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Phosphorus NMR and its application to metabolomics

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Phosphorus NMR and its application to metabolomics

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Introduction

Stable isotopes are routinely employed by NMR metabolomics to highlight specific metabolic processes and to monitor pathway flux. ^{13}C-carbon and ^{15}N-nitrogen labeled nutrients are convenient sources of isotope tracers and are commonly added as supplements to a variety of biological systems ranging from cell cultures to animal models. Unlike ^{13}C and ^{15}N, ^{31}P-phosphourous is a naturally abundant and NMR active isotope that doesn't require an external supplemental source. To date, ^{31}P NMR has seen limited usage in metabolomics because of a lack of reference spectra, difficulties in sample preparation, and an absence of two-dimensional (2D) NMR experiments. But, ^{31}P NMR has the potential of expanding the coverage of the metabolome by detecting phosphorous-containing metabolites. Phosphorylated metabolites regulate key cellular processes, serve as a surrogate for intracellular pH conditions, and provides a measure of a cell's metabolic energy and redox state, among other processes. Thus, incorporating ^{31}P NMR into a metabolomics investigation will enable the detection of these key cellular processes. To facilitate the application of ^{31}P NMR in metabolomics, we present a unified protocol that allows for the simultaneous and efficient detection of ^{1}H-, ^{13}C-, ^{15}N- and ^{31}P-labeled metabolites. The protocol includes the application of a 2D ^{1}H-^{31}P HSQC-TOCSY experiment to detect ^{31}P-labeled metabolites from heterogeneous biological mixtures, methods for sample preparation to detect ^{1}H-, ^{13}C-, ^{15}N- and ^{31}P-labeled metabolites from a single NMR sample, and a dataset of one-dimensional (1D) ^{31}P NMR and 2D ^{1}H-^{31}P HSQC-TOCSY spectra of 38 common phosphorus-containing metabolites to assist in metabolite assignments.

Methods

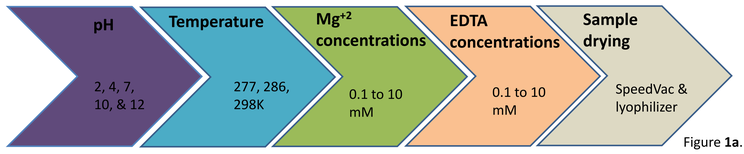

Figure **1a**.

- Selection of 38 phosphorus containing compounds
- Dissolve in 10% D_2O/90% H_2O solvent
- Obtain stock solutions
- Manually adjust pH with diluted NaCl and HCl
- Run NMR data collection of 1D ^{31}P spectrum and a 2D ^{1}H-^{31}P HSQC TOCSY spectrum with adjusted parameters in **Fig. 1a.**
- Data analysis of spectrum using TopSpin 3.6

