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Hyperpolarized NMR metabolomics

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Abstract

Hyperpolarized NMR is a promising approach to address the sensitivity limits of conventional NMR metabolomics approaches, which currently fails to detect minute metabolite concentrations in biological samples. This review describes how tremendous signal enhancement offered by dissolution-dynamic nuclear polarization and parahydrogen-based techniques can be fully exploited for molecular omics sciences. Recent developments, including the combination of hyperpolarization techniques with fast multi-dimensional NMR implementation and quantitative workflows are described, and a comprehensive comparison of existing hyperpolarization techniques is proposed. High-throughput, sensitivity, resolution and other relevant challenges that should be tackled for a general application of hyperpolarized NMR in metabolomics are discussed.

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Introduction

Metabolomics belongs to the family of "omic sciences" (which address the global analysis of pools of biological molecules), and plays an important role for the global understanding of metabolism. It consists of the analysis of the small molecules present in biological samples in the most exhaustive manner. By analyzing those small molecules, the aim is to discriminate different classes of samples under different conditions, to discover new biomarkers [1] and ultimately to map metabolic pathways [2]. Metabolomics is applied in many different fields such as personalized medicine [3], plant sciences [4], and environment [5], among many others. Metabolomics studies can be implemented through untargeted or targeted approaches. An untargeted approach relies on the detection of the largest possible number of metabolites without any *a priori*, to eventually highlight biomarkers after statistical analysis [6]. A targeted approach consists in the accurate quantification of candidate biomarkers to confirm their significance, which explains that targeted analyses are often applied following untargeted ones [7]. Complementarily, stable isotope resolved metabolomics (SIRM) consists in detecting labeled metabolites after incorporation of a labeled precursor to obtain information on specific metabolic pathways [8].

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)—often hyphenated with chromatography for the latter—are the two main analytical techniques in metabolomics [9,10]. MS benefits from an unmatched sensitivity, however, it is limited by its lower repeatability and the ambiguity of feature assignment, whereas NMR is highly reproducible and provides valuable structural information [11]. Consequently, MS and NMR are highly complementary and increasingly combined in multi-platform approaches [12–17]. However, this complementarity is limited, as is the general spread of NMR spectroscopy in the metabolomics field compared to MS techniques, by the low sensitivity of NMR and the ubiquitous peak overlaps that occur in ^{1}H NMR spectra of complex biological mixtures. 2D NMR can address overlap issues by spreading signals over a second frequency dimension and recently, many developments have been carried out to make 2D NMR more sensitive, faster and quantitative, in order to match metabolomics analysis requirements [18–20]. Nonetheless, all conventional NMR methods still suffer from an intrinsic low sensitivity due to the weak energy gap between spin states, leading to a poor nuclear polarization—*i.e.* 4.10^{-4}% for ^{1}H at 300 K at 9.4 T (400 MHz spectrometer). Hyperpolarized NMR makes it possible to increase the nuclear polarization by several orders of magnitude. Many solution-state hyperpolarization techniques exist, such as optical pumping of noble gases [21], optically polarized crystals [22], Overhauser dynamic nuclear polarization [23], dissolution dynamic nuclear polarization (d-DNP) and parahydrogen (p-H_2) hyperpolarization.

While all these methods can address the current sensitivity limitations of NMR, d-DNP and p-H_2 hold most promises for metabolomics and are the focus of this review. These two methods have been applied to a variety of fields such as magnetic resonance imaging or monitoring of (bio)chemical reactions [24]. However, the investigation of their potential for metabolomics is much more recent [25], and the last decade has witnessed many efforts to improve their suitability for the analysis of complex biological mixtures. This review presents the latest developments in hyperpolarized NMR methods and discusses the perspectives that could arise from their broad use in the field of metabolomics.

Making metabolomics more sensitive by d-DNP

DNP is a hyperpolarization method based on polarization transfer from unpaired electrons to nuclei, which provides a close-to-unity nuclear polarization in a non-selective way. Among the different experimental approaches to DNP, dissolution-DNP (d-DNP) is best suited to improve the sensitivity of liquid-state NMR. It consists in dissolving a hyperpolarized frozen sample with a hot solvent prior to a super-sensitive liquid-state NMR acquisition. The experiment benefits from enhanced nuclear polarization while retaining the resolution advantages of liquid-state NMR for complex mixture analysis such as those of interest in metabolomics [26].

