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COMMUNICATION

Hyperpolarized ^{13}C NMR Spectroscopy of Urine Samples at Natural Abundance by Quantitative Dissolution Dynamic Nuclear Polarization

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Abstract: Hyperpolarized nuclear magnetic resonance (NMR) offers an ensemble of methods that remarkably address the sensitivity issues of NMR. Dissolution Dynamic Nuclear Polarization (d-DNP) provides a unique and general way to detect ^{13}C NMR signals with a sensitivity enhanced by several orders of magnitude. The expanding application scope of d-DNP now encompasses the analysis of complex mixtures at natural ^{13}C abundance. However, it has in this area been limited to metabolite extracts. Here, we report the first d-DNP-enhanced ^{13}C NMR analysis of a biofluid -urine- at natural abundance, offering unprecedented resolution and sensitivity for this challenging type of sample. We also show that accurate quantitative information on multiple targeted metabolites can be retrieved through a standard addition procedure.

Liquid-state nuclear magnetic resonance (NMR) spectroscopy is a major analytical tool that provides both unambiguous structural and quantitative information on complex mixtures. NMR is one of the most used analytical techniques in metabolomics, together with mass spectrometry (MS) coupled to chromatography.[1] Metabolomics plays an important role in the general understanding of metabolism and is involved in many different fields, such as plant[2] and environmental sciences,[3] biomedical science[4] or personalized medicine.[5] The vast majority of NMR metabolomic experiments relies on 1D ^{1}H experiments for sensitivity and time reasons.[6] However, strong peak overlap is a major limitation for the analysis of inherently complex biological mixtures. ^{13}C NMR benefits from a wider spectral dispersion and a narrower signal linewidth, which could help to overcome this limitation, but is hardly used in metabolomics since it is three to four orders of magnitude less sensitive than ^{1}H NMR.[7] Being able to record ^{13}C NMR spectra at natural abundance without compromising on sensitivity would open many perspectives for the analysis of complex mixtures.

Hyperpolarization techniques based on Dissolution-Dynamic Nuclear Polarization (d-DNP) make it possible to increase the nuclear polarization by several orders of magnitude and offer a way to increase drastically the sensitivity of ^{13}C NMR.[8] Several recent developments reported the exploration of d-DNP for the untargeted analysis of complex metabolite mixtures. Studies have been reported on labelled ^{13}C biological samples, allowing to access targeted information on metabolic pathways through a stable-isotope resolved metabolomics approach.[9–11] At natural abundance, a preliminary study showed the possibility to detect hyperpolarized ^{13}C signals on plant and cancer cell extracts in a single scan.[12] Later, another study reported the excellent repeatability of this technique (a few percent), as required for metabolomics applications.[13] More recently, ^{13}C d-DNP NMR was successfully incorporated into an untargeted metabolomics workflow on a simple type of plant extracts.[14] However, natural abundance ^{13}C d-DNP NMR has never been applied to the analysis of biofluids, which are probably the most widely studied biological matrices in metabolomics. While the performance of d-DNP has been shown for the analysis of relatively concentrated plant extracts, the analysis of biofluids constitutes a more challenging target, both in terms of concentration and matrix complexity. Recently, we reported a multi-parametric optimization of the d-DNP experimental workflow[15] that paves the way towards such application, by allowing a significant increase in sensitivity and resolution. In particular, this systematic optimization focused on sample preparation (*i.e.* radical concentration and DNP juice composition) and dissolution parameters (*i.e.* dissolution duration, choice of dissolution solvent and stabilisation delay) to ensure i) a high and robust hyperpolarization in the solid-state and ii) intense, narrow and repeatable ^{13}C signals in the liquid-state. [15]

In this communication, we report the first hyperpolarized ^{13}C NMR experiments on biofluid samples at natural ^{13}C abundance in the case of urine, in conditions which are compatible with metabolomics studies. In addition, we show that the quantitative character of NMR can be maintained with these hyperpolarized experiments, leading to the determination of absolute concentrations for metabolites that are difficult to quantify with conventional ^{1}H NMR.

