Optimized Protocol and Evaluation of Referencing Methods in Quantitative 1H NMR Lipid Analysis

Santosh Kumar Bharti[1] and Zaver Bhujwalla[1]

[1]*Div. of Cancer Imaging Research, The Russell H. Morgan Dept. of Radiolog and Radiological science, Johns Hopkins University, School of Medicine, Baltimore, Maryland, United States*

Introduction: Lipid metabolism plays several important roles in multiple cancer processes including invasion, metastasis and proliferation, as well as in normal and pathological conditions [1]. NMR spectroscopy offers rapid and accurate quantitative analysis of lipids and phospholipids isolated from tissue, cells, and plasma. However, quantitative analysis of lipids is a tedious process and its accuracy is largely affected by multistep solvent extraction, sample preparation, and the referencing techniques used. Here we have presented an optimized protocol for sample preparation and referencing techniques for quantitative ^{1}H NMR lipid analysis.

Methods: A concentrated stock solution of lipid was prepared in deuterated chloroform: methanol (2:1 ratio) for evaluation of the sample preparation step and referencing techniques for quantitative analysis. All the lipids were extracted using Folch's lipid extraction methods [2]. All NMR experiments were performed on a Bruker Avance spectrometer operating at 500.13 MHz for ^{1}H and equipped with a 5 mm broadband inverse probe incorporating z-axis gradient coils. Single-pulse 1H NMR spectra were recorded at room temperature with time domain 64K, spectral width of 15 ppm, repetition time of 15.0 sec, 4.36 sec acquisition time, 32 scans and 8 dummy scans, 90° hard pulse. TMS (tetramethylsilane) as an internal standard, and TSP (rimethylsilane propionic acid sodium salt) as a stem co-axial reference capillary and QUANTAS (QUANTification by Artificial Signal) [3] were evaluated for quantitative lipid analysis in chloroform and methanol mixture.

Results and Discussion: TMS is a commonly used internal reference standard in chemical sciences but its application is limited due to its high volatility. Quantitative NMR (qNMR) analysis of lipid samples in a chloroform and methanol mixture using TMS as internal standard can result in erroneous values due the highly volatile nature of TMS. A stem co-axial insert is mainly used to avoid sample and reference contamination and achieve better precision and reproducibility in qNMR analysis [4]. However, using a stem co-axial insert results in reduction of sensitivity of up to 40%, depending upon the diameter of the stem co-axial insert in the RF region. QUANTAS is a pre-calibrated artificial signal with adjusted intensity, used for quantification during spectral processing. QUANTAS referencing requires acquisition of the entire sample set under identical experimental conditions including receiver gain, relaxation delay etc. QUANTAS and a co-axial insert containing TSP in D_2O have shown comparable precision and accuracy in lipid quantitation (**Figure 1**). Furthermore, a comparison between TMS and QUANTAS shows, that due to the volatile nature of TMS, significant quantitative errors get incorporated in the data (**Figure 2**). Taking some precaution during sample preparation significantly reduces the volatility of TMS and improves the quantitative accuracy and precision. These steps include, using a small and narrow diameter test tube for dissolving the sample, using freshly prepared solvent and reference mixture, keeping solvent mixture in cold ice conditions to slow down the evaporation, preparing samples one by one, and acquiring the NMR data as soon as possible. To see the effect of sample storage at qNMR analysis, NMR acquisition was carried out on a set of freshly prepared samples and on the same samples after 6 weeks of storage at 4°C (**Figure 3**). After 6 weeks of storage, in some of the samples TMS evaporated while the lipid quantity remained same. Such sample cannot be further re-used for qNMR analysis. Change in the CDCl3: MeOD ratio was also observed during sample storage that significantly affects the spectral reproducibility **(Figure 4).** In conclusion, optimizing the sample preparation step reduces the quantitative error due to evaporation of TMS and solvent. QUANTAS and stem co-axial insert containing TSP can be alternatively used for better quantitative accuracy and precision. Working with co-axial insert references may create a little difficulty in shimming the sample. Since metabolomics requires the acquisition of a large number of samples, in such conditions QUANTAS can be a good reference for accurate analysis and scaling the raw data for statistical analysis.

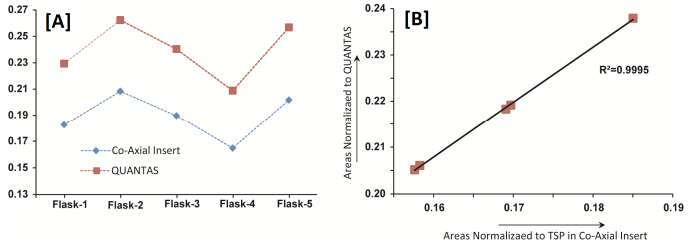

Figure 1: Comparing QUANTAS and stem co-axial insert containing TSP in D_2O, [A] Five cell lipid extract samples with variable concentration were taken and divided in two equal parts. The first set of sample were recorded with the co-axial insert and second set with QUANTAS referencing. QUANTAS qNMR analysis shows excellent correlation with the co-axial insert and a higher S/N ratio. [B] Correlation coefficient obtained from linear regression between QUANTAS and co-axial insert qNMR, shows that both the referencing techniques have very good quantitative similarity.

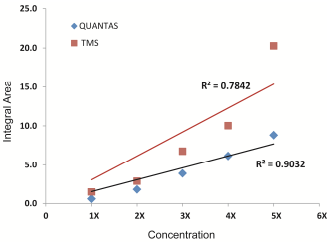

Figure 2: Linearity comparison of QUANTAS and TMS referencing methods. X-axis represents lipid concentration in fold, prepared from stock solution. R^2 value shows that QUANTAS has better quantitative linearity when compared with TMS.

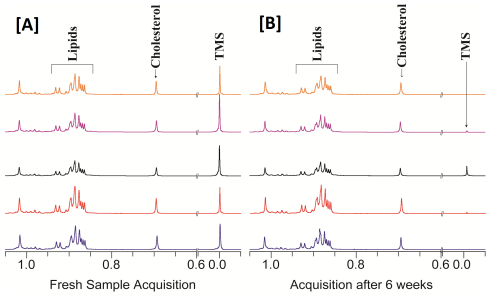

Figure 3: Lipid extract spectra obtained from cells recorded immediately after sample preparation and after 6 week storage at 4°C temperature. TMS from the sample evaporated while lipids remained the same.

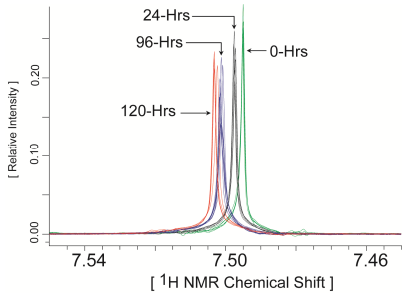

Figure 4: Freshly prepared lipid samples (n=5) recorded at 0h (Green), 24h (black), 96h (blue) and 120h (red). A drift in the NMR signal was observed because of solvent composition changes due to evaporation.

References:

[1]: Fernandis *et al., J. of Chrom.* 2009, 877 (26), 2830-2835; [2]: Folch *et al., J. Bio. Chem.* **1957,** *226* (1), 497-509; [3]: Farrant *et al., Mag. Res. Chem.* 2010, 48 (10), 753-762; [4]: Bharti *et al., TrAc,* ***2012,*** *35* (0), 5-26.

Acknowledgements: This work was supported by NIH R01CA136576 and P50 CA103175.