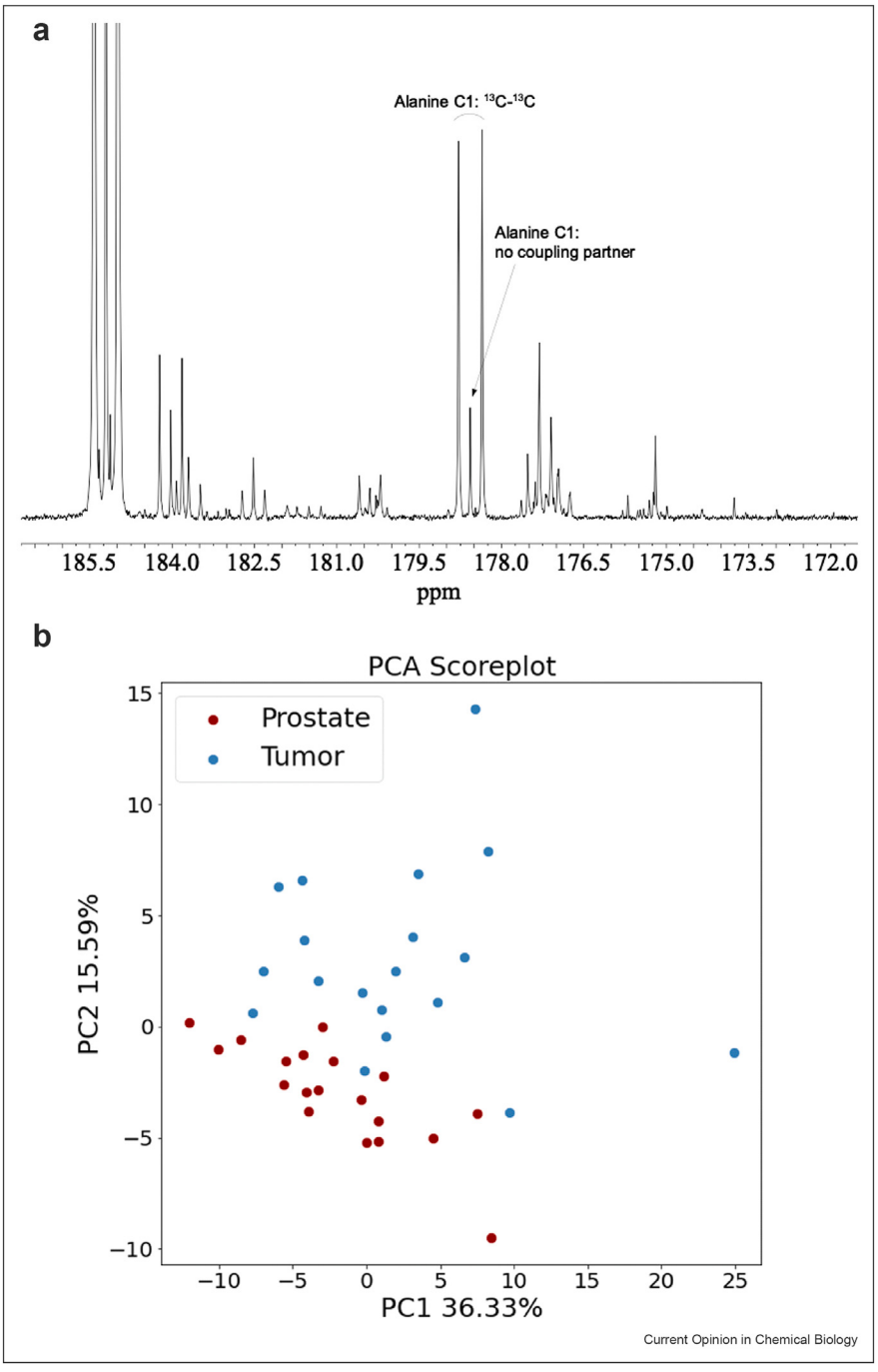
The d-DNP workflow starts by mixing or dissolving the sample of interest in a water-glycerol matrix doped with stable organic radicals used as polarizing agents (PAs). This sample is cryogenically cooled inside a polarizer equipped with a cryostat, forming a glassy matrix. Hyperpolarization takes place inside the polarizer through microwave irradiation for a duration ranging from a few minutes to a few hours, allowing an efficient transfer of polarization from highly polarized electrons to nuclei. This is followed by a rapid dissolution of the sample in a hot solvent and its transfer to a nearby liquid-state NMR spectrometer through a transfer line. After a short stabilization delay, a super-sensitive liquid-state signal acquisition is performed at room temperature. However, the hyperpolarization is short-lived and decreases because of longitudinal relaxation with a time constant T_1 once the sample has been dissolved. Moreover, the irreversible character of the overall process allows recording only a single, or at most a few, scans. The quantitative nature of d-DNP is also a challenging question, since all signals with different T_1 have a different response factor, contrary to quantitative 1D NMR. As a consequence, an accurate knowledge of peak-specific response factor is required to recover quantitative information from d-DNP data [27]. Therefore, d-DNP applications mainly rely on ^{13}C detection since the ^{13}C hyperpolarization survives longer during the transfer between the two magnets due to their longer T_1. Compared to a thermally polarized signal that would be obtained on the same sample by conventional ^{13}C NMR, the signal is typically enhanced by four orders of magnitude. In contrast, ^{1}H d-DNP enhancement is modest—one to two orders of magnitude—due to protons hyperpolarization fast decay [28]. However, recent developments, including faster transfer lines [29,30] and methods to extend the lifetime of hyperpolarization [31,32] could allow a more general use of ^{1}H hyperpolarization. Still, the significant sensitivity achieved by ^{13}C d-DNP NMR, associated with the wider spectral width of ^{13}C and its narrow signal lineshape has recently enabled several key applications of d-DNP in metabolomics.