A single midstream urine sample was collected from the first morning urine (to avoid bacterial contamination) of a healthy volunteer, from whom a statement of informed consent was obtained. The urine sample was centrifugated at 4350 rpm during 30 min. Supernatants were then frozen and stored at -80°C. Samples were thawed at room temperature and freeze-dried overnight to match with d-DNP sample preparation and to minimize the impact on metabolic profiles.[16] Freeze-dried urine samples were stored at -80°C prior to sample preparation (See SI). Freeze-dried urine was dissolved in DNP Juice (d_8-glycerol: D_2O: H_2O, 6: 3: 1, v: v). TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, 50 mM) was added as polarizing agent and TSP-d_4 (sodium 3-trimethylsilylpropionate-d_4, 20 mM) was added as an internal standard for normalization and chemical shift referencing. Concerning standard additions, metabolite standards were directly weighted on a precision balance and

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mixed with the sample. See Supporting Information for detailed sample preparation procedure.

d-DNP experiments were carried out with an optimized semi-automated d-DNP workflow.[15] Samples were polarized at 1.2 K with frequency-modulated microwave irradiation using gated cross-polarization.[13] After 20 min of polarization, 5 mL of hot solvent (D_2O or CD_3OD) were used to rapidly dissolve the sample and bring it to the nearby liquid-state spectrometer through a transfer line equipped with a magnetic tunnel. Acquisition was performed on a 400 MHz spectrometer equipped with direct-detection cryoprobe at room temperature, in a single scan after a total time of 9.2 seconds after dissolution. A 90° excitation pulse was used, and the signal was recorded for 1.72 s with a spectral width of 30 kHz. All spectra were acquired in the same conditions and processed the same way. Apodization (lb = 1 Hz) was applied prior to Fourier Transform. Phase and baseline were corrected automatically. Chemical shifts were calibrated on the TSP methyl group (0 ppm). Signal areas were normalized to the quaternary carbon TSP signals (188.5 ppm). Integration regions were always centered and were identical for all spectra. See Supporting Information for detailed acquisition and processing NMR parameter.

