Original Article

Detection of Metabolomic Profile of Saliva in Healthy Malaysian Adults

Han Hing Lee[*,1], Lee Chee Yen[2], Yoshinobu Shoji[3,4], Chin Hoe Teh[4].

[1]*Hospital Sultan Haji Ahmad Shah, Jalan Maran 28000 Temerloh, Pahang, Malaysia.*

[2]*Bundaberg Base Hospital,Bundaberg West, Queensland 4670, Australia.*

[3] *Faculty of Dentistry, Universiti Teknologi MARA Sg. Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Malaysia.*

[4] *Bruker (Malaysia) Sdn Bhd, 303, Block A, Mentari Business Park, 46150 Petaling Jaya, Selangor, Malaysia.*

Keywords: *NMR, healthy adult, saliva, metabolomics*

DOI: https://doi.org/10.24191/cos.v2i0.17525

Abstract

Background: Saliva is a readily accessible biofluid that is important for the overall quality of life, functionally essential in the chewing, swallowing, tasting, regulation mouth flora and prevention of caries. The aim of this study is to assess the global metabolomic profile of saliva in healthy Malaysian adults. **Methods:** As a first step to determining and understanding the metabolomic profile of saliva in healthy Malaysian adults, we have collected saliva samples of 50 adults and measured the salivary metabolite to establish a profiling metabolite data, Human Metabolome Database (HMDB). Metabolites concentrations of saliva in healthy subjects were measured by using ^{1}H NMR spectroscopy. **Results:** The results showed there was no significant inter-individual variations of the key metabolites observed among the healthy Malaysian adults and there was no significant variation of the metabolites between female and male subjects. **Conclusion:** The metabolomic profile of saliva in healthy Malaysian adults could be used to establish the metabolomic database and used as a comparison for future study of the saliva of specific diseases.

Introduction

Metabolomics provide an analysis of changing metabolite levels in biological samples [1]. Metabolomics have been applied to a number of important areas, which include the discovery of biomarkers as well as mechanistic studies aimed at discovering metabolites or metabolic pathways that regulate cellular and physiological processes [1]. For example, metabolomics has been applied to clinical conditions such as Crohn's Disease, Cancer, Genetic inborn errors of metabolism and cardiovascular studies [2-3]. "Genomics and proteomics tell you what might happen, but metabolomics tells you what actually did happen." Said Bill Lasley,University of California, Davis. Metabolomics is more sensitive than genomics and proteomics as the metabolome measured is the final downstream product of transcription and translation, thus closest to the phenotype. The most important, saliva can be the sample for metabolomics study and has advantages over other biofluids such as blood and urine. Saliva collection is non-invasive and relatively fast compare to other biofluids collection [4-5].

*Correspondence to: Yoshinobu Shoji, Faculty of Dentistry, University Technology MARA Sg. Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Malaysia.
E-mail: shoji@salam.uitm.edu.my
Tel: +603-61266620, Faks: +603-61266103

Saliva is readily accessible complex biological fluid that is produced in, and secreted from the salivary glands. It consists of 99 % water with electrolytes, mucus, proteins, and small molecular weight metabolites making up the rest of the components [6-8]. Unbiased global metabolomic has rapidly enhanced disease characterization and biomarker discovery. Metabolites within a broad range of pathways contributed to the differentiation of healthy from diseased individuals, as well as between disease phenotypes. Some salivary metabolites have been successfully identifies using ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) [9], but to date chemometrics has not been applied to saliva metabolite datasets to find potential markers for disease [10]. Metabolite profiling of saliva remains largely under-explored despite demonstrated potential for biomarker detection in other biofluids such as urine and blood [11-13].

There is no metabolic database of healthy adult available in Malaysia. The aim of this study is to establish the metabolomics profile database of healthy Malaysian adults. With the database, we hope future researches can be done to identify new biomarker for early diagnosis of diseases with more specific to the Malaysia population.

