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Preliminary Results of Human Saliva ^{1}H-NMR

Seizo Takahashi[1], Keiko Imura[1], Kozue Shiraishi[1] and Yukiharu Yamaguchi[2]

[1]Department of Chemical and Biological Sciences, Japan Women's University,
1-1-2 Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan

[2]Division of Clinical Technology, Pfizer Global R & D, Pfizer Japan Inc.
Shinjuku-bunka Quint Bld., 3-22-7 Shibuya, Shibuya-ku, Tokyo 151-8589, Japan

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Abstract: High-resolution ^{1}H NMR spectroscopy of fresh and intact saliva was measured aimed at elucidating the intra/inter variance of healthy young females. Saliva was used as a model system of "drug response" to internal hormones as exemplified by the menstrual cycle. 30 volunteers aged 20 to 24 were recruited and divided into two groups who showed high- or low-basal body temperature at the first stage. Saliva of both temperature stages was collected for the same subject 5 times a day. The ^{1}H NMR spectral pattern exhibited quite a large variance for each sample. The signal intensities were slowly increased but remained within a reasonable range for 4 hours after sampling, followed by a conspicuous signal enhancement for more than 2 days. The intensities did not change if the macromolecules were removed by ultra-centrifuge. The result suggested that the macromolecules in saliva underwent the irreversible denaturation, releasing the bound small molecules. Thus the signal intensities may vary depending on the delay time after sampling when the samples were centrifuged. The pH titration revealed that apparent pKa of some acids shifts more than 0.5 along with varied Hill coefficient, which suggested that the observed small molecules make a rapid chemical exchange between bound and free states in solution. The presence of dynamic magnetic exchange was verified observing the double-quantum filtered spectra. The simultaneous observation of single and double quantum spectra was explored.

Introduction

This research was started at the year 2003 as a joint project of Japan Women's University with Pfizer Inc. and Mitsubishi Space Software Inc. to look for a novel quantification approach to drug research and development. The present paper is the first preliminary report of a part of ^{1}H-NMR spectroscopy. It is well known that most compounds, which looked hopeful at first, end in failure to clinical tests. It is mostly due to the variability of drug response between subjects, which is quite often too large to assess a validity of target drug on a limited number of human subjects.

A purpose of this research was to get an insight into the intra- and inter-subject variability of physiological response to internal hormones as a model of drug. A large number of saliva's were collected of healthy young females for a full menstrual cycle. They were divided into two groups to randomize the subjects as those of high or low basal body temperatures at the first stage. Their saliva's of every morning were frozen immediately after sampling and collected once a week to stock in a laboratory deep-freezer. The subjects were also requested to come over the lab office at both temperature stages to study the daily changes in fresh saliva. Their specimens were collected 4 times a day in the lab in addition to the morning saliva collected at home. The specimens were immediately sampled for ^{1}H-NMR spectroscopy.

The organic acid concentration of human saliva has mostly been analyzed by means of chromatography[1-7]. However, these methods involve the laborious process for sampling, and require the prior-knowledge concerning to the presence of particular bio-molecules in the sample. Consequently, such analytical methods generally offer only a partial characterization of the metabolic status of biofluids.

High resolution NMR spectroscopy has a number of

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advantages over the alternative analytical methods such as: (1) It provides simultaneous multi-component information of biofluids and tissue extracts; (2) It has a high degree of spectral dispersion with reasonable sensitivity: (3) It has components containing more than 1 class of ^{1}H nuclei that give 2 or more connected signal patterns and intensities; (4) It involves dynamic information of molecules such as a chemical exchange; and (4) Molecules which would not necessarily be anticipated to be present in biological samples could be identified. As far as we know, saliva has been largely overlooked as a biofluid for NMR spectroscopic analysis. The reported papers are mostly limited to the spectral assignments, and intensity changes of limited cases[8-12]. To understand the large variation of NMR spectra between samples, the present preliminary report is focused to the characterization of the salivary ^{1}H-NMR as a first step.