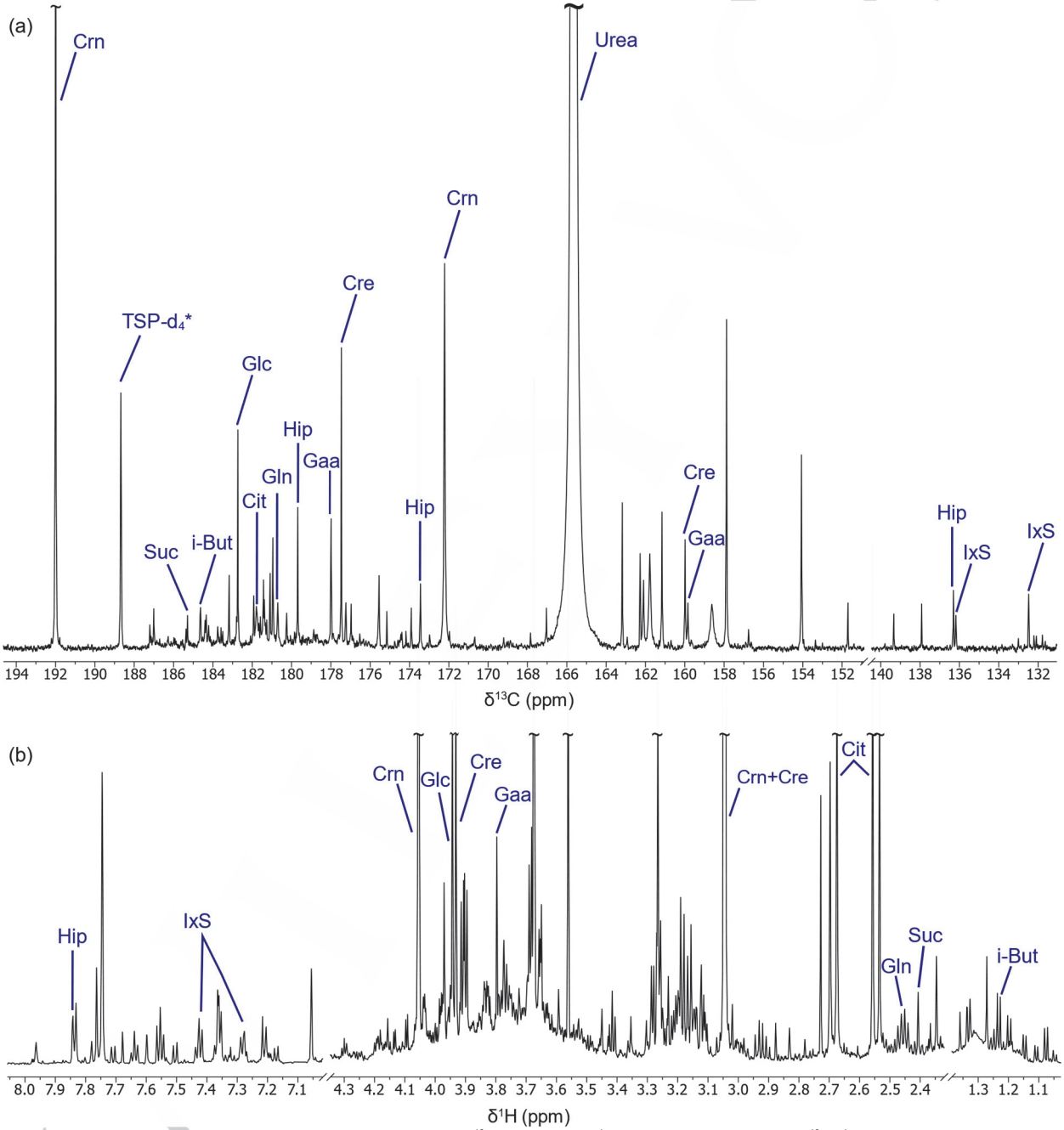

Fig.1: Illustration – in the case of urine – of resolution advantages of ^{13}C NMR versus ^{1}H NMR. (a): hyperpolarized ^{13}C-{^{1}H} spectrum of freeze-dried urine – 150 mg. mL^{-1} before dissolution (30 mg in the sample cup, giving a concentration of ~1.5 mg/mL right before the detection) – using D_2O as dissolution solvent, acquired in a single scan following DNP assisted with cross-polarization, and recorded 9.2 seconds after the dissolution starts, acquired on a 400 MHz spectrometer equipped with a nitrogen-cooled cryoprobe. (b): conventional ^{1}H NMR spectrum recorded in quantitative conditions (32 scans, 36 minutes) of the same freeze-dried urine sample – 25 mg. mL^{-1} (15 mg in the NMR tube) – in D_2O acquired on a 700 MHz spectrometer equipped with an inverse cryoprobe. Internal reference signal of TSP-d_4 is indicated by a star (*). Annotations were performed by internal standard addition experiments in both cases. Crn=Creatinine, Cre=Creatine, Glc=Glycolate, Hip=Hippurate, Gln=Glutamine, Gaa=Guanidinoacetate, Suc=Succinate, Cit=Citrate, IxS=Indoxyl Sulfate, i-But=3-aminoisobutyric acid.

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Fig.1a shows the 1D ^{13}C NMR spectrum of a freeze-dried urine sample recorded in a single-scan after d-DNP, relying on an optimized experimental workflow. The spectral fingerprint is extremely rich with many signals that would not be observable with several hours of conventional ^{13}C acquisition at high field (see Fig. S5).

