et al.* to classify the geographical and botanical origin of olive oil samples (Fig. 7).[121] An excellent precision was achieved in 22 min by relying on a symmetric HSQC pulse sequence that compensated pulse imperfections. More recently, the sensitivity per unit time of this approach was further improved by the incorporation of adiabatic pulses and by the incorporation of a variable recycling time (VRT), opening the way to new applications on smaller sample amounts.[123] Although the analytical requirements and associated scientific questions are different, these developments are in synergy with those achieved in the field of metabolomics.

## 6. Monitoring

### 6.1. Changing samples

The non-destructive nature of NMR makes it an ideal analytical technique to monitor changes occurring in biological or chemical systems over the course of time. The quantitative nature of NMR is central to many monitoring applications, including kinetic studies in chemistry or biochemistry, or the

 

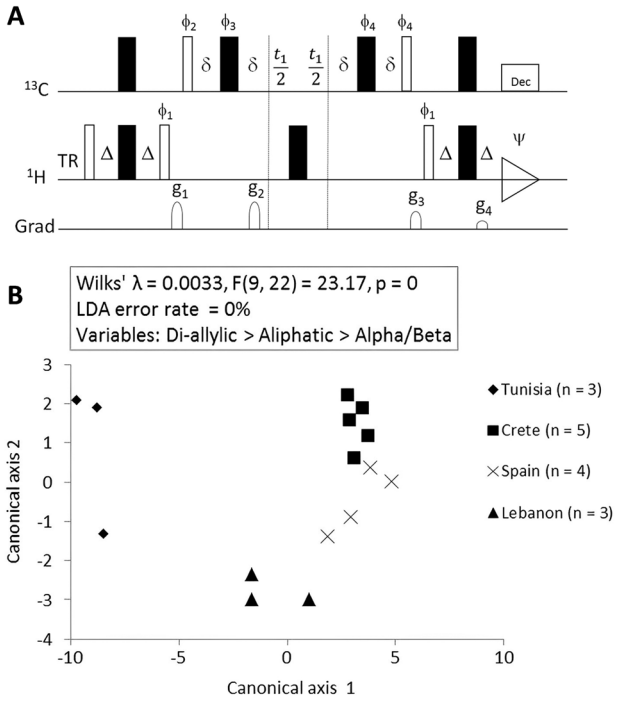


Fig. 7 Application of high precision 2D HSQC NMR to isotopomics. (A) Symmetrized HSQC pulse sequence. Narrow (open) and wide (filled) rectangles represent 90° and 180° pulses, respectively. Pulse phases are x unless indicated. Phase cycling is given by $\phi_1 = y; \phi_2 = y, -y; \phi_3 = x; \phi_4 = x, -x$ and $\Psi = x, -x$. Quadrature detection is obtained in $F_1$ dimension by incrementing $\phi_2$ and $\phi_3$, according to the States-TPPI method. TR is the recovery delay (17.5 s), Δ was set to $^1J_{CH}/4$, with $^1J_{CH}$ corresponding to the average of the $^1H-^{13}C$ one-bond coupling constants (140 Hz), $\delta$ was set to 1.3 ms (minimum value needed to include a gradient pulse and its recovery delay). (B) Classification of vegetable oils using variables from their corresponding HSQC spectra according to their botanical origin. Reproduced with permission from ref. 121.

optimization and control of flow chemistry or biological processes. Is it particularly interesting for the measurement of complex process data in the (bio)chemical industry, where it has emerged as a promising process analytical technology (PAT) capable of providing accurate information on reaction kinetics and yields. In the industry 4.0, reaction monitoring is often carried out with multiple techniques, and the quantitative character of NMR gives it a special role as it provides reference quantitative data for other techniques.[124]

The samples studied by quantitative NMR in the case of monitoring are necessarily complex, since they contain a mixture of reactants, intermediates and products. Moreover, reactions are generally performed in non-deuterated solvents (or mixtures of solvents) that pose additional solvent signal suppression challenges. And of course, an additional complexity arises from the fact that these mixtures evolve in time. This complexity can be circumvented by performing off-line measurements of samples taken from the reaction medium, but in many cases, it is much more interesting to monitor processes in real-time with NMR. Reactions can be performed in the NMR tube,[125] which however strongly limits the experimental conditions and hence the range of processes that can be monitored.[126] Great promises have emerged from the development of flow NMR, where the sample is flown to or through the spectrometer.[127] Such experiments include the *online* approach where a fraction of the reaction medium is continuously circulated through the spectrometer, and the more recent *in-line* strategy where the output of the reactor directly flows through the NMR spectrometer.[128] The former is best suited to the monitoring of batch reactions, while the latter is more adapted to the monitoring of flow chemistry processes.