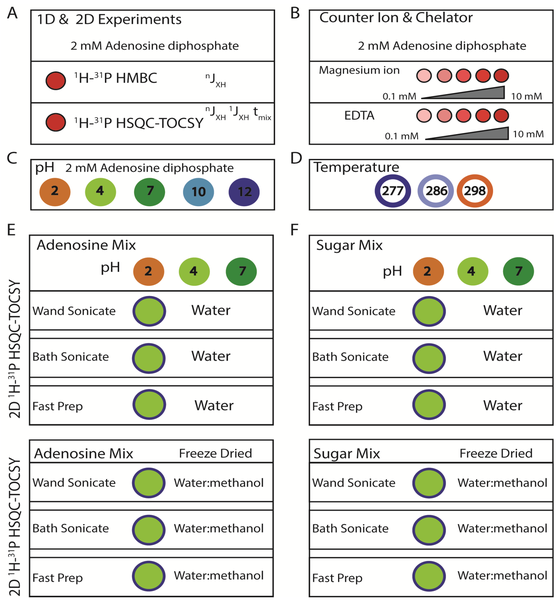

Figure 1b. Summary of the sample preparations, optimization matrix and standard samples used to develop a multi-SIRM (Stable Isotope Resolved Metabolomics) workflow incorporating ^{31}P NMR. (**A**) Identification of the preferred 2D ^{1}H-^{31}P NMR pulse sequence using a standard 2 mM ADP sample. The one-bond $^{1}J_{X\text{-}H}$ coupling constant (160 to 200 Hz), the long range $^{n}J_{X\text{-}H}$ coupling constant (5 to 10 Hz), and the TOCSY mixing time (70 to 120 msec) were optimized. (**B**) The impact of a Mg^{2+} counter ion or a chelator (EDTA) on the ADP ^{31}P NMR spectral quality was examined. Similarly, the impact of (**C**) pH and (**D**) temperature on ADP peak width and intensity in both 1D and 2D ^{31}P NMR spectra were evaluated. (**E & F**) The stability of phosphorylated-metabolites were investigated across a range of experimental conditions using a series of 2D ^{1}H-^{31}P HSQC-TOCSY experiment collected on two different metabolite mixtures (adenosine analogs and sugar phosphates). A range of pH values, extraction solvents, and cell lysing techniques were investigated.

Impact of pH, Temperature, and Counter ion on ^{31}P shifts

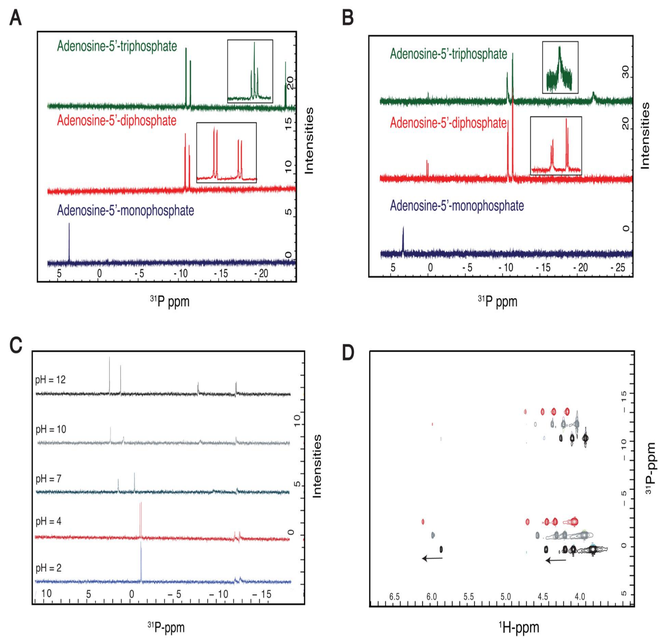

Figure 2. (**A**), (**B**) The chemical shifts in the 2D ^{1}H-^{31}P HSQC-TOCSY spectrum with the addition of Mg^{+2} in AMP, ADP, and ATP NMR samples. (**C**) 1D ^{31}P spectra for 90 mM solution of ADP in 90:10 water:D_2O solution at pH values of 2, 4, 7, 10, and 12. (**D**) Overlay of the 2D ^{1}H-^{31}P HSQC-TOCSY spectra for ADP at three different temperatures (277, 286 and 298K)

Impact of pH, Temperature, and Counter ion on ^{1}P shifts

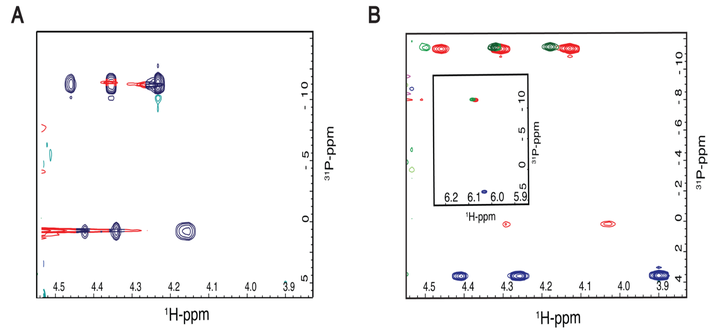

Figure 3. Representative examples of 2D ^{1}H-^{31}P NMR spectra. (**A**) Overlay of 2D ^{1}H-^{31}P HSQC-TOCSY (blue) and 2D ^{1}H-^{31}P HMBC (red) spectra acquired for a 1 mM solution of Adenosine Triphosphate (ATP) in 90:10 water: D_2O solution at pH 4. (**B**) Overlay of 2D ^{1}H-^{31}P HSQC-TOCSY spectra with an 80 msec TOCSY mixing time for 2 mM solution of Adenosine monophosphate (AMP) (blue), Adenosine diphosphate (ADP) (red) and ATP (green) in 90:10 water: D_2O solution at pH 4