Fig.1b shows a conventional ^{1}H NMR spectrum acquired from the same original sample. The comparison with Fig.1a highlights the advantages of high-resolution ^{13}C spectra. Indeed, ^{13}C NMR benefits from a wider spectral width and narrow singlets (average linewidth 1.5 Hz after apodization) while ^{1}H NMR multiplets are highly overlapped -even if the ^{1}H NMR spectrum was recorded at 700 MHz, the highest field available in our lab. Solving such overlap issue is crucial in metabolomics, since the biologically relevant variations of key metabolites may be obscured by surrounding, less informative peaks, making biomarker discovery and quantification difficult. Although peak deconvolution tools exist, these solutions are often limited by the need to rely on databases that depend on the studied biological matrix and on the sample preparation conditions, and by strong peak alignment issues arising from pH variations.[17,18]

The ^{13}C NMR spectrum shown in Fig.1a was recorded after a relatively long delay following dissolution (9.2 seconds in total including dissolution, transfer and stabilization of the sample), deliberately chosen to ensure a stable and repeatable procedure. This duration only allows detection of the signals from quaternary ^{13}C nuclei, for which the polarisation survives longer thanks to their higher longitudinal relaxation times T_1 (a few tens of seconds for quaternary carbons versus a few seconds for aliphatic carbons). Nevertheless, the vast majority of urinary metabolites show at least one quaternary ^{13}C.[19] The development and implementation of fast transfer lines (< 2 seconds) could decrease this transfer time and allow an efficient detection of aliphatic carbon signals.[20,21] An alternative solution is to rely on a dissolution solvent that makes it possible to work with a shorter transfer time. For instance, methanol-d_4 has shown interesting physico-chemicals properties for dissolution and transfer.[15] Deuterated methanol was tested for both urine metabolic fingerprinting and quantitative analysis in this study with a shorter transfer time (4.2 seconds in total). Hyperpolarized spectra obtained in both solvents are compared and discussed in SI. On the one hand, methanol-d_4 has interesting features: it provides a higher sensitivity and a better experimental robustness, although its use is limited by the modest solubility of some metabolites in this solvent. On the other hand, D_2O is a cheaper solvent, which offers a much higher solubility for metabolites and as it can be used for buffer solution, it provides more reproducible chemical shifts due to smaller pH variations. In addition, most metabolomics databases provide chemical shifts in D_2O, thus making peak annotation easier.

The quantitative measurement of metabolite concentrations is one of the advantages offered by conventional ^{1}H NMR for metabolomics. Indeed, quantitative information offers the opportunity to confirm the significance of putative biomarkers and allow precious cross-study comparisons.[22] In conventional 1D NMR, quantitative measurements are straightforward to achieve, as there is a virtually identical proportionality factor between signal area and concentration for all spins in a given spectrum, provided that careful acquisition and processing conditions are respected. This makes absolute quantification possible with a single reference signal. However, many factors in the d-DNP experiment make such proportionality factor peak-dependent, including nuclei-dependent polarization efficiency in the solid-state, and site-specific polarization losses during the transfer time. Modelling such peak-specific response factors would require an accurate determination of all polarization and relaxation parameters involved in the d-DNP experiments, that would be highly impractical. We rather suggest to determine such response experimentally by relying on standard addition method combined with a normalization to a reference signal.