The development of commercial flow NMR settings has led to a significant growth of NMR monitoring experiments over the last few years. There are two main experimental strategies for flow NMR monitoring. The first one consists in bringing the reaction medium close to a high-field NMR spectrometer and performing a flow monitoring experiments that benefits from the high sensitivity and resolution of a high-field magnet.[127] However, this approach is limited by the ability to bring the reaction medium in an NMR room, which may require the installation of a fume hood, and has little compatibility with industrial environments. A second approach relies on compact (or ''benchtop'') NMR spectrometers relying on permanents magnets and operate at medium field (1–2 *T*), that can be brought close to any chemical or biochemical reactor thanks to its portability and ease of use.[129] However, NMR spectra recorded on such apparatus are hampered by strong peak overlap and second order coupling effects arising from the limited frequency range, making the extraction of accurate quantitative data much more difficult than at high field. A compromise could arise from emerging high-temperature superconducting magnets, which have recently shown promises for online reaction monitoring.[130]

In terms of analytical target, most monitoring applications seek to achieve a good precision (typically a few percent) while trueness is not always a primary goal. The relative evolution of NMR signals from reactants, products and intermediates is monitored over the course of time and can be normalized to a starting or end point, or to the signal of a compound that does not evolve with time. Still, obtaining precise values remains difficult owing to the difficulties mentioned above. Two directions have been adopted to extract precise NMR signals from reacting components in mixtures. The first one pertains to the field of signal processing, and relies on advanced signal decomposition and modelling methods to extract accurate peak areas from overlapped spectra. The second one relies on the use of fast 2D NMR methods which are compatible with samples that evolve in time.

### 6.2. Advanced data processing

Data processing approaches have been mainly applied to the monitoring of reactions with benchtop NMR spectroscopy, where the peak overlap issue makes conventional peak integration not very adapted. Two data processing approaches, PLS-R (partial least squares regression) and IHM (indirect hard modelling) have been mainly employed. Both methods have their advantages and drawbacks, which have been extensively discussed in the literature.[131] Briefly, IHM is more easily

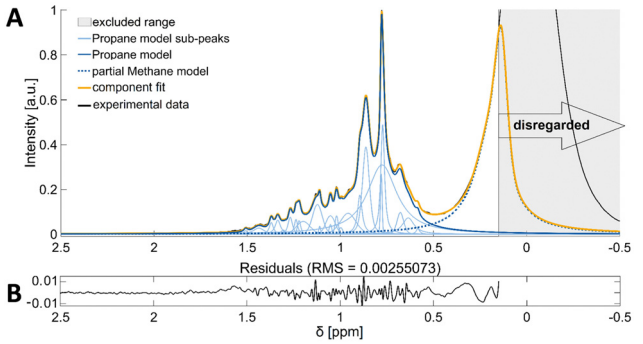


Fig. 8 Analysis of a $^{1}$H NMR gas spectrum. (A) Hard model for propane (7.7 mol%) mixed with methane (92.3 mol%) at 200 bar total pressure. (B) Fit residual showing that the model represents the measured signal with high accuracy. Reproduced with permission from ref. 135.

automated and more robust towards changes in experimental conditions, but PLS-R can yield a higher precision. Nevertheless, both methods are promising for quantitative reaction monitoring with benchtop NMR, and both have been applied to a variety of reaction monitoring situations such as the monitoring of esterification[132] or lithiation reactions.[133] Alternative methods have also emerged, such as the modeling of 1D benchtop NMR spectra based on quantum mechanical parameters determined from high-field spectra,[134] or the use of artificial neural networks.[124] The latter was applied to predict the evolution of concentrations on a moderately complex problem with four reactants. Note that these methods are also interesting for the extraction of quantitative data from benchtop NMR spectra obtained on static mixtures. Although not a purely "monitoring" situation, Fig. 8 illustrates a very original application of supervised analysis to the quantitative analysis of natural gas mixtures.[135] In this study, benchtop NMR data obtained on pressurized (1–200 bar) natural gases was processed with an IHM to determine gas concentrations with excellent accuracy and precision.