Determination of isotopic ratios can be easily carried out by integration of isotopic patterns of labeled samples in 1D ^{13}C NMR thanks to its resolution advantages (Figure 1) [33]. Lerche's group reported a method combining ^{13}C d-DNP with NMR-based SIRM using labelled [U-^{13}C, d_7]glucose as a tracer and benefiting from the sensitivity boost offered by d-DNP [27]. With this method, the authors were able to distinguish different cancer cell types from ^{13}C-enriched tissues. Also, the authors proposed a method to provide quantitative information by d-DNP for identified labeled metabolites. To account for inhomogeneous signal decay during transfer, a signal to loss coefficient (SLC) was determined relative to an internal standard and compared to the ratio measured by thermal ^{13}C NMR [27]. In 2020, Frahm et al. applied a similar approach to the differentiation of aggressive and less-aggressive prostate cancer cell extracts, and managed to highlight biomarkers of this discrimination [34]. In 2021, mice tissues were analyzed for the first time with d-DNP, and the same group observed a tendency to separate healthy and cancer tissues by principal component analysis (PCA) (Figure 1b) and supervised analysis [35••].

Natural abundance analysis by d-DNP is more challenging in terms of sensitivity due to the lower abundance of ^{13}C (1.1%), but it still offers great perspectives for metabolomics. Indeed, ^{13}C NMR metabolomics, which is barely accessible without hyperpolarization for sensitivity reasons—would offer significant advantages compared to its ^{1}H NMR counterpart, such as reduced peak overlap that directly facilitates metabolite assignment and biomarker discovery.

A few years ago, a proof-of-concept work highlighted experimental conditions that make it possible to record hyperpolarized d-DNP ^{13}C NMR spectra of biological extracts at natural ^{13}C abundance, relying on cross-polarization from ^{1}H to ^{13}C nuclei in the solid state. In 2015, Dumez et al. reported hyperpolarized ^{13}C NMR spectra acquired on plants and cancer cell extracts at natural abundance [36]. In 2016, Bornet et al.

Figure 1



(**a**) Carbonyl region of hyperpolarized 1D-^{13}C NMR spectrum of prostate extract from mouse injected with [U-^{13}C]glucose 30 min prior to tissue harvest. (**b**) Full spectrum analysis of hyperpolarized 1D ^{13}C NMR spectra by principal component analysis (PCA) each point represents a single sample of which are determined by the collective contributions of all sample corresponding integrals. Data was mean centered and unit variance scaled before PCA was applied. Adapted from reference [35••].

reported an average repeatability of 4% on normalized signal areas for such extracts, demonstrating the suitability of the d-DNP experimental setting for metabolomics [37].

In 2020, ^{13}C d-DNP was successfully incorporated into a full metabolomics workflow, allowing to separate red-ripe and mature-green tomatoes extracts (Figure 2) [38••]. Authors highlighted known biomarkers of two

