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# Effects of inter-day and intra-day variation on salivary metabolomic profiles

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ABSTRACT

*Background:* Salivary secretion is an important parameter reflecting the health status of an individual and has been used clinically for the diagnosis of various oral diseases, such as xerostomia. Salivary metabolomic profiling is considered an emerging potential tool for the detection of various systemic diseases. To our knowledge, this is the first study to investigate the quantitative relationship between salivary secretion volume and salivary metabolomic profile.

*Methods:* To evaluate inter- and intra-day variations in salivary secretion, 234 saliva samples were collected three times per day for three days from 13 subjects and analyzed. Capillary electrophoresis-mass spectrometry was used for non-targeted quantification of water-soluble metabolites.

*Results:* No significant inter- or intra-day variations were observed in salivary secretion volume. No significant inter-day variations were observed in metabolomic patterns. In contrast, significant intra-day variations were observed in salivary metabolomic profiles. The difference was more obvious for stimulated saliva than for unstimulated saliva. These profile changes were independent of salivary secretion volume.

*Conclusions:* Our results indicated that diurnal change had a greater effect on salivary metabolomic profiles than the other factors. Hence, sampling time should be tightly controlled to minimize unexpected bias in the clinical use of salivary metabolomics.

## 1. Introduction

Salivary secretion has been clinically used as an indicator of various diseases [1–4]. Low salivary secretion increases the risks of dental caries, periodontal diseases, and candidiasis; causes pain, affects oral functions, such as eating and swallowing [5]; and causes infection and increase in the risk of pneumonia. Thus, salivary secretion is an indicator of systemic quality of life [6], which is one of the most important parameters in the dental field for evaluating the health status of the oral cavity of an individual. However, salivary secretion is just one parameter and it is difficult to show disease specific features.

Saliva contains various molecules including enzymes, hormones, antibodies, antimicrobial components, growth factors, proteins, DNA, and (importantly) metabolites [7–10]. Salivary metabolites include secretions from oral bacteria, substances that pass from the blood into the salivary gland, and components of gingival crevicular exudate. Salivary metabolomic profiles have been correlated with various metabolic diseases, such as cancers [11–16], diabetes [17], and periodontal diseases [18,19].

Before the use of salivary metabolomic profiles for clinical applications, standard operating procedures, such as sample collection and storage conditions, should be developed [20]. Diurnal variation of salivary components is a concern with respect to the establishment of sample collection protocols. For example, salivary secretion depends on circadian rhythms [21]. Metabolomic profiles are correlated with circadian rhythms in various biofluid samples, particularly in blood samples [22–26]. With respect to saliva, such data are limited and only few studies have been reported (e.g., the time-course of a few

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polyamines [27] and differences between morning and night [28]). There have also been few comparative studies on metabolites in saliva and blood [29]. Diurnal oscillations of microRNA [30] and the microbiota [31] may change salivary metabolomic profiles.

The purpose of this study was to investigate the relationship between salivary secretion and salivary metabolomic profiles. The comparisons included (1) stimulated and unstimulated saliva and (2) inter- and intra-day variations, using matched samples.

## 2. Materials and methods

### 2.1. Study participants

This study was performed after obtaining approval (No.243) by the Kanagawa Dental University Ethics Committee. Written informed consent was obtained from subjects who agreed to serve as saliva donors. Subjects with a normal occlusion, who had undergone no treatment of occlusion or were not under current treatment for oral cavity conditions, were selected.

### 2.2. Sampling method

Unstimulated and stimulated whole saliva samples were collected from 13 subjects. First, unstimulated saliva was collected by a passive drool method [32–34]. Subjects swallowed residual saliva present in the mouth before the beginning of the collection; then, with their mouths slightly open, subjects allowed saliva to drip from the lower lip into a container (Corning Inc., Tokyo, Japan). During collection, swallowing of saliva during sampling was prohibited and naturally secreted saliva was collected into a container (Corning Inc., Tokyo, Japan) cooled on ice for a period of 15 min. Then, stimulated saliva was collected using the gum method. Saliva secreted while chewing paraffin gum (Eiken Chemical Co., Ltd., Tokyo, Japan) was collected for a period of 10 min in a container (Corning Inc., Tokyo, Japan) cooled on ice. Eating and drinking, except for water intake, was restricted at least 2 h before the sampling. In order to prevent stimulation of the oral cavity, unstimulated saliva samples were collected before stimulated saliva samples. Unstimulated and stimulated saliva samples were collected consecutively from the same subject within a specific period of time to minimize intra-day variations between samples. The volumes of unstimulated and stimulated saliva samples were measured at each sampling time-point and on each day. Differences among samples collected in the three time periods on the same day were analyzed to determine intra-day variation; differences among the sample groups collected at the same time-point on days 1, 3, and 5 were analyzed to determine inter-day variation.