### 6.3. Fast 2D NMR methods

While all the above-mentioned methods can yield precise quantitative data, they require *a priori* knowledge on the spectra of pure compounds. In the case of unknown analytes, unsupervised multivariate processing methods can be used as an alternative.[136] But a more general solution relies on a better separation of overlapped signals through multi-dimensional methods, as for the previously mentioned application fields.

Conventional 2D NMR methods can in principle be used for reaction monitoring, but this requires that the acquisition timescale of 2D spectra matches the reaction timescale. Therefore, accelerated 2D acquisition methods are often used for reaction monitoring, and a number of applications that rely on NUS have been recently reported.[137] In that case, particular care must be brought to the choice of NUS parameters and sampling schedule so that the precision on 2D peak volumes is not impacted. For instance, COSY spectra recorded with NUS were used to monitor a Meerwein–Ponndorf–Verley reaction in order to determine reaction kinetics, which required relative quantification only.[138] More recently, Steimers *et al.* showed that 2D NUS HSQC spectra could be used to obtain absolute quantitative data on the evolution of concentrations in a reaction involving two reactants and five products, by calibrating the HSQC response factors for each peak of interest.[137]

When reaction timescales are shorter than the acquisition timescale of 2D NMR spectra, alternative methods should be used. This is the case of time-resolved non-uniform sampling (TR-NUS),[139] where a single 2D experiment is acquired during the progress of the reaction following a randomized NUS schedule, then processed with a sliding window approach, leading to a series of 2D spectra with improved time resolution compared to the classical NUS approach. The TR-NUS approach has been applied at high magnetic field to the monitoring of a fermentation process[140] and to the kinetic study of an aza-Michael reaction.[141] Its potential has also been demonstrated on a benchtop spectrometer equipped with a flow cell, to monitor a hydrogenation reaction, using $p$-$H_2$ for sensitivity enhancement.[142] A different approach to shorten the duration of 2D NMR experiments during reaction monitoring consists in relying on fast-pulsing experiments, such as the ASAP (acceleration by sharing adjacent polarization) method, which was recently used to monitor a proton deboronation reaction.[143]

Alternatively, UF 2D NMR, which completely departs from the $t_1$-incremented scheme of classical 2D NMR experiments, provides a general solution to real-time monitoring.[81,144] Indeed, 2D spectra can be recorded in a single-scan, which makes it ideally suited for the study of very fast processes. Signal averaging can be necessary for sensitivity reasons, but it does not impact the spectra quality since the entire 2D spectrum is recorded at each scan. In addition, UF 2D NMR is a general approach that can be applied to any pulse sequence, although sensitivity and hardware considerations also impact the choice of experimental conditions. UF 2D NMR spectroscopy has been applied to the monitoring of various reactions in the NMR tube, including the study of multi-step reactions involved in the synthesis of pyrimidines,[145] the kinetic study of a mutarotation equilibrium,[146] or the monitoring of redox reactions generated by an *in situ* electrochemical cell.[147] UF 2D spectroscopy was also applied to the monitoring of batch organic reactions with a flow cell, both at high field[148] and on a benchtop spectrometer.[149] The case of flowing samples poses specific challenges to the implementation of UF 2D NMR due to the interference between the sample motion and the spatial encoding of the NMR sensitive region. A solution consists in using spatial encoding that is orthogonal to the flow, either by the use of triple-axis gradient probes (at high field)[150] or by using a spectrometer where the $B_0$ field is naturally orthogonal to the flow (in the case of some benchtop spectrometers).[149] UF 2D COSY on flowing samples has been applied to monitor the conversion of aniline over time, in a Pd-catalyzed Heck-Matsuda reaction monitored with a benchtop spectrometer.[149] At high field, UF 2D COSY was also applied to monitor the saponification of ethyl acetate.[148] In both cases, the resulting time resolution is significantly higher than the one that could

 