Materials and Methods

Participants

50 healthy individuals (30 female and 20 male), aged 12 to 64 (median age of 38 years) , were recruited in this study. Informed consents were obtained from patients according to the IMU RCEC guideline. A total of 5 ml of saliva collected for analysis and immediately kept in the -80 degree freezer. The subjects have been screened by measuring the blood pressure, glucose level, Body Mass Index, questionnaire regarding to health condition, diet habit and medication. The screening process was carried out to ensure the healthy subjects were collected.

Saliva samples preparation

For acquisition of salivary NMR data, the frozen harvested saliva will be thawed, and spun at 3000 rpm to remove particulate matter. All saliva samples were prepared with addition of deuterated phosphate buffer for biofluid pH stabilization (pH 7.4). Additionally, sodium 3-trimethylsilyl- (2,2,3,3-$^{2}H_4$) -1-propionate (TSP) was added to serve as an internal standard and the deuterated solvent served as a field frequency lock. for the NMR spectrometer. Sodium azide was added as preservative.

^{1}H NMR metabolomic profiling

All samples was analysed using a Bruker Avance III NMR spectrometer operating at 500 MHz ^{1}H observation frequency using standard 1 dimensional acquisition parameters at UiTM Malaysia. All resulting NMR spectra were phase - and baseline-corrected. The data were Fourier transformed, and spectra were referenced to the TSP signal at 0 ppm. The results were then referenced using Bruker Topspin (version 3.1, Bruker, Fallanden, Switzerland) software.

Multivariate data analysis

Characterisation of spectral modulations by application of multivariate statistical data analysis was used to reduce complexity of these data and to facilitate visualisation of inherent patterns within the data. Data was normalised and scaled prior to multivariate analysis. The multivariate statistical analysis such as principal

components analysis (PCA) were applied. The key metabolites were further validated statistically through permutations methods (with 10000 resampling) to ensure reliability and reproducibility of the results.

Results

A sample ^{1}H NMR spectrum of unstimulated saliva from healthy Malaysian adults with several resonances identified is shown in Figure 1 and 2. Resonance assignments were made using Bruker Topspin software. All results were phased and baselined corrected. We have identified a small number of key metabolites that were present as part of the human saliva metabolome of healthy Malaysian adults. The metabolites found were Proprionate, Acetate, Alanine, Lactic acid, Tyrosine, Phenylalanine and Formate as shown in Table 1. Spectra derived from saliva of healthy Malaysian adults were dominated by Acetate and Propionate as shown in Table 1.

There is no significant inter-individual variations of the key metabolites were observed among the healthy Malaysian adults. To determine differences between male and female saliva, 20 healthy female subjects and 5 healthy male subjects were recruited. PCA scores plot of ^{1}H NMR spectra derived from saliva of healthy female and male control showed there was no significant variation of the metabolites between female and male, as shown in Figure 3.