The saliva samples were maintained on ice during sampling, and the analytical saliva samples were then stored at −20 °C.

### 2.3. Collection conditions

Unstimulated and stimulated saliva samples were collected within the same time periods. To investigate intra-day variation, samples were collected three times: in the morning (8:00–9:00), daytime (12:00–13:00), and evening (17:00–18:00). To investigate inter-day variation, samples were collected every other day on 3 days (days 1, 3, and 5). Accordingly, a total of nine saliva samples were collected from each subject.

### 2.4. Metabolomic analysis

The protocol of sample processing has been described elsewhere [13]. Briefly, frozen saliva was thawed at 4 °C for approximately 1.5 h. Subsequently, the sample was dissolved using a Voltex mixer at room temperature and centrifuged through a 5-kDa cutoff filter (Millipore, Bedford, MA) at 9100 × *g* for at least 2.5 h at 4 °C. Then, 45 μL of each sample was added to a 1.5-mL Eppendorf tube, with 2 mM of methionine sulfone, 2-[N-morpholino]-ethanesulfonic acid, D-Camphol-10-sulfonic acid, sodium salt, 3-aminopyrrolidine, and trimesate. Then, 150 μL of filtrate was lyophilized and dissolved in 25 μL of Milli-Q water containing a reference compound (200 μM of 3-aminopyrrolidine and trimesate) prior to CE-time-of-fight (TOF)-MS analysis.

The instrumentation and measurement conditions used for CE-TOF-MS have been described elsewhere [35] with slight modification including the different version of the software. All samples were measured within a single batch in order to eliminate unexpected bias caused by different batches. Internal standards were added to eliminate unexpected bias caused by fluctuation of specificity of mass spectrometry, and the peak area of each metabolite divided by that of the internal standard (relative peak area) was used. The relative peak areas of each metabolite and those of the standard mixture were compared to calculate the absolute concentrations.

### 2.5. Data analysis

Differences in the mean volumes of unstimulated and stimulated saliva at all sampling time-points and on all days were analyzed using the Wilcoxon matched-pairs signed-rank test; the *P*-values were adjusted by Bonferroni correction.

Metabolite concentrations of unstimulated and stimulated saliva at all sampling time-points and on all days were analyzed using the Wilcoxon matched-pairs signed-rank test. The Steel-Dwass test was used for the comparison of sampling time-points and sampling days. *P*-values for metabolites were adjusted by the false discovery rate (FDR) (Benjamini-Hochberg method) to control for the α error caused by multiple independent tests, yielding Q-values.

The collected saliva samples were subjected to metabolomic analysis and then principal component analysis (PCA). The absolute concentrations of quantified metabolites, except for metabolites not detected among all samples, were used for the PCA. The other attributes, e.g., the stimulated and unstimulated saliva, were visualized on the score plots using different colors. Here, Pearson correlation was used during the computations in the correlation matrix.

The differences of principal components (PC) were evaluated by using the Wilcoxon matched-pairs signed-rank test, and *P*-values were corrected by the FDR. The Euclidean distance between the PC values at score plots was calculated for quantitatively evaluating the influence of each sampling condition. Comparison among PC values at each collection point were conducted, e.g., between the values in the morning and daytime. In addition, the combined data were also compared, e.g., between the values in the morning and daytime and daytime and evening.