Materials & Methods

Sample collection and preparation

The basal body temperature change is a well-established biomarker of the menstrual cycle. Among 100 volunteers, 30 students were screened as the healthy women of aged 20-24(average 21.5+/-1.2) who showed clear and stable cycle on this biomarker as well as the acceptance/compliance of controlling life style for this study. None of these subjects had any active dental caries and carry any chronic complains or disorders. To void any interferences arising from the introduction of exogenous agents into the oral environment, the subjects were asked to collect all saliva available immersing into the cotton roll of Sallivet™ (ca. 2 m*l*), and put the cotton into the plastic tube without touching hand immediately after waking in the morning, each day for 30 days. Each subject was also requested to refrain from oral activities (i.e. eating, drinking, tooth-brushing, oral rinsing, smoking, etc) during the short period between awakening and sample collection (<5 min). Specimens were frozen in a home refrigerator, and transported to the laboratory once a week in a cooling bag. They were stored in a laboratory deep freezer for future measurements.

The subjects were also requested to come over the lab office to collect the fresh saliva and voices on the day after 7+/-3 days after menstruation and estimated ovulation. The visit days were estimated from the recorded body basal temperatures via Madonna™. Specimens and voices were collected in the laboratory 4 times a day at about 10 : 30, 12 : 00, 13 : 00 and 15 : 00 in addition to the morning saliva. They were immediately sampled for NMR measurements.

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.

Measurements of Basal Body Temperature Change

The basal body temperature was measured using a "My Sophia II™" made by Nishitomo Co. Ltd. It is capable to store the body temperature together with the measured date to show on a graph, and also to transfer to a personal computer (PC) via a USB 2.0 connector equipped with a dedicated attachment with the aid of a program Madonna™.

The way to measure a basal body temperature was followed by an attached flyer. Namely, get up constantly in the morning at a fixed hour, and watch on a "My Sophia II™" to put a temperature probe under the tang for 5 min keeping still on a bed.

The 100 volunteers were requested to come over a lab office once a week to watch the history of basal body temperature as a pre-screening. The temperature was chased for 4 weeks to cover a one full menstrual cycle. The screened 30 volunteers were requested to continue for another 4 weeks, till the end of the test. The latter data were transferred and stored in a PC, while the previous data were erased when the pre-screening was finished.

pH and Bleeding Measurements

pH titrations were performed at room temperature (ca. 27°C) on a Horiba F-52 pH meter equipped with a long-thin combination pH electrode (3 × 200mm) (Sanwai Kagaku Inc.). The electrode was designed by one of the authors to improve the response and stability. For other experiments, pH measurements were performed on the pH test papers (Spezialindikator, pH6.5-10.0 and pH4.0-7.0, Merch). Bleeding was checked on a commercial urine test paper Hema-Combistix™ (Bayer Medical Ltd.).

NMR Measurements and analysis

NMR spectra were acquired on a Bruker AMX-400WB spectrometer operating at a frequency of 400.13 MHz with a probe temperature at 290K. Saliva was

taken 0.48 m*l* and added 0.02 m*l* deuterium oxide in a standard 5mm NMR sample tube for a field frequency lock. Homo-gated pulse was applied 3 second prior to the observation pulse to suppress the solvent signal and accumulated 64 times the FIDs of 16K data points. Receiver gain was set 64 to save enough margin against overload. Total run time was 5′45″ for single-quantum (SQ) and 6′30″ for double-quantum filtered (DQF) spectrum, respectively. Collected data were transferred to a remote workstation or personal computer, and analyzed using a home made software written by MATLAB™.

Results

Intensity Change after Sampling (Fig. 1)

The intact salivary sample (added phosphate buffer) showed the apparent spectral intensity of small molecules successively increased over a couple of days. Increasing rate was larger at 310K than at 290K. It was also found that the intensity remained fairly constant when measured within 5 hrs after sampling, followed by a conspicuous increase. After measurement, white precipitation was formed in the sample, while intact fresh sample looked rather cloudy. When specimen was ultra-centrifuged to remove the macromolecules, the spectral intensity remained constant. The results suggested that (1) the proteins in saliva undergoes the irreversible denaturation after excretion from the salivary glands, and (2) proteins binds small molecules and release when denatured.

It was not fully tested yet the effect of Salivet™ as an equipment to collect saliva. It absorbs the specimen to a cotton roll, followed by a gentle filtration by centrifuge. Lately we found that saliva without phosphate buffer exhibits fairly constant spectral intensity throughout 3 days. If this is true, the spectral increase found above could be due to the interaction of phosphate and macromolecules in saliva. It obviously needs further studies to know what happens when saliva was excreted from the salivary glands.