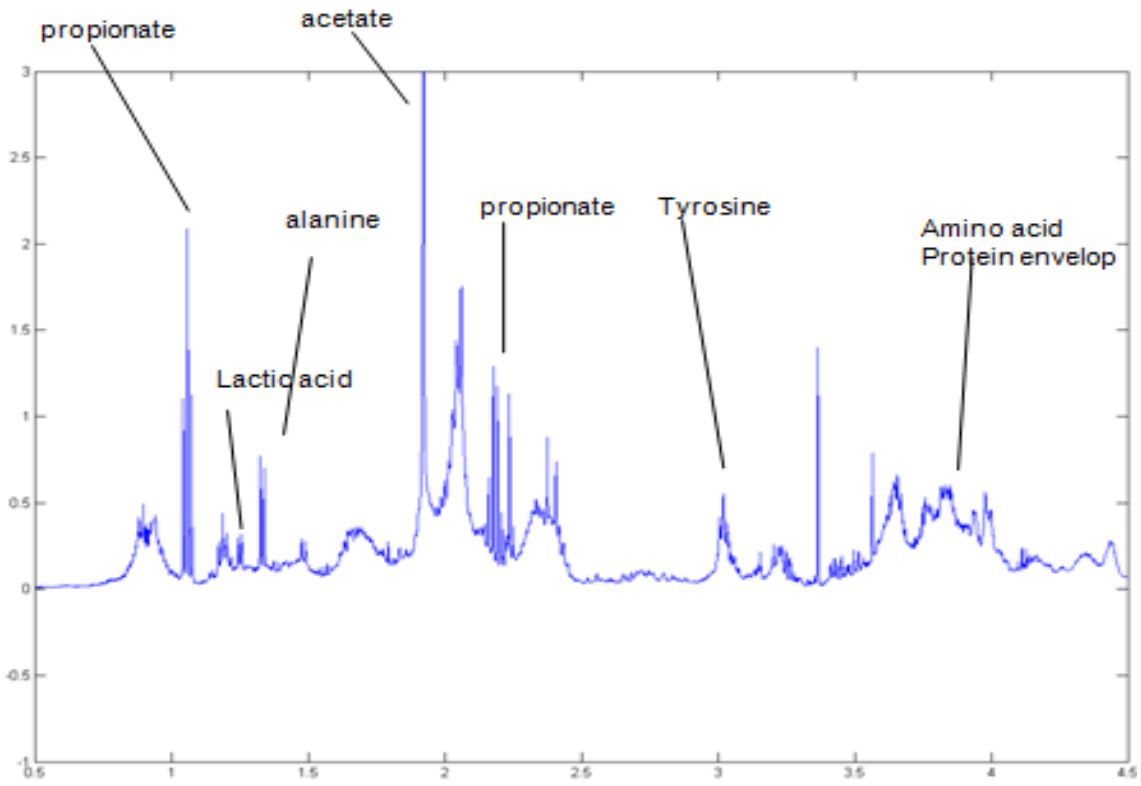

Figure 1: Median ^{1}H NMR spectra derived from saliva of healthy controls (aliphatic region).

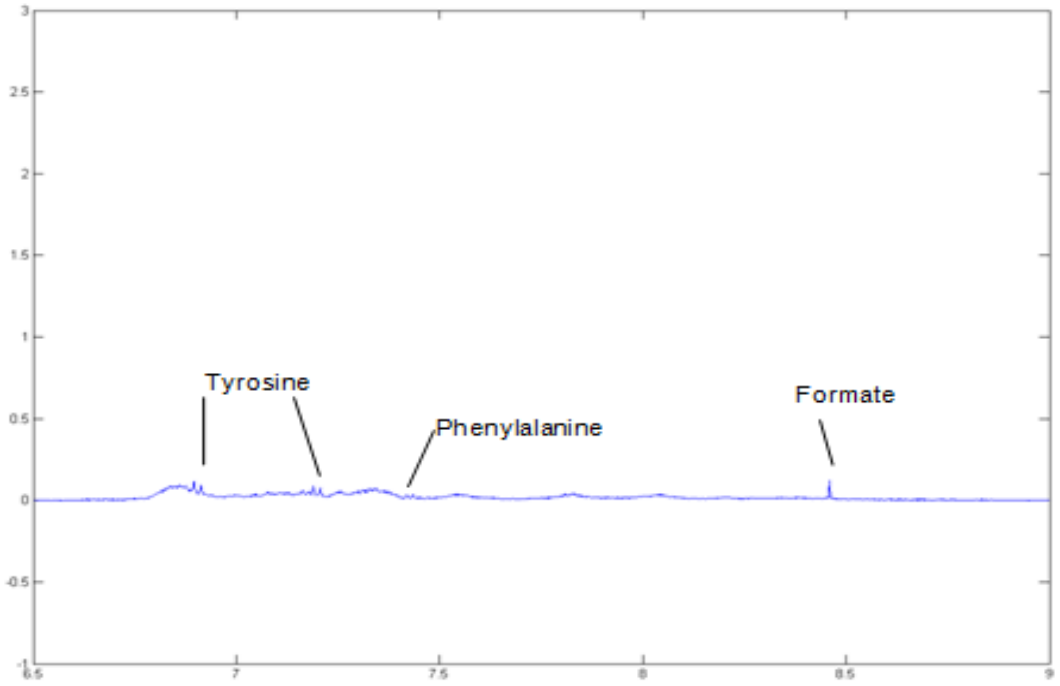

Figure 2: Median ^{1}H NMR spectra derived from saliva of 25 samples (aromatic region)