The quantified metabolites were also analyzed by clustering and visualized in a heatmap. The attributes of the assessors (age, sex, and smoking and drinking habits) were also mapped on the hierarchical cluster analysis. The absolute concentrations of each metabolite were converted to *Z*-scores and were visualized in heat map form; clustering of metabolites and samples was conducted using the Euclidean distance. The analyses were conducted with XLSTAT (ver. 2014.1.04, Addinsoft, Paris, France), MeV TM4 (ver. 4.9.4, http://mev.tm4.org), R (ver. 3.5.1, The R Foundation for Statistical Computing, Vienna, Austria), and GraphPad Prism (ver 5.04, GraphPad Software Inc., La Jolla, CA, USA).

## 3. Results

### 3.1. Saliva volume

There were 11 male and 2 female subjects (13 subjects in total). The age was 29.09 ± 3.85 years (mean ± standard deviation [S.D.]) in male and 33.50 ± 6.50 years in female subjects; overall, the age of the subjects was 29.77 ± 4.64 years. Comparisons of the unstimulated and

**Table 1**
Saliva volume (mean ± S.D., ml).

| Sampling day | Volume | | | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Intra-day variation | | | Inter-day variation | | |
| | Sampling time | | | Morning vs Daytime | Morning vs Evening | Daytime vs Evening | Day-1 vs Day-3 | Day-1 vs Day-5 | Day-3 vs Day-5 |
| | Morning | Daytime | Evening | | | | | | |
| Unstimulated | | | | | | | | | |
| day-1 | 5.89 ± 1.82 | 6.65 ± 1.31 | 7.61 ± 1.72 | 0.142 | 0.039 | 0.046 | 0.700 | 0.363 | 0.675 |
| day-3 | 6.17 ± 1.99 | 6.78 ± 2.51 | 7.36 ± 2.71 | 0.552 | 0.091 | 0.410 | 0.756 | 0.382 | 0.443 |
| day-5 | 6.78 ± 3.33 | 7.34 ± 2.54 | 7.96 ± 2.98 | 0.294 | 0.263 | 0.410 | 0.875 | 0.753 | 0.695 |
| Stimulated | | | | | | | | | |
| day-1 | 13.8 ± 5.60 | 15.5 ± 6.34 | 13.2 ± 4.20 | 0.255 | 0.701 | 0.034 | 0.039 | 0.039 | 0.576 |
| day-3 | 11.3 ± 3.32 | 11.8 ± 6.04 | 11.0 ± 3.82 | 0.844 | 0.753 | 1.000 | 0.023 | 0.033 | 0.675 |
| day-5 | 10.7 ± 3.79 | 11.9 ± 4.06 | 12.0 ± 3.71 | 0.147 | 0.155 | 0.937 | 0.030 | 0.386 | 0.123 |

Note: Unstimulated and stimulated saliva samples were collected within 15 and 10 min, respectively.
Wilcoxon matched-pairs signed-rank test, followed by Bonferroni correction, was used.