Figure 2

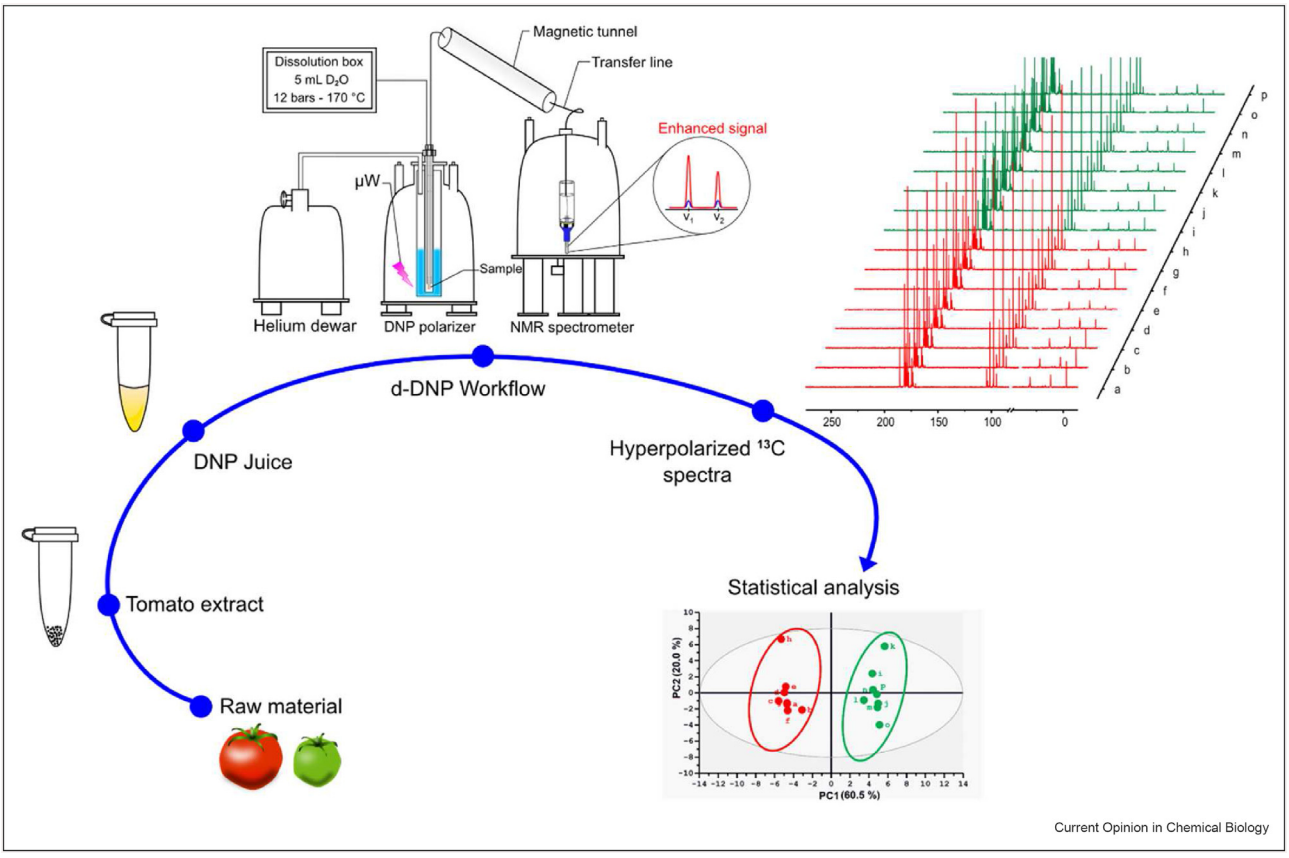


Illustration of untargeted metabolomics workflow on tomato extracts using ^{13}C d-DNP. Tomato extracts were prepared from a single pooled sample of tomato powder and mixed in a DNP juice (d_8-gly: D_2O: H_2O, 6:3:1, v/v). 200 μL of solution were polarized at 1.2 K and 7.5 T using cross-polarization. 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 with permission from reference [38••].

ripening stages of tomato, previously determined by ^{1}H NMR but so far inaccessible by ^{13}C NMR [38••]. This proof-of-concept study represents an important step towards hyperpolarized ^{13}C NMR metabolomics. However, in spite of the high polarization reached in the solid-state — *c.a.* 60% — part of the polarization was lost during the transfer time (12.8 s in this study), and the sample was diluted by a factor 25 during dissolution. This led to a limit of detection in the millimolar concentration range for ^{13}C d-DNP, roughly comparable to ^{1}H NMR. Nonetheless, the resolution advantages of ^{13}C detection increase the overall data quality and can facilitate the discovery of biomarkers. Moreover, the same authors recently reported that a fine optimization of the whole ^{13}C d-DNP workflow could further increase sensitivity and resolution, that could enlarge the application perspectives of d-DNP to metabolomics at natural ^{13}C abundance [39]. The ability of ^{13}C d-DNP to provide relevant information for both labelled and unlabeled samples is now proven, yet, there is plenty of room for improvement before its more general use in metabolomics. As discussed previously, d-DNP sensitivity is limited by the loss of hyperpolarization during the transfer between the two magnets—accelerated by paramagnetic phenomena induced by radicals—and by the non-negligible dilution factor. Several recent developments could help circumventing these limitations.