Compound	^{1}H chemical shift (ppm)
Propionate	1.04, 2.18
Acetate	1.92
Alanine	1.47
Lactic acid	1.32
Tyrosine	6.89, 7.18
Phenylalanine	7.32, 7.37, 7.42
Formate	8.45

Table 1: ^{1}H NMR assignments for Human Saliva at 500MHz

Discussion

In the present study, the use of human saliva samples for NMR-based metabolomics was evaluated. High-resolution ^{1}H NMR spectra was obtained, and the major signals were clearly assigned consistent with earlier studies. Technical advances in NMR spectroscopy, it is now possible to identify and quantify hundreds of metabolites from many different types of biological samples in relatively short orders [9]. However, the procedure from sample collection to analysis is sensitive. Cellular debris from both human and bacterial sources may contribute various metabolites, and products of bacterial metabolism may also be present. Steroids, some other hormones, and many drugs and antibodies can diffuse from serum into saliva [13]. Filtration of saliva samples using centrifuge provides an effective method of removing both proteins and particulate matter from saliva samples. Analysis of the sample will be easier when the protein is removed from the sample [13]. It also depends on the method of collection, food components may be directly observed during targeted profiling of saliva [14]. For example, caffeine can be solutes already dissolved in the food, and rapidly dissolve into the saliva. All of these factors provide the important information for consideration when collecting saliva sample. The result only showed of 25 samples out of the total of 50 samples collected, 20 females and 5 males. This smaller sample set might be potentially affecting the result. The reason for reduced sample set was mainly due to the inhomogeneities in its field of the samples, outliers with weird peak that may be due to sugar, food or underlying systemic disease. Some studies indicated that there are age-associated alterations in certain aspects of salivary gland function [15]. In this study, the samples recruited were from age range from 19 to 64. Studies enclosing a larger number of subjects and specific age group are needed to minimize the potential confounders.

The samples collected in this study were only from around the Klang valley area. Samples from other states should be covered in order to establish a database that can represent the healthy adults in Malaysia. There are few unknown peaks needed to be further investigated. Some metabolites in the saliva that were previously identified in other studies such as Butyrate, Choline, Ethanol, Glycine, Histidine and Pyruvate were not found in this study. This might be due to the small sample size and thus inadequate of some metabolite concentration to be quantified. In cases where quantitation was not reliable for a particular compound, concentrations were either not used (for generation of a model using multivariate statistics), or reported as being less than 1 μM. It is known that gender has an influence over urinary metabolite concentrations [11, 12]. To determine differences in salivary composition due to gender, 20 females and 5 males was obtained and analysed using ^{1}H NMR spectroscopy coupled with targeted profiling. Figure 3 revealed no significant variation between male and female saliva metabolites. As compare to the study of Takeda et al. [9], the male subjects has higher in concentration of nearly all to metabolites compared to female subjects, included acetate, formate, glycine, lactate, methanol, propionate, propylene, glycol, pyruvate, succinate, and taurine. The sample size was obviously imbalance, as 20 female samples versus 5 males samples only. Thus, a larger sample set is needed for further validation and future clinical application.