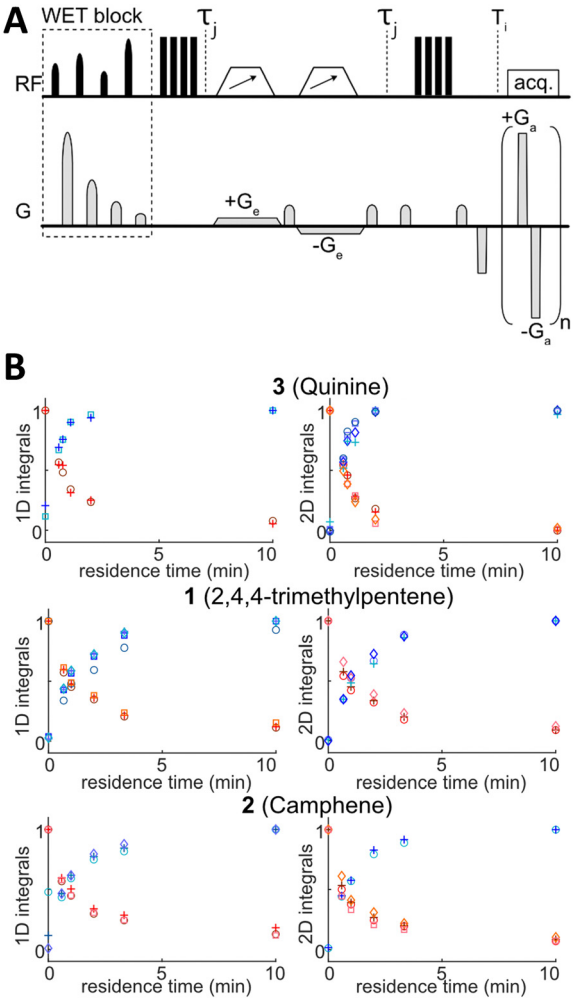


Fig. 9 Illustration of the potential of UF 2D COSY for the in-line monitoring of flow photochemistry reactions, consisting of the thiol-ene addition of 2-mercaptoethanol to three unsaturated organic compounds: 2,4,4-trimethyl-1-pentene **1**, camphene **2** and quinine **3** (A) UF COSY pulse sequence used in this work, including a WET solvent suppression module combined with composite 90° pulses. (B) Integrals of selected reagent (red) and product (blue) correlations as a function of the residence time for the 6 reaction runs on compounds **1–3**. Reproduced from ref. 151.

be obtained with conventional COSY, and the quality of kinetic data highlights the very good precision of the method. Very recently, Bazzoni *et al.* also demonstrated that UF COSY combined with multiple solvent suppression schemes could be used for the in-line monitoring of a flow photochemical reaction (Fig. 9).[151] UF 2D NMR was found to provide kinetic data that was in very good agreement with 1D data, while offering improved signal dispersion, thus providing an efficient and reliable approach to screen reaction conditions in organic synthesis.

Finally, in addition to 2D spectroscopy, DOSY also offers a promising approach to the monitoring of reactions involving compounds with different molecular weights. A proof-of-concept experiment showed that UF DOSY could be applied to the monitoring of a deamination reaction in an NMR tube, yielding reliable diffusion coefficients and molecular weight estimates.[152] Very recently, two other fast and flow compatible diffusion experiments were also developed and applied to the monitoring of organic reactions.[153,154]

## Conclusions

The above-mentioned developments and applications show the incredible diversity of analytical methods that NMR spectroscopists have been able to develop in recent years for the quantitative analysis of complex mixtures. They also underline how crucial it is that these developments include considerations of analytical chemistry, and a clear vision of the objective to be reached in terms of trueness and/or precision. Some of the mentioned developments have reached an advanced level of maturity from an analytical point of view, allowing their application to problems imposing a very constrained analytical framework. Others are at the proof of concept stage. But in any case, there is no doubt that the field of quantitative NMR is extremely dynamic, both from the point of view of methodological developments and applications, and that it should continue to meet many analytical challenges in the years to come.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

The author warmly acknowledges past and present members of the MIMM research team in the CEISAM lab, including special thanks to Jean-Nicolas Dumez for critical reading of the manuscript. This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no 814747/SUMMIT). The author also acknowledges the French National Infrastructure for Metabolomics and Fluxomics MetaboHUB-ANR-11-INBS-0010 (www.metabohub.fr) and the Corsaire metabolomics core facility (Biogenouest).

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