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Improved NMR detection of phospho-metabolites in a complex mixture

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Abstract

Phosphorylated metabolites are omnipresent in cells, but their analytical characterization faces several technical hurdles. Here, we detail an improved NMR workflow aimed at assigning the high-resolution sub-spectrum of the phospho-metabolites in a complex mixture. Combining a pure absorption J-resolved spectrum (Pell & Keeler, *J. Magn. Res.* 2007) with alternate on- and off-switching of the ^{31}P coupling interaction during the t_1 evolution with a Pure-In Phase (PIP-) HSQMBC experiment (Castañar et al., *Angew. Chemie* 2014) without or with TOCSY transfer during the INEPT transfer gives access to selective identification of the individual subspectra of the phosphorylated metabolites. Returning to the initial J-res spectra, we can extract with optimal resolution the full trace for the individual phospho-metabolites, which then can be transposed on the high-resolution quantitative 1D.

Introduction

The metabolome of a living organism is defined as the collective set of small molecules that are produced by cells, and provides as hence a direct snapshot of cellular activity and/or physiological status. In the era of -omics approaches of biology, Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy are currently the techniques of choice for its analytical characterization. Both techniques are complementary, with MS profiting from a superior sensitivity, bringing single-cell metabolomics within range,¹⁻³ whereas NMR allows to study molecules directly in solution and can for example resolve isomers from the same molecule⁴⁻⁶. Ideally, the latter technique might also give direct access to the metabolome characterization inside the cells.^{7,8} Over the recent years, this exciting perspective has come closer due to major advances towards improved NMR analyses of cell suspensions⁸ in particular with the development of ^{13}C hyperpolarization based on the DNP technique^{9,10} together with fast acquisition methods¹¹⁻¹³.

1 Because life has chosen ATP as the general energy currency, the phosphate moiety can be
2 found in a large fraction of biomolecules.¹⁴ A genome-scale model of the model bacteria *E. coli* for
3 example estimated that 43% of its metabolome would carry at least one phosphate group.¹⁵
4 However, the identification of the phospho-metabolome is rather challenging. Especially for the
5 many phosphorylated carbohydrates that act as intermediates in for example the glycolytic¹⁶ or the
6 pentose phosphate¹⁷ pathways, both mass and NMR spectroscopy face severe technical difficulties.
7 Their hydrophilicity hinders physical separation by reverse phase liquid chromatography before mass
8 analysis, whereas their analysis as a mixture by MS/MS is plagued by the presence of various
9 structural isomers with similar fragmentation patterns.¹⁸ For NMR, early studies of the metabolism of
10 different organisms extensively used direct detection of the 100% abundant spin-1/2 ³¹P nucleus,^{7,19–}
11 ²¹ but its decreased sensitivity compared to proton NMR (due to its lower gyromagnetic ratio and
12 generally larger line widths) and the important influence on chemical shift of external factors such as
13 pH and Mg²⁺ concentration all have contributed to its reduced attractivity in metabolomics studies.
14 But proton NMR equally has its limits. For the glycolytic intermediates of interest here, for example,
15 both the ³¹P and ¹H signals are all clustered in a narrow range of chemical shift values.²² As a result,
16 molecular databases contain relatively few phospho-metabolites. Only spectra of Glucose-6-
17 phosphate (G6P) are present in all major databases,^{23–27} whereas Fructose-6-phosphate (F6P) or
18 fructose-1,6-phosphate (FBP), the next intermediates, only have their spectra recorded in the BMRB
19 database.²³ Finally, the above mentioned condition-dependent values of the chemical shift hamper
20 database searches on the sole criterium of chemical shift, and alternatives will be clearly needed.