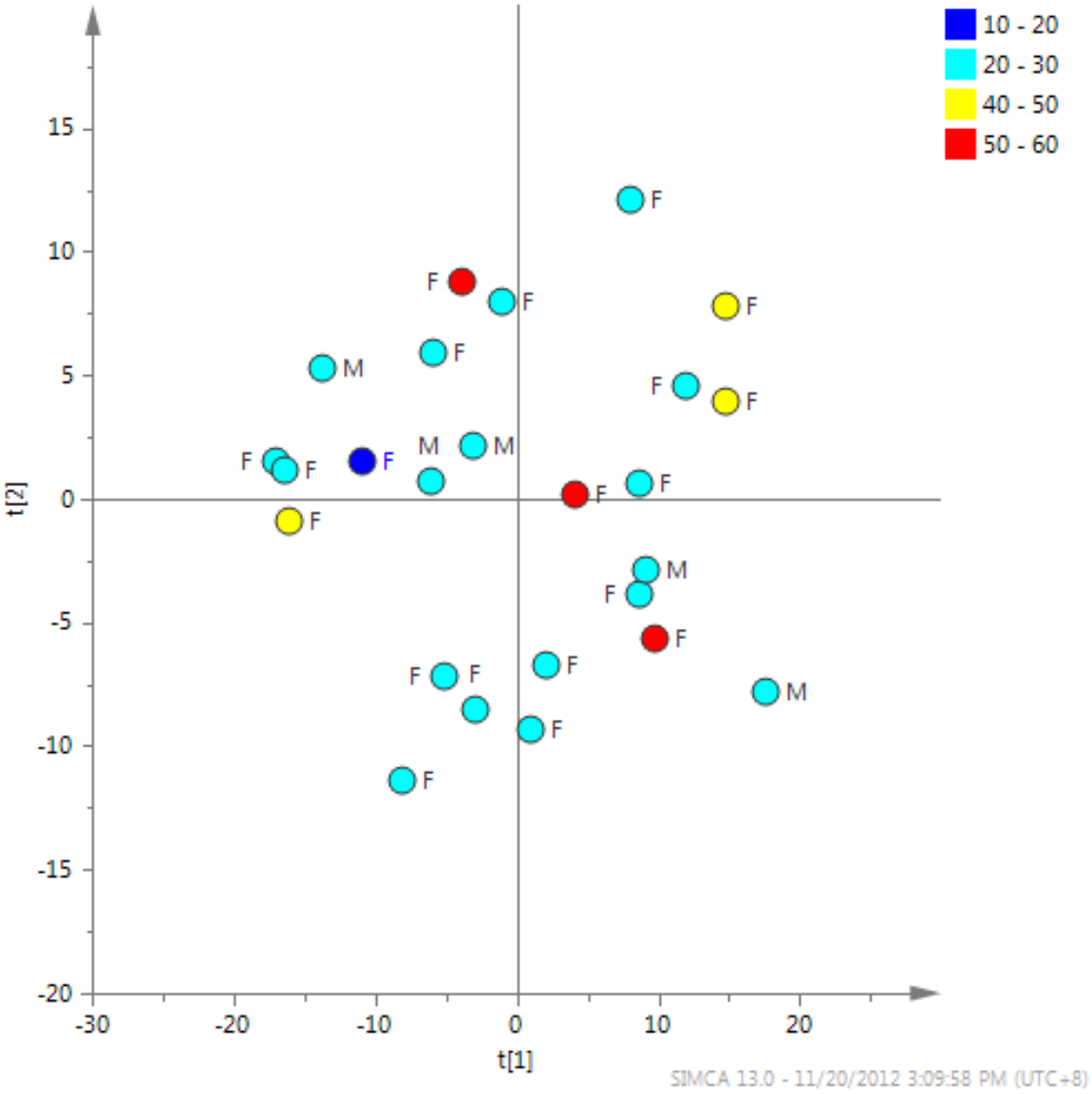

Figure 3: PCA scores plot of ^{1}H NMR spectra derived from saliva of healthy controls. (Labelled according to gender, coloured according to age groups). Comparison of male and female saliva.