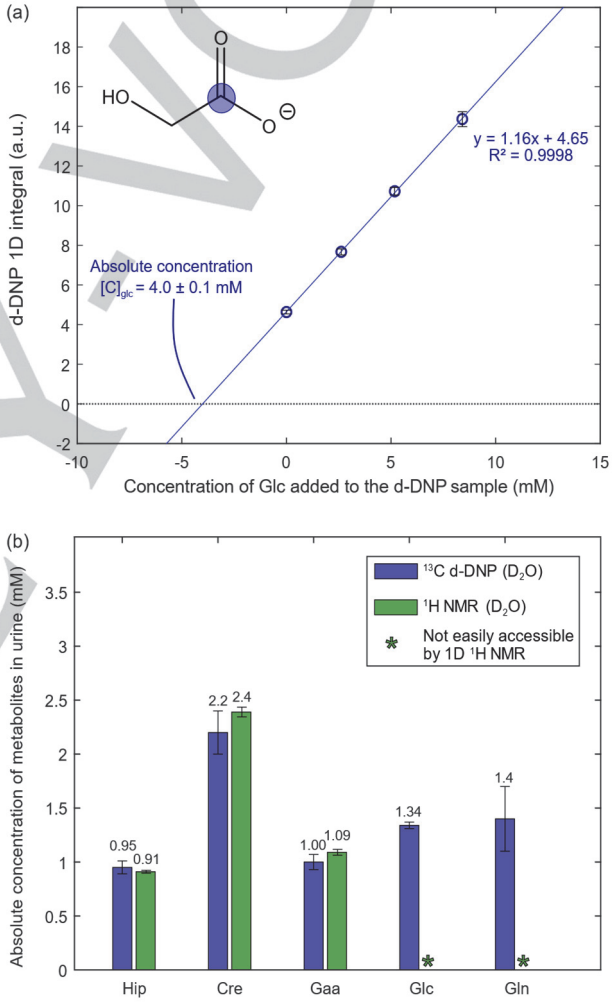

Fig.2: (a) Standard additions curve for the glycolate signal (Glc, 182.7 ppm) from ^{13}C d-DNP spectra in urine. Normalized integral (average of triplicate) is plotted as a function of added concentration. The initial absolute metabolite concentration is obtained by the ratio of the slope to the y-intercept. (b) Quantitative results obtained simultaneously by standard additions with d-DNP using D_2O as dissolution solvent. The absolute concentration values shown in panel b account for the dilution (respectively concentration) factor of the samples for ^{1}H 1D (respectively d-DNP) measurements (see SI). A deconvolution tool was used for conventional 1D ^{1}H NMR at 700 MHz. Error bars on the values measured by d-DNP are given by the quality of the linear fit and illustrate the good repeatability of the method. Errors bars on the values measured by ^{1}H NMR are determined by the average of three experiments.

Standard additions consist in performing a few (typically three) measurements where the sample has been previously spiked with known amounts of targeted metabolites. We previously demonstrated the efficiency and robustness of this method in quantitative 2D NMR, which is also characterized by the same kind of site-specific response factors.[23,24] The additional normalization to a reference signal (here the quaternary TSP peak) is crucial to reduce the variability arising from experimental variations between successive d-DNP experiments.[13,14]

Fig.2 illustrates the efficiency of this method on the example of five metabolites whose concentrations were determined simultaneously with a single standard addition procedure. For metabolites whose peak area is also accessible by deconvolution of 1D ^{1}H NMR peak areas (Hippurate, Creatine and Guanidinoacetate), results illustrate the excellent match between ^{13}C d-DNP and 1D ^{1}H NMR quantification. Moreover, we report absolute concentration values for Glutamine and Glycolate which were not accessible through deconvolution of 1D ^{1}H NMR spectra because of significant peak overlap (Fig.1 and Fig.S9). The average coefficient of variation (CV) for normalized signals of quantified metabolites was 4%, while the average CV for all detectable signals (SNR>6) was 9%. These results are obtained across the 12 experiments of the standard addition procedure and demonstrate the good repeatability of the hyperpolarized method. Another standard additions series using CD_3OD as a dissolution solvent showed comparable results (see SI), again with elements of comparison pointing in favor of the use of D_2O in practice.