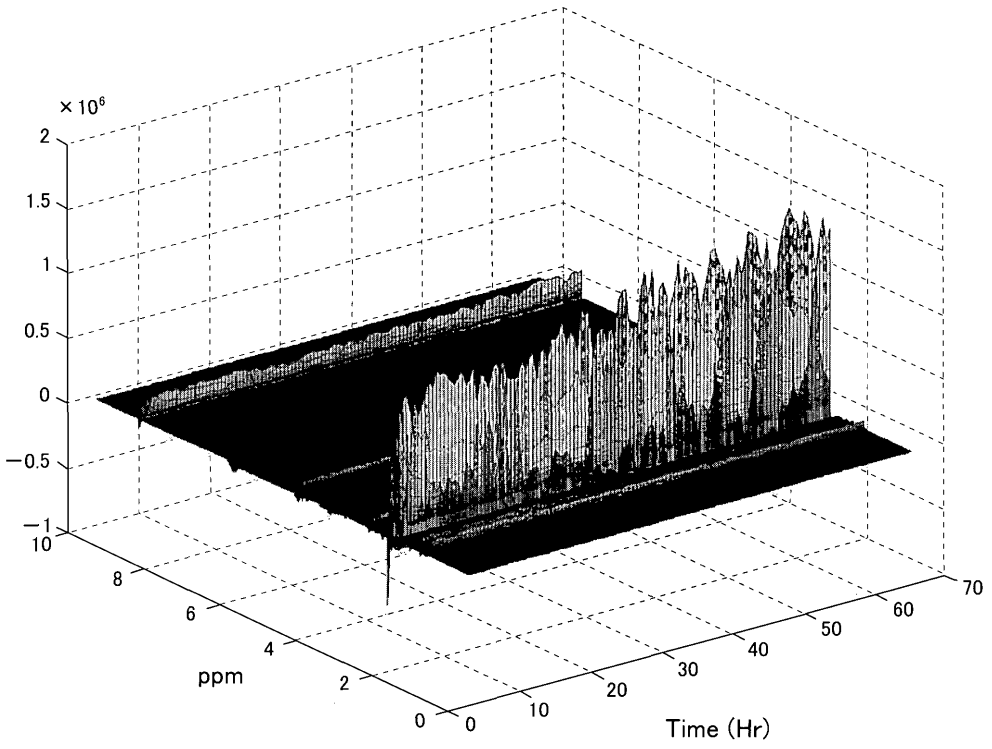

Fig. 1 Time Dependent Spectral Change of Intact Saliva

Fresh intact human saliva was chased the time dependency of ^{1}H-NMR spectrum at 310K. The saliva was sampled using Salivet™. X-, Y-, and Z-axes represent frequency, time and intensity, respectively. The residual solvent water signal was suppressed by software for the spectra applied homo-gated solvent suppression pulse. The highest peak is derived from the acetate methyl protons, and small peak at lowest field is from the formate proton. Note that relatively slow but steady increase in intensity over the whole range of 3 days. The intensity increase may be either due to the degradation or release from macromolecules.

pH Titration (Fig. 2)

Change in chemical shifts was monitored against pH to see if the spectral pattern depends on the variation of $NaHCO_3$ concentration in the specimen. It was found that the apparent pKa's were 5.26+/-0.06 for formate proton and 4.39+/-0.07 for acetate methyl protons. The observed pKa's are shifted 1.51 for formate and -0.38 for acetate from the reported ones. Note that the pKa shifts are of different direction which suggests that protonated forms are either stabilized or destabilized in the macromolecules. The Hill's coefficients were 0.92+/-0.09 and 1.43+/-0.19, respectively. The results suggest that (1) these acids are bound or adsorped to macromolecules for a short time, (2) they make the rapid chemical exchanges between free and bound states, and (3) formate and acetate are

