# Human Saliva 1H-NMR Study Characterizing Macromolecule/Metabolites Interaction

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**Abstract**

High-resolution $^1$H NMR spectra of fresh and intact saliva samples were measured. The $^1$H NMR spectral intensities of small molecules increased with time under the presence of macromolecules. However, the change in signal intensities remained within a reasonable range for 4 hours after sampling. The pH titration revealed that the Hill coefficients were less than 0.5, which suggested that the observed small molecules make a rapid chemical exchange between bound and free states in solution. The presence of dynamic magnetic transfer from small molecules to macromolecules was verified observing the double-quantum filtered spectra. The simultaneous observation of single and double quantum spectra were also explored.

**Introduction** Clinical application of NMR metabonomics has been explored using urine or serum samples. In this study, fresh saliva was investigated as an alternative to the excretory biofluid like urine, or the functional biofluid like serum. High resolution NMR spectroscopy has advantages such as: (1) It provides simultaneous multi-component information of biofluids; (2) It has a high degree of spectral dispersion and has a reasonable sensitivity: (3) Single molecule has often more than one resonance which have connected patterns and intensities; and (4) Molecules which would not necessarily be anticipated to be present in biological samples could be identified. In particular, interaction between macro-molecules and metabolites can be characterized by inter-molecular multi quantum coherence phenomena. In our knowledge, salivary NMR has not been so extensively studied as a biofluid with a few exception(1,2). Clinical saliva metabonomics will be utilized for (1) prognosis of dental disease, such as pyorrhea, (2) aid of disease management influenced by lifestyle, (3) predicting blood drug concentration and pharmacokinetic profiles.

**Materials & Methods** Fresh saliva was collected into a Sallivet™ (ca. 2 mL), and 0.48mL was transferred to a standard 5mm NMR tube with 0.02mL deuterium oxide. The use of human materials conformed to an informed consent protocol that was approved by the Research Ethics Committee of the Japan Women's University.

$^1$H-NMR spectra were acquired on a Bruker AMX-400WB spectrometer operating at 400.13MHz and at 290K. The solvent signal was suppressed using a 3s homo-gated pulse and collected single-quantum (SQ) and double-quantum filtered (DQF) spectra after the accumulation of 64 scans. The data were analyzed using homemade software written by MATLAB™ on a remote workstation.

**Results** The salivary sample exhibited the spectral intensity of small molecules successively increased over a couple of days. Their rates were larger at 310K than at 290K. The increase rate was within a reasonable range till 4hrs after sampling, followed by conspicuous spectral enhancement. The intensity remained constant on removing the macromolecules by ultra-centrifuge. The results suggest that (1) the proteins in saliva denaturate irreversibly after excretion from the salivary glands, and (2) the proteins bind small molecules and release when denatured.

Chemical shifts of organic acids in saliva did not make an appreciable change above pH 6.5. However, titration curve below pH 6.5 was found anomalous. For example, methylene protons of iso-butylate showed a Hill coefficient of 0.4, while proton of formate was 0.2. The result suggested that some molecules make the rapid chemical exchange to certain protein(s).

Double quantum spectrum was taken to verify that the small molecules gave rise to the dynamic magnetic exchange to the protons of macromolecules. The result showed that (1) the signals of macromolecules were not observable within the signal-to-noise ratios observed, (2) most peaks in a single quantum spectrum were un-observable, and (3) the acetate methyl signal was the most intensely observed and the resonance of formate was weakly observed. They should not be seen if the molecules are free in the solution. Thus, it is concluded that some small molecules binds to macromolecules in saliva, and that the observed intensities are attenuated variously according to the amount of binding.

**Discussions** The difficulty to infer the metabolite concentrations in blood from that in saliva was uncertain so far. However, the present result suggests that the physiological interaction of macromolecules and metabolites in fresh saliva sample can be characterized by certain NMR techniques and data processing. NMR spectra of intact sample suggest that ultra-centrifuge to get rid of proteins from the system would give an equivocal result to the estimation of small molecules. Sample freezing would give rise to the precipitation of some proteins, the effect of which is awaited for further study.

We have tried to measure the DQF spectrum immediately after the SQ spectrum to get an insight into the dynamic behavior of small molecules in saliva. The result indicated the presence of a rapid chemical exchange between free and bound states. The signal intensity ratios between SQ and DQF resonance could give a measure of bound species in the rapid exchange system.

In order to improve the S/N ratios under a limited time, the simultaneous measurement of SQ and DQF spectrum was explored. The method was to acquire the spectrum of each phase combination of DQF and made another combination to extract the SQ spectrum. It was confirmed that the scheme works although a further refinement was necessary against the imperfection of instrument. We also intend to address inter-intra individual variability of saliva NMR measures using certain statistical approach.

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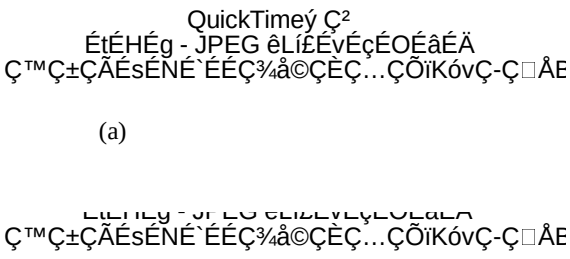


Fig. (a) A typical single quantum $^1$H-NMR spectrum of intact human saliva at 400MHz. Water signal was removed by software in addition to homo-gated spectrum.
(b) Double quantum filtered spec-trum of same sample, displayed in magnitude mode. Strong singlet at 2ppm is from acetate methyl protons. Note that it should not be seen if the molecule were free in