Sensitivity limits could be addressed by using fast-transfer lines, since the polarization decreases exponentially through longitudinal relaxation. Shortening the transfer time can improve the limits of detection for quaternary ^{13}C ($T_1 \approx 40$ s) but could also drastically facilitate the detection of aliphatic ^{13}C ($T_1 \approx 10$ s) and even ^{1}H ($T_1 \approx 2$ s). Different fast transfer lines have been described in the literature [29,40–42]. In particular, an automated liquid-driven system described by Ceillier et al. showed efficient transfer of hyperpolarized liquid within 2.1 s [3]. Nowadays, ^{1}H d-DNP is quite restricted to aromatic ^{1}H ($T_1 \approx 10$ s) [28,43•] but with fast-transfer lines and recent developments, general implementation of ^{1}H d-DNP could represent a major

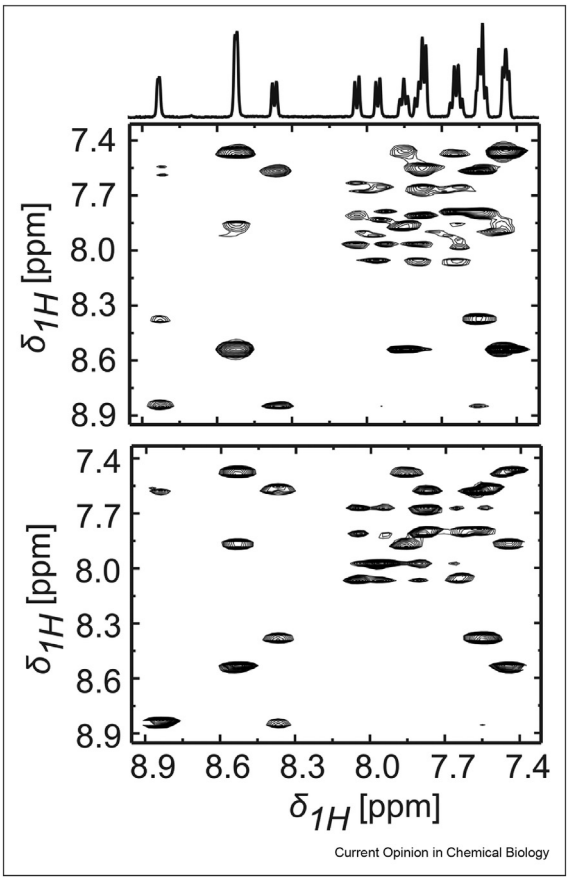
advance for hyperpolarized metabolomics as it would benefit from the higher intrinsic sensitivity of ^{1}H spectroscopy, albeit at the cost of stronger peak overlap. The loss of hyperpolarization between the two magnets is accelerated by the presence of paramagnetic radicals thus, limiting significantly the signal sensitivity. In order to prevent this phenomenon, chemical scavengers can be added to neutralize radicals after dissolution [44,45]. Also, porous material can be used to polarize metabolites efficiently while keeping away radicals from the sample [32].

High throughput and reasonable experimental cost are also very important practical points for a general use of an analytical method in metabolomics. At the time of writing, there is a limited number of automated commercial instruments for d-DNP and research is curtailed by an important consumption of liquid He. However, cryogen-consumption free d-DNP prototypes were already reported in the literature, opening promising perspectives concerning throughput and experimental cost of d-DNP experiments [46–48].

The ability to provide valuable structural information is one of the most important features of NMR. 1D NMR experiments do provide structural information thanks to reliable chemical shifts, J-couplings values and peak integrals. However, they are not always sufficient and often limited by peak overlap.

Unambiguous structural information is generally obtained from 2D NMR experiments, either relying on homonuclear (COSY, TOCSY, …) or heteronuclear couplings (HSQC, HMBC, …), or on couplings through space (NOESY, ROESY) [49]. 2D NMR also offers an additional peak dispersion which is of great interest to overcome 1D signal overlap and facilitate metabolite identification in metabolomics [19,20]. Unfortunately, the irreversible character of d-DNP experiment is incompatible with conventional 2D NMR which requires multiple scans and long experiment times. Although small-angle strategies have been suggested in specific cases to record conventional 2D NMR spectra after hyperpolarization [50], a more general solution relies on Ultrafast 2D NMR (UF), which allows to record a full 2D spectra in a single-scan, replacing the time incrementation of conventional 2D NMR by spatial encoding using frequency-swept pulses and gradients [51,52]. The single-scan character of UF 2D NMR is compatible with the short-lived hyperpolarization of d-DNP [53] and proof-of-concept work on simple mixtures of natural products was demonstrated in 2009 [54] by analyzing natural abundance terpenes at 1 mM with entangled UF HMBC and UF HSQC, following a single d-DNP experiment. In 2015, Dumez et al. reported the first UF long-range heteronuclear correlation spectra of breast cancer cell extracts at natural abundance [36]. More recently, ^{1}H-^{1}H d-DNP enhanced UF 2D spectra were reported by Singh et al. on a mixture of aromatic compounds, observing a signal enhancement higher than 150 (Figure 3) [43•].