21 As a result of these combined technical challenges, only very recently attention has shifted to
22 an extensive characterization of this sub-set of metabolites. Selective immobilization of the phospho-
23 metabolites via metal ion affinity²⁸ or the use of ion exchange chromatography^{29–31} have improved
24 the coverage by mass spectrometry. For NMR, indirect detection schemes have been
25 developed,^{22,32,33} that effectively lead to a selective detection of only those protons coupled to a
26 phosphorus nucleus. However, these experiments are mostly run with ³¹P decoupling during
27 acquisition, which not only limits the overall resolution but also modifies the coupling patterns in the
28 slices extracted from the ¹H-³¹P correlation spectrum. Comparing those slices to the high resolution
29 proton 1D NMR spectrum, which remains the standard experiment when multiple samples have to
30 be analysed, thereby becomes complex. Indeed, the ³¹P decoupling obtains selective removal of the
31 hetero- but not the homonuclear coupling constants, and contrary to the case of ¹³C spectroscopy,
32 ¹H-¹H and ¹H-³¹P couplings are of the same order of magnitude. Accordingly, in our recent NMR study
33 of the metabolome of the zebra mussel *Dreissena polymorpha* as a model system for ecotoxicological
34 monitoring, we could only assign a couple of phospho-metabolites³⁴.

1 The issue of ^1H - ^1H couplings as a limiting factor for resolution has in recent years been treated by the
2 resurrection of the J-resolved NMR technique^{25,35,36} together with a boom in pure-shift NMR
3 techniques.³⁷⁻³⁹ However, both techniques address the issue of homonuclear couplings as a
4 problematic effective broadening leading to significant signal overlap in spectra of complex mixtures.
5 Still, these same patterns carry valuable information that is notoriously condition-independent⁴⁰ and
6 can help in the identification of the metabolites. Ideally, one would like to retain a selective detection
7 of the phospho-metabolites and their intricate coupling pattern as observables in a high-resolution
8 1D NMR spectrum. Although not (yet) a general practice, providing a high-resolution annotated
9 proton spectrum with the full multiplicity for any given proton has the potential to accelerate further
10 exploitation of the data for future metabolic studies.^{34,41} In the present work, we detail our novel
11 workflow which brings closer this aim for the sub-spectrum of the phospho-metabolites (Figure 1). A
12 first entry point is a pure absorption J-resolved spectrum⁴² with alternate on- and off- switching of
13 the ^{31}P coupling interaction during the t_1 evolution. Subtraction of both sub-spectra leads to the
14 selective observation of the ^{31}P coupled protons while maintaining the complete homo- and
15 heteronuclear coupling patterns. The Pure-In Phase (PIP-) HSQMBC experiment introduced by
16 Castañar and co-workers⁴³ can give the same information at the condition that the heteronuclear ^{31}P
17 decoupling is switched off during acquisition, allowing lengthening of the acquisition time and hence
18 resolution enhancement at no cost at all. To identify the additional proton signals of the detected
19 phosphorylated metabolites, we implemented during the INEPT transfers a TOCSY like transfer from
20 the ^{31}P coupled protons to the other protons of the metabolite.^{44,45} The resulting experiment thereby
21 selectively identifies the whole trace of the metabolite at the given ^{31}P frequency, and proton
22 resolution is maintained by again omitting ^{31}P decoupling during the detection. Finally, returning with
23 this trace to the initial J-res spectra, we can extract with optimal resolution the full trace for the
24 individual phospho-metabolites, which then can be transposed on the high-resolution quantitative
25 1D. We demonstrate the workflow on a simple standard solution containing selected phospho-
26 metabolites to discuss different practical considerations. We then apply it to a complex biological
27 sample that is an active bacterial cell extract, wherein the addition of glucose leads to accumulation
28 of the different phospho-metabolites from the glycolysis pathway.^{46,47}

29

30 **^{31}P selective J-res pulse sequence**

31 In our previous effort to establish an easy measurement of the ^{13}C flow from labelled precursors into
32 its cellular products,⁴⁸ we developed a pure-shift ^{13}C heteronuclear J-resolved pulse sequence that
33 maintains solely the ^1H - ^{13}C heteronuclear coupling patterns. As an intermediate step, we had
34 adapted the Pell-Keeler homonuclear 2D J-resolved sequence⁴² by the addition of ^{13}C π pulses during
35 the t_1 delay, thereby dispersing both ^1H - ^1H and ^1H - ^{13}C couplings along the 45° diagonal⁴⁸. This