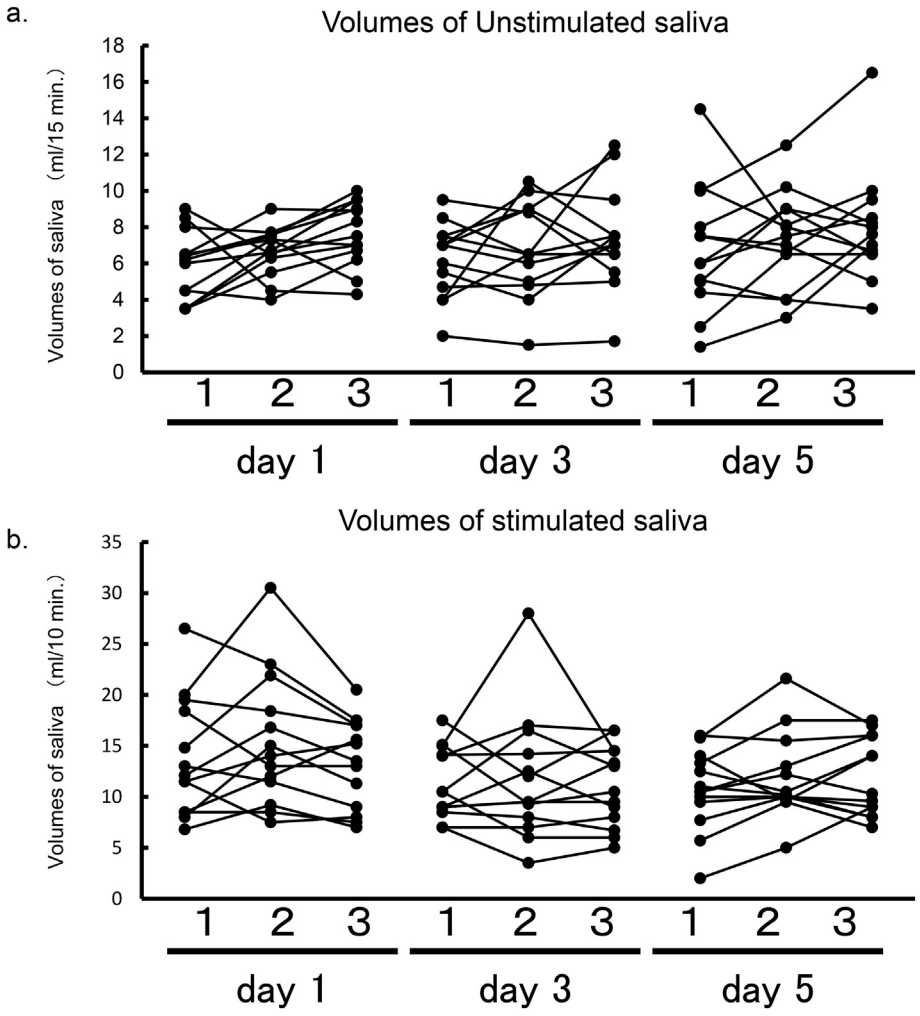


**Fig. 1.** Changes in salivary secretion using samples collected nine times from the same subjects ($n$ = 13). Numbers 1, 2, and 3 represent the sampling time-points in the morning, daytime, and evening, respectively. (A) Unstimulated salivary secretion (mL/15 min); (B) stimulated salivary secretion (mL/10 min). Wilcoxon matched-pairs signed-rank test and Bonferroni correction were used. No comparison showed significant differences ($P$ < .05). On the x-axis, 1, 2, and 3 indicate respective sample collection times in the morning (8:00–9:00), daytime (12:00–13:00), and evening (17:00–18:00), respectively. The y-axis indicates the volume of collected saliva.

stimulated saliva volumes are presented in Table 1. The volumes were compared among saliva samples obtained in the morning, during the day, and in the evening on each sampling day, i.e., both inter- and intra-day variations were evaluated. No statistically significant difference was observed for both unstimulated (Fig. 1a) and stimulated (Fig. 1b) saliva ($P$-value < .05, Wilcoxon matched-pairs signed-rank test with Bonferroni correction).

### 3.2. Overview of metabolite concentration patterns

In total, 47 metabolites were detected, quantified, and used for subsequent analyses. Score plots and loading plots of PCA are depicted in Fig. 2a and Fig. 2b, respectively. Each point on the score plots represents one saliva sample and the points with shorter distances indicate greater similarity between salivary metabolite concentration patterns. In the loading plots, one point represents one metabolite