Figure 3



2D ^{1}H-^{1}H hyperpolarized UF TOCSY spectrum obtained in a single scan on model mixture of pyridine, quinoline and benzophenone at 85 mM before dissolution (top). Conventional 2D ^{1}H-^{1}H TOCSY spectrum on the same sample after rethermalisation and shimming, in 4 h and 25 min using 128 t_1 increments with 8 scans per increment (bottom). The data for obtaining the conventional spectra were processed with same acquisition (t_2) and evolution (t_1) times as used for its corresponding 2D hyperpolarized UF spectra. Shown on top of the 2D sets is the conventional 1D spectrum for this mixture. Adapted with permission from reference [43•].

While not applied yet in a metabolomics context, these approaches could facilitate the identification of biomarkers in hyperpolarized metabolomics studies, or the determination of isotopic patterns in hyperpolarized SIRM. Challenges remain to be addressed, such as the impact of post-dissolution turbulences effects which can significantly affect the quality of spatial encoding in UF experiments [55].

Enhanced ^{1}H sensitivity with para-hydrogen

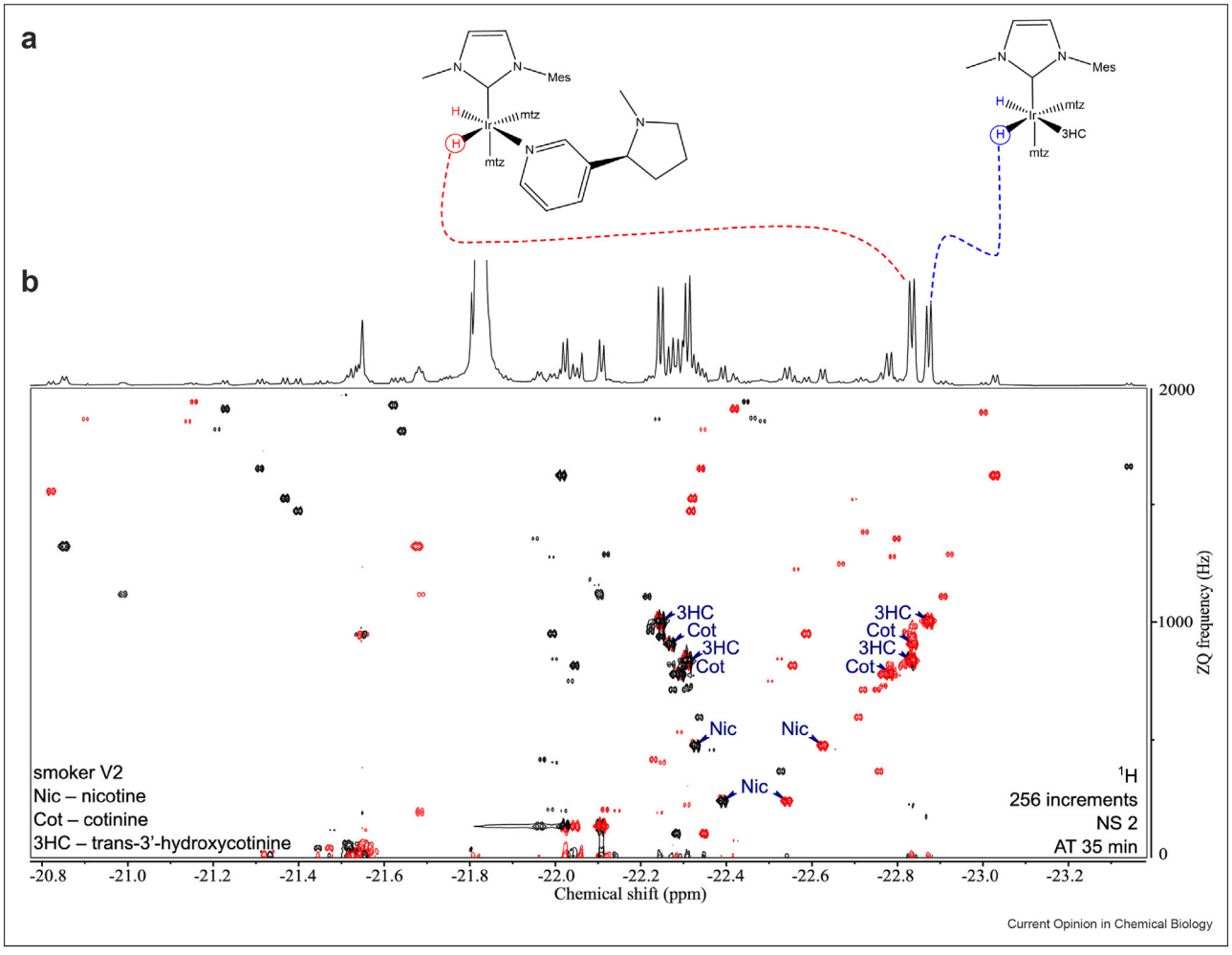
Hyperpolarization techniques based on p-H_2 can overcome the sensitivity barrier of conventional ^{1}H NMR

using the large and long-lived spin order of dihydrogen gas in its para form, and metal-based catalysts that allow a hyperpolarized detection of specific analytes.

