CHAPTER: 06

# HRMAS (HIGH RESOLUTION MAGIC ANGLE SPINNING) NMR (NUCLEAR MAGNETIC RESONANCE) SPECTROSCOPY AND EFFECT OF PHYSICAL VARIABLES

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HR-MAS (High Resolution Magic Angle spinning) NMR (Nuclear Magnetic Resonance) is a technology which combines the advantages of both solid- and liquid-state NMR by allowing to concomitantly measure intact and non-manipulated samples. Based on both 1D and 2D homo- and heteronuclear NMR spectra, HRMAS evaluates the composition of fresh semi-solid samples with a similar resolution as that of classical liquid-state NMR techniques. The enhanced spectral quality still obtained for semi-solid samples is mainly due to the MAS system, whose rapid spinning and sample orientation minimize the anisotropic processes that prevent the acquisition of meaningful NMR spectra for non-liquid materials. The HRMAS NMR has also showed promising results in identifying biomarkers in different diseases. Although few physical variables may effect the spectral quality and data. Therefore, in this chapter we will discuss those factors for efficient data acquisition.

**Storage:** Tissues are rich source of enzymes due to the presence of different metabolic compartments. These enzymes cause perturbations in biochemical composition of tissues even after their resection, therefore, minimization of enzymatic processes after tissue resection is of paramount importance for removing any pre-analytical bias. The optimum temperature for enzymatic activity ranges from 40-45$^{o}$C and therefore the storage should be at lower temperatures. The room temperature storage should be avoided as the enzymatic reactions keep occurring with slower rate of reaction. The best way of storing a resected tissue is snap-freezing under liquid nitrogen environment as it solves both the purposes of minimizing enzymatic activity and biochemical degradation (Beckonert, Coen et al. 2010; Srivastava, Roy et al. 2011). The long term effect of storage at -80$^{o}$C on prostate cancer tissue biopsies were studied for 32 months and it was found that frozen storage induced metabolite perturbations were insignificant statistically and are less critical than the influence of pathological heterogeneities present in a tissue (Jordan, He et al. 2007).

The storage of prostate tissue biopsies in glycine buffer solution was evaluated by Bourne et al. for estimation of leakage of metabolites of tissue in buffer solution after its storage and freeze thaw cycle. The biopsy was suggested to be removed from buffer while MR measurements as the leakage of metabolites result in 55 to 98% loss of metabolites of tissues in buffer solution (Bourne, Dzendrowskyj et al. 2003). Therefore, the biopsies should be snap-frozen in its native state in an eppendorf tube with no buffer solution. This will assure only the morphological changes in cellular structures of tissue and no leakage of metabolites from the subject of interest.

**Freeze thaw cycles:** The freeze thaw processes have a strong role in increased concentration of various metabolites. Middleton et al. had performed a study to assess

the possible role of freeze thawing processes on metabolic composition of tissues. The authors had acquired a proton HR MAS NMR spectrum from freshly resected samples obtained from renal cortex of rats and then to observe the effect of freezing and thawing processes, the same sample was frosted in liquid nitrogen for several minutes and then thawed to room temperature, followed by another acquisition. Lactate, alanine and N-methyl functionalities became quite prominent in spectrum of once freeze-thawed tissue. Water soluble metabolites like alanine, glycine, valine, leucine, isoleucine, aspartate and lysine were found to be increased after freeze-thaw cycle while decrease in resonance intensities of lipids and fatty acids occurred. The similar trend in increase in metabolic concentration was found in medullary tissues as well but it was as pronounced as in cortical tissues. The authors have suggested that the freeze-thawing of tissues cause some physical disruptions in cellular compartmentalization and protein degradation which results in reducing the number of binding sites of small molecules and the consequent release from their binding sites which were otherwise invisible to NMR due to the macromolecular bonding. Also, the extent of freeze-thawing differs from tissue to tissue (Middleton, Bradley et al. 1998; Opstad, Bell et al. 2008). The study was further evaluated by Wu et al. for the effect of freeze-thaw cycle and concluded that sample freezing processes hamper with the physical state of tissue cellular metabolites and environment of tissue water. Freezing causes disruption in bonding among cellular metabolites with macromolecules and water resulting in change in different viscosities of metabolites and tissue water (Waters, Garrod et al. 2000; Wu, Taylor et al. 2003). Consequently, relaxation times and diffusion coefficients vary with freezing. The freeze thaw process does not affect the resonance line-widths of metabolites but it does cause the structural disruption in tissues. The alterations in metabolites are less than 50% after one freeze thaw cycle as compared to the fresh biopsies (Wu, Taylor et al. 2003). Waters et al. had proposed that snap-frozen tissue spectra are highly reproducible despite of minor metabolic perturbations (Waters, Garrod et al. 2000).

**Sample Preparation:** The minimum weight of tissue was proposed to be 0.1μmol $g^{-1}$ wet weight for efficient spectral resolution with high signal-to-noise ratio. The tissue samples should be prepared on ice for minimising the enzyme induced metabolism in tissues. Samples should be prepared at the same temperature and the time interval of sample preparation and its HRMAS NMR spectroscopic acquisition should be kept constant. The constant time interval assures the minimal data variability occurring in highly metabolically active tissues due to different degradation mechanisms. The samples should be carefully washed with normal saline to remove the paramagnetic blood impurities from the tissue, which may hinder the spectral analysis by reducing the spin-lattice relaxation times and induces the line broadening. While sample preparation,

D2O with 0.9% NaCl should be introduced in the rotor: 0.9% NaCl, for maintaining the near physiological conditions (due to saline) and D2O for deuterium field frequency lock during experiment (Beckonert, Coen et al. 2010). The biochemical variations including a prominent increase in glycerophosphorylcholine resonance due to temporal lipid degradation and a deviation of 1:1 alanine doublet to a singlet due to α-CH deuteration were observed in renal cortex samples of rats which were prepared and kept on ice for 4hr (Waters, Garrod et al. 2000).

The rotor should be packed appropriately for proper spinning otherwise it may get damaged itself and, in some cases, may damage the coil as well. The use of spherical rotor-inserts in sample preparation requires sample volume as low as 12µl (usually 65µl in absence of insert) and provides narrower line-widths and consequently improved spectral dispersion and peak heights with well resolved spin-coupling multiplicities. These spherical inserts simply introduce more homogeneity in the system by symmetrically distributing the tissue sample within the rotor and thus better spatial biochemical information (Waters, Garrod et al. 2000).