1 experiment suffers from limited sensitivity, as only that part of the sample which is on-resonance
2 with the selective π pulse (here implemented as a PSYCHE element³⁷) contributes to the signal⁴².
3 Based on signal intensity in the first increment of the 2D experiment, we found a relative S/N ratio
4 for the isolated H2 protons of β -D-glucose of 7% when compared to the same signals in a 1D proton
5 spectrum recorded with identical acquisition parameters. This limited sensitivity influences directly
6 the quantitative character of the experiment. In a 1D proton spectrum, quantitative information can
7 indeed be obtained by using a relaxation delay of 10 or more seconds, but this is not compatible with
8 the reduced sensitivity of the J-res experiment. Still, although not realistic on real-world samples in
9 view of the inferior sensitivity, we have run both the 1D and J-resolved spectra with 1s or 10s
10 relaxation delay on our mix of standards. As a result, we find exactly the same ratio $I(10s)/I(1s)$ for
11 the signals in both spectra (Figure S10). This suggests that a correction factor deduced from a
12 comparison of both 1D spectra can be extrapolated to the intensity of the signals in the 1s J-resolved
13 experiment. A second source of error, more unexpected, comes from the bandwidth of the PSYCHE
14 pulse element. In their original study, the Manchester group preconized a 30 ms PSYCHE pulse
15 composed of the vectorial sum of different CHIRP pulses with a 10 kHz sweep bandwidth in the
16 presence of a 1.5% gradient pulse. While exploring the quantitative character of this experiment, we
17 noted that the TSP resonance was not fully excited on our 800 MHz experiment. Increasing the
18 sweep bandwidth of the CHIRP pulses to 20 kHz solved this problem (Figure S11). Despite these
19 shortcomings, the superior pure absorption line shapes provide a distinctive advantage to the J-res
20 experiment when unravelling signals and coupling patterns in crowded regions.

21 Applying the π pulses on the ³¹P rather than on the ¹³C channel (Figure 2, Figure S1) disperses
22 the heteronuclear ¹H-³¹P coupling along the same 45° axis as the homonuclear couplings. However,
23 the comparable value of the ¹H-³¹P hetero- and ¹H-¹H homonuclear coupling constants makes
24 selective detection of the latter less obvious. One way around this is to compare two spectra without
25 and with the ³¹P π pulses. In the first case, the ¹H-³¹P coupling will be refocused during the t_1 delay,
26 only appearing during the direct t_2 detection period. In the second case, it will evolve during both
27 evolution times, thus showing up along the diagonal. Distinguishing the geminal protons for FBP at
28 the C1 or C2 position (Figure 2) thereby becomes straightforward, as the isolated 1 and 1' protons
29 show up as a homonuclear doublet with an additional ³¹P coupling. The 5 and 5' protons additionally
30 couple to the H4 proton, and the resulting doublet of doublet combines with the additional coupling
31 to the ³¹P nucleus and leads to an apparent quintuplet (Figure 2).

32 After subtraction of both spectra, the remaining positive contours represent only the ³¹P-
33 coupled protons, and hence acts as a selectivity filter for those latter (Figure S2). In order to improve
34 the robustness of the subtraction, we encoded an interleaved pulse sequence by alternate recording
35 of the 1D traces for every t_1 increment without or with the ³¹P π pulses (FigureS1). Zeroing the

1 trapezia left and right of a diagonal corresponding to a particular proton before projecting the
2 remaining diagonal as a positive sum generates the ^1H spectrum of this ^{31}P coupled proton (Figure 3).
3 Importantly, we do not tilt the spectra at any time, thereby maintaining the excellent line shape of
4 the experiment which is important in complex mixtures.⁴⁹ Reminiscent to the distant dipolar field
5 (DDF) resolution enhanced J-res sequence,⁵⁰ we found that the resolution is even better than that of
6 the 1D spectrum (see for example the G6P H3 proton Figure 3, right panels). However, deducing
7 metabolite concentrations from this J-resolved spectrum suffers from various factors including the
8 short recycling delay and the bandwidth of the PSYCHE pulse (see Supplemental Information).
9 Without spectral overlap, the same processing can be applied on both the decoupled and non-
10 decoupled spectra, resulting in two identical spectra except for their opposite phase. After phase
11 inversion of one, both can be summed, thereby increasing the signal to noise ratio by a factor of $\sqrt{2}$
12 (Figure S2). To simplify and increase the throughput of this processing, we developed a python script
13 to be used in topspin (<https://github.com/MetaSys-LISBP/zserter>). Proton signals coupled to a ^{31}P
14 nucleus can thereby be easily extracted, yielding subspectra whereby these particular signals appear
15 as isolated peaks.