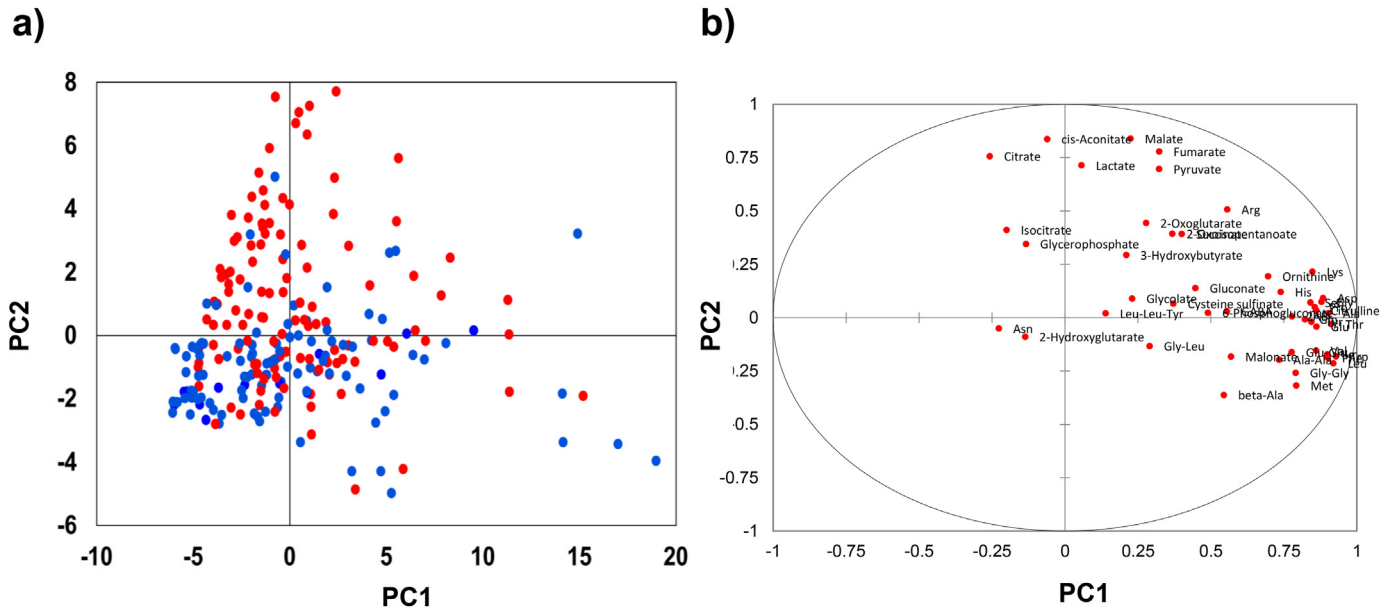


**Fig. 2.** Principal component analysis (PCA) of salivary metabolites. (A) Score plots; (B) Loading plots. (A) The blue and red dots of the score plots indicate unstimulated and stimulated saliva. The horizontal and vertical axes represent the first PC (PC1) and the second PC (PC2), respectively; these are dimensionless. The contribution ratios of PC1 and PC2 were 40.80% and 11.64%, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showing its contribution to the PC; for example, the metabolite concentrations on the right side (the value of the PC1 [first PC] is large) of the loading plots are higher in the sample plotted at the right end (the value of the PC1 is large) of the score plots.

Mount of the metabolites were plotted in the right half of the loading plots (Fig. 2b); for example, all amino acids excluding asparagine (Asn) were plotted at PC1 > 0. In addition, the contribution ratio of PC1 (40.80%) was larger than that of PC2 ([second PC], 11.64%), suggesting that PC1 reflects the overall concentration in the saliva sample and the overall concentration increases with shifting rightward (as the PC1 value increases). In comparison of the unstimulated and stimulated states on the score plots, many points were distributed in the lower region (PC2 < 0) in unstimulated saliva and upper region (PC2 > 0) in stimulated saliva. The PC1 orthogonally intersects PC2, which suggests that change in a part of the overall concentration occurred in the metabolites in the unstimulated and stimulated saliva. Additionally, PC1 predominantly reflects amino acids, while PC2 captures more components of the citric acid cycle.

### 3.3. PC-based comparison of metabolites between unstimulated and stimulated saliva

Score plots (Fig. 2a) shows the space of PC1 and PC2 based on the results of the PCA. The samples plotted within a showed distance each other indicates the metabolite concentration patterns are similar in the corresponding samples. Data representing comparisons between morning and daytime or between evening and daytime from saliva collected on day 1 ($P$ = .168 and 0.216, respectively, Wilcoxon matched-pairs signed-rank test (Fig. 3a and b) revealed no significant difference. Data representing comparisons between the morning and daytime and daytime and evening in terms of overall day 1 analysis ($P$ = .0637, Wilcoxon matched-pairs signed-rank test, Fig. 3c) revealed no significant difference. No significant difference was found in the comparison of saliva collected in the morning between days 1 and 3 or between days 3 and 5 ($P$ = .893 and $P$ = .839, respectively, Fig. 3d and e). Similarly, comparisons between the combined data of days 1 and 3 and combined data of days 3 and 5 ($P$ = .819, Fig. 3f) revealed no significant differences. Differences in metabolite concentrations detected in unstimulated and stimulated saliva samples are summarized in Table 2. Among 47 metabolites, 20 showed significant differences ($Q$-value < 0.05, Wilcoxon matched-pairs signed-rank test, corrected by FDR). Notably, all organic acids (with the exception of succinate) showed significant differences.