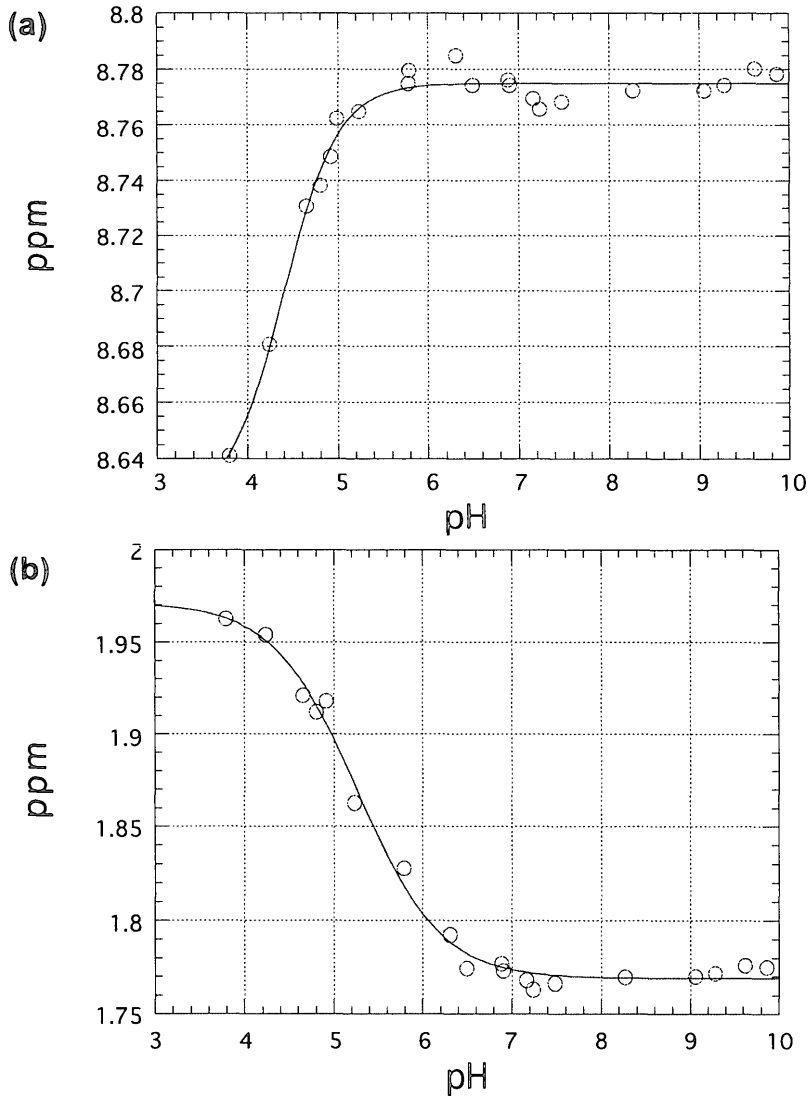

Fig. 2 pH Titration Curve of Intact Saliva

pH and chemical shifts are plotted together with a fitted curve. pH was read using a Horiba F-52 pH meter equipped with a long thin combination pH electrode (3 mm×20cm, Sanwai Kagaku Inc.). Chemical shift was read the point at peak top referencing to the solvent peak as 4.86 ppm. Curve fitting was used a non-linear fitting routine of Kaleida Graph™ with a Hill coefficient as a parameter. Fig. 2 (a) shows that apparent pKa is 5.26+/-0.06 for formate proton with Hill's coefficient 0.92+/-0.09, and (b) shows that the apparent pKa is 4.40+/-0.07 for the acetate methyl protons with Hill's coefficient 1.43+/-0.19. The reported pKa's for free solution are 3.75 for formate and 4.77 for acetate, respectively. This discrepancy suggests that these acids are perturbed their acid-base equilibrium on binding to macromolecules

bound to different environment in macromolecule (s). It was noted that the resonance of these molecules did not make much broadening.

DQF spectrum (Fig. 3)

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

Simultaneous Observation of SQ and DQF Spectrum

A pulse of DQF spectrum is composed of 90°-180°-90° pulse trains added by 90° coherence selection pulse. The first pulse combination is known as a driven-echo