16

17 **Combination with ^1H , ^{31}P correlation experiments**

18 Heteronuclear ^1H , ^{31}P correlation experiments have been commonly used for NMR analysis of
19 DNA and/or RNA^{51,52} and were more recently applied to the identification of phospho-
20 metabolites^{22,34}. Because of the importance of resolving homo- and heteronuclear coupling patterns
21 for the protons in the different phospho-metabolites, we applied the Pure In-Phase (PIP-) HSQMBC
22 sequences previously developed to detect both short- and long-range ^1H , ^{13}C correlations⁴³ without
23 decoupling in the acquisition. This experiment again delivers only the signals of protons directly
24 coupled to a ^{31}P nucleus, but with a better sensitivity than the J-res experiment. We found that S/N
25 for the same glucose signals as described above in the first increment of increases to 27% of that of
26 the comparable 1D proton spectrum. As signal intensity depends crucially on the coupling
27 interactions, the experiment claims no quantitative character (see SI). We next implemented a π
28 pulse train in a XY-expansion scheme⁵³ during the INEPT transfers, thereby obtaining a TOCSY like
29 transfer from the ^{31}P coupled protons to the other protons of the metabolite (PIP-HSQMBCxy16,
30 Figure 3).^{44,45} A coupling constant of 6 Hz was found as the best compromise between the transfer
31 between the ^{31}P nucleus and its coupled protons on one side, and the long range ^1H - ^1H transfer on
32 the other side (figure S3). Extracting a row of the latter spectrum at a given ^{31}P chemical shift (orange
33 line Figure 2.a) shows all proton signals of the corresponding metabolite, but their intensities
34 correspond to the diagonal and cross peaks in a TOCSY experiment and hence are not representative
35 of the concentrations of the respective protons. However, with this extracted 1D spectra we can

1 easily return to the corresponding signals of the J-resolved experiment and measure coupling
2 patterns and constants. The trapezia extraction processing proposed above can be used to isolate the
3 spectrum of the whole molecule (Figure 3, Figure S4) and label the identified molecule in the high
4 resolution 1D spectrum (Figure 3, Figure S4). Although we did not detect nucleotides or nucleotide
5 sugars in our cell extract (*vide infra*), the same workflow remains valid to characterize a mixture of
6 these metabolites as well (Figure S5).

7

8 **Identification of phosphorylated metabolites in a complex biological sample**

9 We next turned to a cell extract of *E. coli* as a real-world sample. Similarly as found for a
10 yeast extract by Professor Buchner more than a century ago,⁴⁶ an important number of enzymes
11 remain active in such a cell extract and produce notably the glycolytic intermediates after
12 supplementing the extract with 5 mM glucose.^{47,54} Glycolysis in such a cell extract has been followed
13 in real-time by multiple-reaction monitoring with an ESI-MS quadrupole mass spectrometer⁴⁷, but
14 isomers such as G6P/F6P or 2PG/3PG could not be distinguished. Whereas the ultimate aim of our
15 NMR set-up is to follow the complex reaction network in real time, possibly through simultaneous ¹H
16 and ³¹P detection schemes,⁵⁵ we here aim to assign as far as possible the 1D proton spectrum in
17 terms of phospho-metabolites. Can we already extract selectively the 1D ¹H spectra for these
18 phosphorylated glycolytic metabolites after quenching the extract by a standard⁵⁶
19 acetonitrile/methanol protocol? A recent effort to detect those same metabolites by NMR in a
20 synthetic mixture of the purified enzymes concluded that proton NMR leads to too many overlapping
21 signals to allow identification, and the authors therefore returned to ³¹P NMR with its
22 aforementioned disadvantages.⁵⁷ It therefore is a suitable sample to evaluate the potential for our
23 workflow.