Conclusion

We can readily apply the metabolic profile to identify the key metabolites in saliva by using ^{1}H NMR, following sample preparation and filtration to remove interfering protein and particulate matter to ease the analysis procedures. The median of the 25 samples show the peaks and key metabolites were identified, therefore these metabolomic profiles of saliva is potential for the future study of adults with the disease.

Acknowledgement

This research project was funded by a grant from the Postgraduate and Research Department, International Medical University, Bukit Jalil, Malaysia.

References

1. Vinayavekhin N, Homan E, Saghatelian A. Exploring disease through metabolomics. **ACS Chem Biol. 2010 Jan 15;5(1):91-103**. doi: 10.1021/cb900271r.
2. Mamas M, Dunn W, Neyses L, Goodacre R. The role of metabolites and metabolomics in clinically applicable biomarkers of disease. **Arch Toxicol. 2011 Jan;85(1):5-17.** doi: 10.1007/s00204-010-0609-6. Epub 2010 Oct 16.
3. Rafael MN. Saliva as a tool for oral cancer diagnosis and prognosis: a review. **Oral Oncol. 2009 Dec;45(12):1006-10.** doi: 10.1016/j.oraloncology.2009.07.005. Epub 2009 Oct 13.
4. Bertram HC, Eggers N, Eller N. Potential of Human Saliva for Nuclear Magnetic Resonance-Based Metabolomics and for Health-related Biomarker Identification. **Anal Chem. 2009 Nov 1;81(21):9188-93.** doi: 10.1021/ac9020598.
5. Yamada-Nosaka A, Fukutomi S, Uemura S, Hashida T, Fujishita M, Kobayashi Y, et al. Preliminary Nuclear Magnetic Resonance Studies on Human Saliva. **Arch Oral Biol. 1991;36(9):697-701.**
6. De Almeida PDV, Gregio AMT, Machodo MAN, de Lima AAS, Azevedo LR. **Saliva Composition and functions: a comprehensive review. J Contemp Dent Pract. 2008 Mar 1;9(3):72-80**.
7. Lingstrom P, Moynihan P. Nutrition, saliva, and oral health. **Nutrition. 2003 Jun;19 (6):567-9.**
8. Wishart DS, Knox C, Guo AC, Esner R, Young N, Gautam B. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 2009 Jan;37(Database is sue):D603-10. doi: 10.1093/nar/gkn810. Epub 2008 Oct 25.
9. Takeda I, Stretch C, Barnaby P, Bhatnager K, Rankin K, Fu H, et al. Understanding the human salivary metabolome. **NMR Biomed. 2009 Jul;22(6):577-84**. doi: 10.1002/nbm.1369.
10. Issaq HJ, Nativ O, Waybright T, Luke B, Veenstra TD, Issaq EJ, et al. Detection of bladder cancer in human urine by metabolomic profiling using high performance liquid chromatography/mass spectrometry. J Urol. 2008 Jun;179(6):2422-6. doi: 10.1016/j.juro.2008.01.084. Epub 2008 Apr 23.
11. Kochhar S, Jacobs DM, Ramadan Z, Berruex F, Fuerholz A, Fay LB. Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabonomics. **Anal Biochem. 2006 May 15;352(2):274-81**. Epub 2006 Mar 20.
12. Psihogios NG, Gazi IF, Elisaf MS, Seferiadis KI, Bairaktari ET. Gender-related and age-related urinalysis of healthy subjects by nmr -based metabonomics. **NMR Biomed. 2008 Mar;21(3):195-207.**
13. Slupsky CM, Rankin KN, Wagner J, Fu H, Chang D, Weljie AM, et al. Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. **Anal Chem. 2007 Sep 15;79(18):6995-7004.** Epub 2007 Aug 17.
14. CL P. The role of saliva in maintaining oral health and as an aid to diagnosis. **Med Oral Patol Oral Cir Bucal. 2006 Aug;11 (5):E449-55.**
15. Dodds MWJ, Johnson DA, Yeh CK. **Health benefits of saliva: a review. J Dent. 2005 Mar;33(3):223-33.** Epub 2004 Dec 19.