Although standard additions require successive experiments, several compounds can be quantified simultaneously by including all targeted analytes in the same spiking mixture, as it was the case in the example of Fig.2. As a perspective, the approach could be potentially reduced to a single measurement per sample by calculating signal loss coefficients (SLC) for each metabolite, as suggested by Lerche *et al*. in the case of labelled metabolites.[9] However, the generalization of such method to natural abundance samples will require a full analytical validation which is out of the scope of this study.

We have shown the ability of d-DNP experiments to provide rich and highly metabolic fingerprints on urine at natural ^{13}C abundance. Such sensitive and repeatable results on a biofluid could not have been achieved without a fine optimization of experimental settings.[15] On the one hand, this optimization is quite general and does not need to be conducted each time a new sample is analyzed. In addition to the high resolution and sensitivity of the d-DNP method, the combination of standard additions and normalization makes it possible to recover accurate quantitative information on metabolites whose concentration is difficult to access by conventional ^{1}H NMR. On the other hand, we estimate that the limit of detection is around 100 µmol/L (for a linewidth of 1.5 Hz), while the limit of quantification is around 1 mmol/L (concentrations in the sample cup, before dissolution). This is typically an order of magnitude higher than required for the analysis of more dilute biofluids such as plasma or serum (where the concentration of many relevant metabolites is rather in the order of tens of µmol/L). The application to more diluted biofluids will either require an optimization of sample preparation or an optimization of the experimental design to reduce the significant dilution factor occurring during dissolution and shorten the transfer time between the two magnets, enhancing sensitivity even further. Finally, it should be highlighted that an alternative hyperpolarization approach based on para-hydrogen also allows the quantification of metabolites in biofluids.[25,26] While it is highly sensitive, this method remains limited to certain classes of metabolites and can be seen as complementary to the present approach.

Overall, this first report of d-DNP for urine analysis at natural ^{13}C abundance paves the way to the exploration of both analytical and applied research questions, building on the emergence of a new analytical technique in the metabolomics workflow. Possible applications include (pre-)clinical studies on patient samples. However, routine applications of this new approach will require efforts to increase the throughput and decrease the cost of d-DNP experiments. Perspectives also include further methodological input such as the implementation of 2D experiments compatible with d-DNP to facilitate biomarker identification, as well as the need for standard operating procedures to allow building databases and comparing data between instruments. Such developments will be key to enable broadband applications of d-DNP to metabolomics of urine and other biofluids.

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Keywords: NMR spectroscopy • Hyperpolarization • Dissolution Dynamic Nuclear Polarization • Metabolomics • Quantitative analysis

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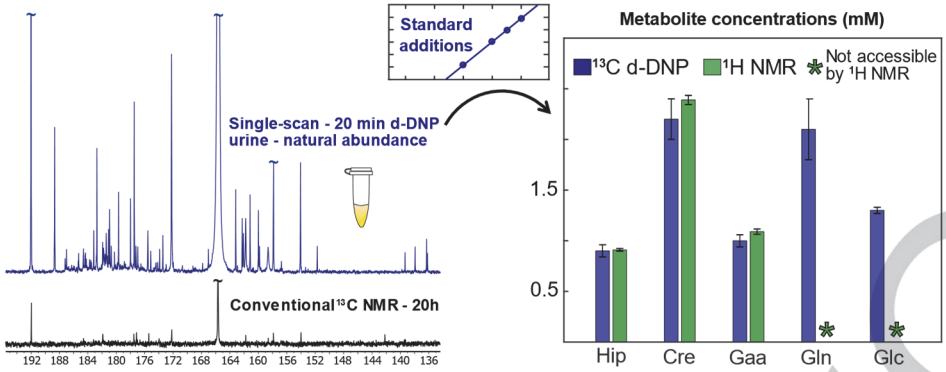
Graphical abstract:



Hyperpolarized ^{13}C NMR spectroscopy by dissolution dynamic nuclear polarization provides a rich and resolved ^{13}C metabolic profile on urine samples at natural abundance while retaining precious quantitative information using a standard addition workflow.

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