24 To identify glycolytic metabolites, an aliquot of *E. coli* extract (prepared as described
25 previously, see SI) was thawed, and supplemented with 5 mM glucose (lyophilized). After 20 min, the
26 extract was quenched by addition of 800 μl of cold (-20 °C) acetonitrile/methanol solution (1:1
27 vol/vol), vortexed, let a -20 °C for one hour before centrifugation (15000 x g, 15 min, 4 °C). The
28 supernatant was dried by speedvac and stored dry at -80°C until analysis. For NMR analysis, the dried
29 sample was resuspended in D2O with 1mM TSP. Sample pH was of 6.6 (uncorrected pD
30 measurement). Figure 4 demonstrates the workflow to identify one representative phospho-
31 metabolite (3-phosphoglycerate, 3PG) of the pathway with as entry point the J-res difference
32 spectrum. The projection of its isolated ¹H proton signal in the J-res difference spectrum corresponds
33 to a trace from the PIP-HSQMBC spectrum, and extracting the trace at the same ³¹P frequency in the
34 PIP-HSQMBCxy16 spectrum yields the full trace for this metabolite. This trace allows identification of
35 the same signals with an optimal resolution in the original J-res spectrum. The signal of the H2'

1 proton at 3.95 ppm thereby unambiguously is a doublet of doublets of doublets. However, when we
2 consider the same signal in the other J-res spectrum without the ^{31}P π pulses, we immediately see it
3 is a doublet of doublets further split by a ^1H - ^{31}P coupling (Figure 4). Equally, the lineshape of the H2
4 proton is slightly different in both sub-J-spectra, which we assign to a small (0.5Hz) but non-vanishing
5 coupling constant between this proton and the ^{31}P nucleus (Figure S6). This is confirmed by a weak
6 cross peak in the PIP-HSQMBC spectrum for this same proton (Figure S6). Interrogating the Human
7 Metabolome Database²⁴ (hmdb), the Biomagresbank²³ (bmr) or the Birmingham Metabolite
8 Library³⁶ (Figure S7), we could assign the three lines to 3PG through matching of the coupling
9 patterns. However, the experimental conditions varying slightly between our extract and the
10 standard conditions used to record the database 3PG entry, chemical shift values are close but not
11 identical (Figure S7), and this will be a recurrent issue for database mining with spectra of phospho-
12 metabolites. Another potentially confounding factor is that the J-coupling values when expressed in
13 ppm become field dependent,⁵⁸ and can thereby distort the apparent pattern. The Birmingham
14 Metabolite library³⁶ or SpinCouple²⁵ avoid this issue by using tilted spectra, and assign thereby all
15 lines of a multiplet to a single chemical shift. However, as stated before, this comes at the cost of a
16 deteriorated lineshape,⁴⁹ which we avoid due to our projection procedure. The assignment of the
17 different phospho-metabolites in the extract is detailed in Figure S8, and gives rise to a fully assigned
18 1D spectrum (Figure S9).

19 FBP is an example where we cannot use the J-resolved subtraction spectrum as entry point,
20 for reasons of limited sensitivity (for a detailed analysis of this parameter, see Supplemental
21 Information) and of presence of its signals in an overly crowded region. However, its signals show up
22 in the PIP-HSQMBC spectrum, where they profit moreover from the spreading due to its particular
23 ^{31}P frequency. Importantly, even though the chemical shift values for FBP between our standard and
24 the cell extract do not coincide perfectly (Figure S8.2), the quadruplet structure of the C1 linked
25 protons (Figure 2) gives good confidence to the assignment, that we finally verified by spiking of FBP
26 in the cell extract. Taking into account chemical information available in the literature, such as GA3P
27 being predominantly present in its diol form⁵⁹ or the presence of both a keto- and gem-diol form for
28 DHAP,⁶⁰ we could assign with certainty 6 of the 9 phosphorylated glycolytic intermediates in our
29 spectrum. No distinctive signal suggesting the presence of the three other intermediates (F6P, 2PG,
30 FBP) could be observed in the quenched cell extract. Finally, returning with these assignments to our
31 quantitative 1D experiment, we estimated the phosphometabolite concentrations in this sample as
32 follows: [G6P] 237 μM ; [DHAP] 260 μM ; [3PG] : 789 μM ; [PEP] : 450 μM .