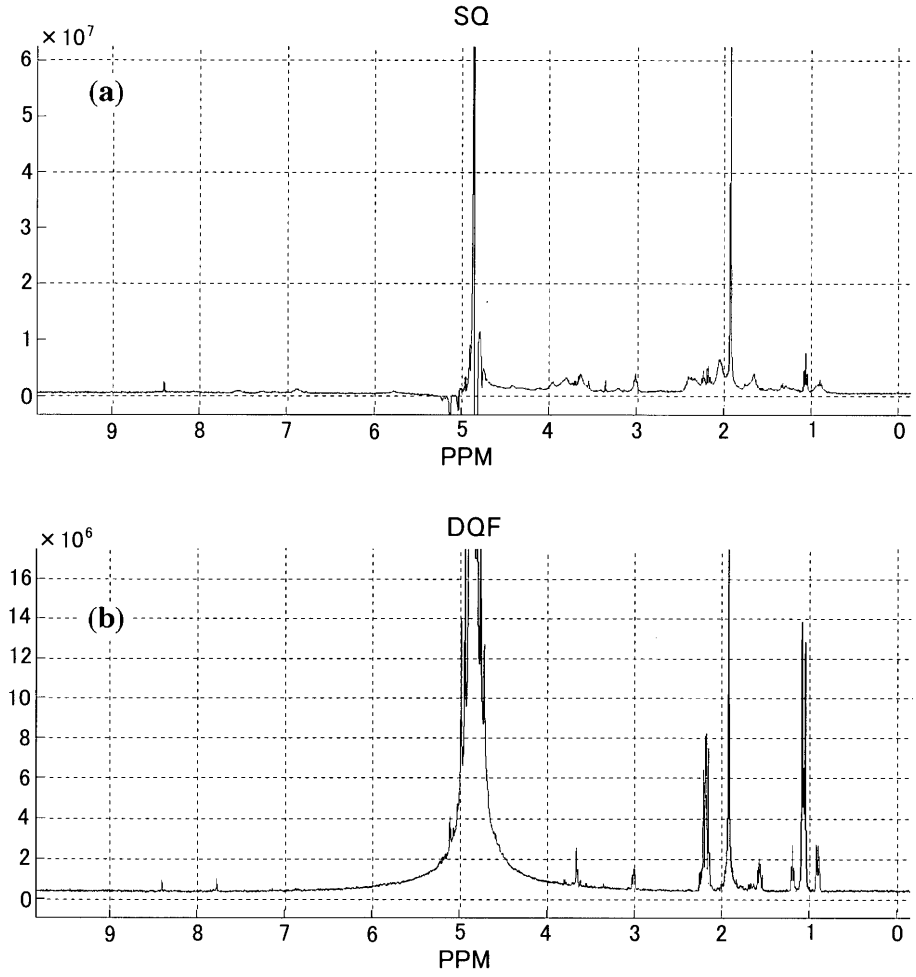

Fig. 3 SQ and DQF ^{1}H-NMR Spectrum

(a) SQ (single quantum) ^{1}H-NMR spectrum of intact saliva was taken at 400MHz with the aid of 3s homo-gated saturation pulse. An NMR micro-tube of symmetric geometry was used with 200 μl sample volume doped with 10% D_2O. Note that acetate methyl protons are most intense and formate resonance appears at the lowest field.

(b) DQF (double quantum filtered) spectrum was taken immediately after. Note that the two singlets, which originate from acetate and formate, were obviously observable. Another singlet close to formate resonance is most likely from histidine C-2 proton. It was not seen in SQ spectrum because of a relatively strong broad resonance. Note also that many resonance observed Fig. 3 (a) were not seen in (b).

pulse sequence which is used for the selective suppression of sharp resonance. DQ coherence is selected taking a certain phase combination out of single-quantum (SQ) coherence. Thus another phase combination should result in a SQ spectrum. To take this alternative phase combination from a set of data, the FID of each phase combination has to be saved independently. Testing several pulse programs suggested that the best strategy is to fix the receiver phase, minimize the hardware switching, and make an auxiliary software correction. It was noted that the phase and timing instability of the instrument is far from being disregarded and hard to control.

Discussions

Drug concentration in blood provides essential information to predict drug effects in individuals. Although there could be some level of correlation between drug concentration in plasma and saliva as a population, it will be difficult to predict individual plasma concentration from saliva sample. Several factors could contribute to increasing these individual variability, *e.g.* concentration of sampled saliva itself, bleeding, protein interactions secreted in saliva, metabolic condition, *etc.* NMR could measure entire profile of metabolites without separating its constituents (metabonomics). Since we expected rather large inter/intra individual variability of saliva itself, we plaanned to analyze relatively large number of subjects having similar demographic background, repeatedly. At the same time, basal body temperature was measured as an established marker of physiological changes during menstrual cycle of young female.

The present result suggests that macromolecules in saliva interact to small molecules making rapid chemical exchange between bound and free states. Small molecules are released after excretion from the salivary glands under the denaturating process of macromolecules. Thus the observed concentration may depend on the time when the macromolecules are removed after sampling. This phrnomena may also happen in blood sample. However, blood plasma contains much higher drug concentration than in saliva, resulting in a relatively small variation due to sampling.