Among all the variants of p-H_2 techniques, non-hydrogenative para-hydrogen induced polarization (nh-PHIP) is particularly relevant and developed for mixture analysis. This is notably the case of signal amplification by reversible exchange (SABRE), which consists in using the reversible binding of p-H_2 and the substrates to a metal complex, to transfer spin order from p-H_2 to substrates through J-couplings, followed by the spontaneous conversion of spin order into observable magnetization, allowing the detection of substrates at low-µM detection in a single-scan [56,57]. SABRE is compatible with a wide set of conventional 2D NMR experiments (COSY, UF COSY and HMBC) [58]. However, SABRE techniques are quite restricted to organic solvents since water-soluble catalysts provide—at the time of writing—poorly efficient polarization transfer [59,60], limiting their application for biological sample analysis and metabolomics.

Several non-hydrogenative PHIP (nh-PHIP) based techniques rely on a direct observation of hyperpolarized hydrides (Figure 4). Indirect detection of metabolites is possible since hydride chemical shifts are sensitive to bound metabolites. However, it makes signal assignment more challenging and—in the absence

Figure 4



(**a**) Illustration of hyperpolarized hydrides showing different chemical shifts in function of bond metabolites, Cotinine (left, red) and 3HC (right, blue) (**b**) 2D ZQ p-H_2 spectrum of urine extract in $CDCl_3$. Each complex of an analyte gives rise to two opposite phase doublets at the same ZQ frequency. Chiral analytes (i.e., Nic, Cot, and 3HC) can combine in two ways due to stereogenic center on iridium in the complex, forming two diastereomers with different physical properties. Signals were assigned by internal standard addition during method development. Upper 1D trace recorded separately with the 1D Selective excitation of polarization using PASADENA (SEPP) sequence. Adapted from reference [63••].

Figure 5

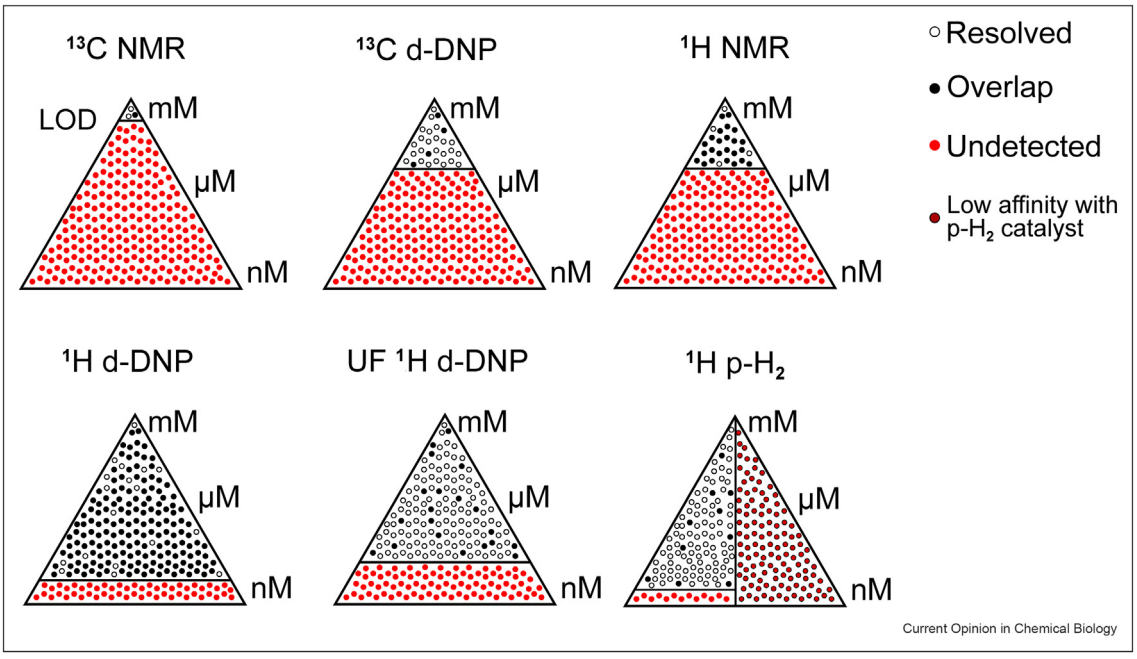


Illustration of the sensitivity, resolution and selectivity of main hyperpolarized techniques for metabolomics. White, black and red dots represent respectively resolved, overlapped and undetected metabolites signals. Dark red dots represent concentrated metabolites that are not detected by para-H_2 NMR because of its selectivity. For d-DNP schemes, estimated LOD are related to metabolite concentration before dilution at natural abundance. For conventional NMR schemes, estimated LOD are given for a 15 min acquisition time on a 700 MHz equipped with a cryoprobe in quantitative conditions. For p-H_2, the triangle is split in half to illustrate that compounds with a low affinity with the p-H_2 catalyst are not detected, regardless of their concentration.

of rich libraries—internal standard additions are often mandatory for signal assignment [61•].