^{1}H-^{31}P HSQC-TOCSY Identification of Sugar Phosphates

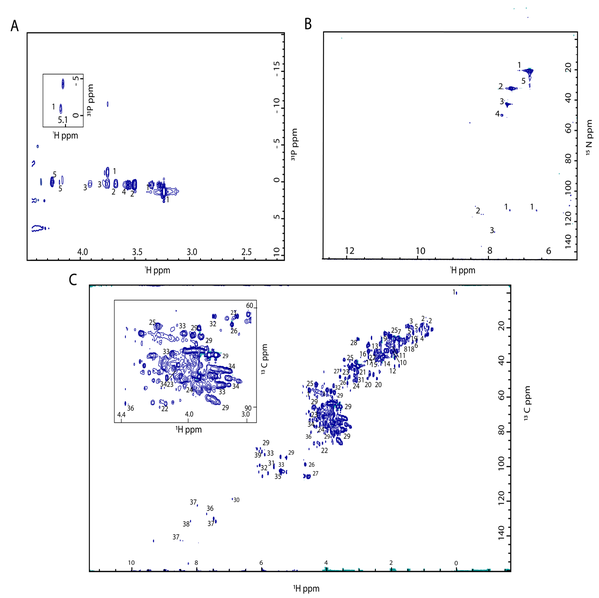

Figure 4. (**A**) 2D ^{1}H-^{31}P HSQC-TOCSY, (**B**) 2D ^{1}H-^{15}N HSQC and (**C**) 2D ^{1}H-^{13}C HSQC spectra collected from an *E. coli* cell lysate grown in M9 minimal media supplemented with $^{15}NH_3Cl$ and $^{13}C_6$ glucose. Cells were harvested during the log phase. The metabolite assignments correspond to 1: Glucose-1,6-biphoshphate, 2: Glucose-6-phosphate, 3: Ribose-5-phosphate, 4: AMP, and 5: Glycerol phosphate in (A) 1: Arginine, 2: Glutamine, 3: Asparagine, 4: 5-azacytidine, 5: Glutamate in (B) The metabolite assignments in (C) correspond to 1: Internal standard TMSP, 2: Valine, 3: Alanine, 4:N-acetyl alanine, 5: leucine, 6: beta-leucine, 7: lactate 8: 2-hydroxy-3-methyl butyrate, 9: acetyl carnithine 10: N-acetyl aspartate, 11: N-acetyl glucosamine, 12: ornithine, 13: aminobutyrate, 14:acetate , 15: arginine, 16: N-acetyl ornithine, 17: cystathionine, 18: aminohexanoic acid ,19: 2-hydroxy butyrate 20: malate, 21: pyruvate, 22:lysine, 23: glutamate, 24: glutamine , 25: N-acetyl glutamate, 26: succinate, 27: citrulline, 28: coenzyme A, 29: glucose ,30: fumarate, 31: proline , 32: isoleucine, 33: fructose, 34: glycerol, 35: phosphoenolpyruvate, 36: UDP-glucose, 37: AMP, 38: NAD 39: Glucose-6-phosphate.

Conclusion

- ^{31}P NMR offers a significant benefit to the metabolomics field by expanding the coverage of the metabolome, and by enabling the detection of metabolites integral to critical cellular processes, such as energy metabolism and cell signaling.
- A multi-SIRM protocol was successfully demonstrated that allows for the easy inclusion of ^{31}P-NMR into a metabolomics study.
- A database of reference 31P NMR spectra was generated to facilitate the automate assignments of 38 common phosphorus-containing metabolites.

References and Acknowledgments

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