In order to get a reproducible result by NMR spectroscopy, the present results suggest that the sample should be measured within 4 to 5 hrs after sampling if the macromolecules were not removed. However, centrifuge would not give a solution to estimate the accurate concentration of small molecules in saliva, since a fair amount of them exists in a bound state to macromolecules. Similar problem resides in sampling using Salivet™. It may remove some part of macromolecules on a cotton roll. In fact, it was found that the intensity change with time was diminished comparing to the direct sampling in an NMR tube and that many sharp resonance from small molecules appeared more clearly. The effect of sample freezing may cause further problem, since it is expected to give rise to the precipitation of some proteins selectively.

It is desirable to measure the dynamic behavior together to get an insight into the salivary NMR. We have tried to measure the DQF spectrum immediately after the SQ spectrum. Because of the limitation of spectrometer time available for many samples, the total time was set within 20min for each sample including the preparation time. As a result, the number of scan was set 64 for the study of intra-/inter-variance of subjects. Although the S/N ratios of DQF spectra were not so good, it was enough to calculate signal intensity ratios of some resonance between SQ and DQF spectrum. The data 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 observations of SQ and DQF spectrum were 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 mostly for the present purpose although the further refinement was necessary to cure the imperfection of the instrument.

The partial least squares and statistical analyses of the accumulated data are underway. By inspection, it was obvious that the conventional approach does not work which compares the intensities of small molecules with some parameters such as basal body temperature, before and after meal, and daily change. We believe that we have to make a challenging approach in order to draw some meaningful correlation which will be appeared on a second report. In short, we are going to try a kind of multivariate analysis, *i.e.*, a high order multivariate correlation matrix projects onto a lower dimension and analyze the pattern for grouping.

References

1) J.E. Tyler: *J. Dent. Res.*, (1971) **50**, 1189-1194
Quantitative estimation of volatile fatty acids in carious enamel by gasw chromatography of their methyl esters.

2) J.E. Lambert, and J.K. Moss: *J Chromatogr.*, (1972) **74**, 335-338
Gas-liquid chromatography of short-chain fatty acids on Dexsil 300GC.

3) G.A. Botta, L. Radin, A. Costa, G. Schito and G. Blasi: *J. Periodontal Res.*, (1985) **20**, 450-457
Gas-liquid chromatograpy of the gingival fluid as an aid in periodontal diagnosis.

4) S.M. Vratsanos and I.D. Mandel: *J. Dent. Res.*, (1982) **61**, 465-468
Comparative plaque acidogenesis of caries-resistant vs. carie-susceptible adults.

5) H.A. Linke and S.J. Moss: *Zeit für Ernahrung* (1992) **31**, 147-154
Quantitation of carbohydrate sweeteners and organic acids in human oral fluid using HPLC analysis.

6) H.A. Linke and S.J. Moss: *Zeit für Ernahrung* (1997) **36**, 191-197
Intra-oral lactic acid production during clearance of different foods containing various carbohydrates.

7) H.A. Linke and L.H. Birkenfeld: *Zeit für Ernahrung* (1999) **43**, 131-139
Clearance and Metabolism of starch foods in the oral cavity.

8) H. Harada, H. Shimizu and M. Maeiwa: *Foren. Sci. Int.*, (1987) **34**, 189-195
^{1}H-NMR of human saliva. An application of NMR spectroscopy in forensic science.

9) A. Yamadanosaka, S. Fukutomi, S. Uemura, T. Hashida, M. Fujishita and Y. Kobayashi *et. al.*: *Arch. Oral Biol.*, (1991) **36**, 697-761
Preliminary nuclear magnetic resonance studies on human saliva.

10) K. Dan, K. Kawano, Y. Terada, F. Iga and R. Hirayasu: *Jpn. J. Oral Biol.*, (1989) **31**, 324-328
High resolution proton nuclear magnetic resonance studies of human saliva.

11) C.J.L. Silwood, E.J. Lynch, S. Seddon, A. Sheerin, A.W.D. Claxson and M.C. Graclveld: *NMR Biomed.*, (1999) **12**, 345-356
^{1}H-NMR analysis of microbial-derived organic acids in primary root carious lesions and saliva.

12) C.J.L. Silwood, E.J. Lynch, A.W.D. Claxson and M.C. Graclveld: *J. Dent. Res.*, (2002) **81**, 422-427
^{1}H and ^{13}C NMR spectroscopic analysis of human saliva.