Fortunately, nh-PHIP based techniques are also compatible with 2D NMR experiments, especially 2D zero-quantum (ZQ) experiments. ZQ spectroscopy shows several advantages compared to more conventional 2D experiments, indeed ZQ coherences are not altered by unwanted inter-hydride J-couplings ($J_{AX} \approx 7.5$ Hz), moreover, they offer a linear pattern for similar metabolite structures that can aid for signal assignment (Figure 4) [62].

Recently, Reimets et al. showed that 2D ZQ nh-PHIP based methods (Figure 4) were suited to determine accurately the concentration of nicotine and its derivatives in smoker urine at sub-μM concentrations—using a standard additions workflow—as suitable for pharmacokinetics and targeted metabolomics [63••]. However, both H_2 and parahydrogen catalysts are poorly soluble in aqueous solvents, limiting direct analysis of biofluids. Moreover, in the case of urine, the presence of urea and ammonium leads to intense unwanted signals and must be removed from the sample. As a consequence, a solid phase extraction (SPE) with an organic solvent (MeOH or $CHCl_3$) of urine sample is necessary prior to the analysis.

Hyperpolarization induced by p-H_2 provides an impressive ^{1}H enhancement—typically two orders of magnitude—and the recent developments focusing on biofluids make it a promising hyperpolarization technique for metabolomics thanks to its inexpensive and straightforward workflow despite the required extraction step. However, p-H_2 techniques are still limited by their selective character, indeed, only a few functional groups can efficiently bind to the organometallic complex and be subsequently detected, like amines, diazirines, amino-acids or nitrogen and sulfur heteroatomic compounds (Figure 5). This limitation could be addressed by developing new chemosensors—including water-soluble ones—with different affinity to a wider range of chemicals. Predicting hydride chemical shifts values as a function of bound metabolites is another very challenging task, and only internal standard additions are currently used to assign nh-PHIP signals, however, chemical shifts are very trustful and libraries could be built to overcome this issue [61•].

Conclusion

Hyperpolarization NMR techniques offer great opportunities to break the sensitivity barrier of NMR. The last decades have witnessed many efforts to make hyperpolarized NMR suitable for metabolomics. In particular, dissolution-dynamic nuclear polarization and

parahydrogen-based methods have been extensively explored.

These hyperpolarized techniques and their perspectives are compared in Figure 5 to better understand their advantages and drawbacks in terms of sensitivity, resolution and selectivity. ^{13}C d-DNP benefits from ^{13}C resolution advantages while allowing to detect metabolites at sub-millimolar concentrations. In contrast to ^{13}C, ^{1}H d-DNP will benefit from higher sensitivity of ^{1}H nuclei, improving theoretically the limit of detection by 3 orders of magnitude at the cost of stronger peak overlap. Moreover, homonuclear UF ^{1}H DNP (TOCSY, COSY) can reduce significantly strong peak overlap by spreading ^{1}H signal over a second dimension. p-H_2 also benefits from the natural sensitivity of ^{1}H nuclei and from 2D spectroscopy, an association that already demonstrated sub-µM limits of detection. However, p-H_2 methods are currently limited by their inherent selectivity, indeed, only selected functional groups of compounds can efficiently bind to organometallic complexes. Note that some degree of selectivity may actually be considered as a strength to observe accurately metabolites at very low concentration, especially if the selectivity is controlled.

At the time of writing, dissolution-dynamic nuclear polarization has shown its potential for both SIRM and metabolomics at natural abundance with super-sensitive ^{13}C detection. The implementation of ^{1}H d-DNP, multi-dimensional d-DNP and cryo-free systems are among most promising perspectives in the field as they can increase respectively sensitivity, resolution and through-put to another level. Parahydrogen-based methods are straightforward, cheap and naturally compatible with multi-scan approaches, allowing multi-dimensional NMR and an impressive sub-µM limit of detection. Signal assignment and intrinsic selectivity of parahydrogen-based methods are the most challenging limits to overcome and can be addressed respectively by parahydrogen chemical shifts libraries and a wider diversity of organometallic complexes. Along with the emergence of alternative hyperpolarization methods such as optical crystals, we expect hyperpolarized NMR, especially based on d-DNP and p-H_2 techniques, to be a source of both new developments and impacting applications for NMR metabolomics in the coming years.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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- •• of outstanding interest

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