33

34 **Conclusions**

1 The workflow presented above demonstrates that detailed information on the phospho-metabolome
2 can be extracted from a complex sample by using the ^{31}P nucleus as a selectivity filter, overcoming
3 thereby the need to physically separate this particular class of metabolites. Its limitations are
4 obviously related to the low sensitivity of NMR spectroscopy when compared to mass spectrometry.
5 However, when we consider that the main aim of the workflow is to provide an accurate annotation
6 of the 1D spectrum, the extraction of the traces from the J-resolved spectrum via the procedure
7 described proved perfectly transposable to the 1D spectrum (Figure 3), thereby clarifying the sub-
8 spectrum of the phospho-metabolome. Quantitative measurements can be obtained from the
9 annotated 1D proton spectrum, where the issue of differential relaxation times can be solved by a
10 long recycling delay. Our results on the cellular extract further highlight a number of important
11 points when characterizing the phospho-metabolome. First, chemical shift values alone are not easy
12 to use for an automatic assignment procedure, as they are very sensitive to the exact sample
13 conditions. Secondly, coupling patterns are more reliable, and can be determined with excellent
14 resolution by the here described ^{31}P selective J-resolved difference sequence and/or the PIP-
15 HSQMBC sequence without decoupling. Further development of database query algorithms that give
16 access to the precise coupling patterns⁶¹ would therefore be of great use. Finally, our study shows
17 that NMR spectroscopy remains a valuable complementary technique to mass spectrometry in the
18 effort to characterize the phospho-metabolome.

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ASSOCIATED CONTENT

Supporting Information

Details of samples and NMR experiments. Analysis of sensitivity and quantitative character. Assigned spectra of phospho-metabolites. Additional information as indicated in the text. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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Notes

The authors declare no competing financial interest.

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1 **Figure Legends**

2

3 **Figure 1.** Schematic representation of the NMR workflow to detect phospho-metabolites.

4

5 **Figure 2a.** Pulse sequence for the Pell-Keeler ^1H , ^{31}P J-res experiment. The ^{31}P π pulses (in blue) are
6 applied in an alternating manner with full or zero power, thereby generating two separate spectra. **b.**
7 Superposition of 2D J-res spectra without (black) or with (blue) ^{31}P π pulses and hence without or
8 with ^1H - ^{31}P coupling evolution during t_1 . The spectrum was acquired on a 5mM fructose-1,6-
9 bisphosphate (FBP) standard in phosphate buffer pH 7.

10

11 **Figure 3.** Identification of G6P in a standard mix (10mM ATP DHAP G6P Threonine). **(a)** The J-res
12 difference spectrum (red) selects the signal of proton groups coupled to a ^{31}P nucleus. Extraction of
13 the 45° strip of the spectrum gives the individual 1D spectrum of the selected proton, shown here as
14 a projection. **(b)** This signal serves as a starting point for identification of the protons in the 2D ^1H , ^{31}P
15 PIP-HSQMBC correlation spectrum without (pink) or with (green) a TOCSY transfer during the INEPT
16 delay and hence full assignment of the phospho-metabolite signals. **(c)** Returning with this trace to
17 the J-res spectrum with ^{31}P J coupling evolution during t_1 gives access to a high-resolution spectrum
18 with the full coupling patterns and constants, thereby helping the identification of the molecule
19 (here β -D-glucose 6-phosphate). **(d)** Finally, this information can be readily transposed to the high
20 resolution 1D spectra. Full assignment and coupling constants of the different phospho-metabolites
21 obtained with this workflow are detailed in figure S5.

22

23 **Figure 4.** Assignment of 3-phosphoglyceric acid in an *E. Coli* extract. The workflow is identical as in
24 Figure 3.

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