### 3.4. Clustering-based comparison of metabolites between unstimulated and stimulated saliva

The quantified metabolites were analyzed by clustering and visualized in a heatmap form (Fig. 4). Overall, unstimulated saliva (light blue) and stimulated saliva (pink) were heterogeneously distributed. Metabolites with higher concentrations were clustered at the right side of the heatmap. Most of the amino acids, such as citrulline, valine, and methionine, were included in another cluster with dipeptides, including Ala-Ala and Glu-Glu. Most organic acids, such as pyruvate, lactate, fumarate, malate, *cis*-aconitate, and citrate, were also included in this cluster; notably, the presence of this cluster was enhanced in samples that exhibited higher concentrations of amino acids. Concomitantly corrected attributed, such as sex, age, and smoking habits, were mapped on this heatmap; however, no clear features were observed, indicating independent relationship among these clusters and these attributes.

### 3.5. Changes associated with the sampling time and day

The distance between the points of unstimulated saliva on the score plot was compared among the sampling time-points and among the sampling days (Fig. 5). Comparisons of unstimulated saliva between morning and evening ($P$ = .0048; Wilcoxon matched-pairs signed-rank test, Fig. 5b) revealed a significant difference, but no significant differences were observed in any other comparison. For stimulated saliva, comparisons between morning and daytime ($P$ = .0043; Wilcoxon matched-pairs signed-rank test, Fig. 5d) and between daytime and evening ($P$ = .0312; Wilcoxon matched-pairs signed-rank test, Fig. 5d) revealed significant differences. The metabolite concentrations for unstimulated and stimulated saliva are summarized in Tables S1 and S2, respectively. Unstimulated saliva showed 12 significant differences

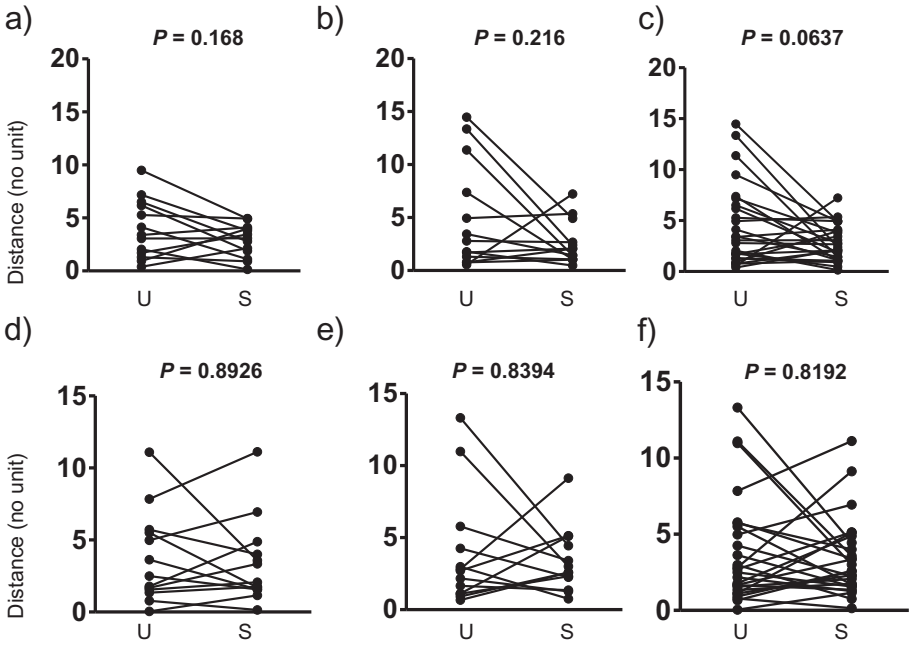


**Fig. 3.** The Euclidean distance of each point was calculated to express the influence of each sampling condition. Unstimulated saliva (U) and stimulated saliva (S) were analyzed using the Wilcoxon matched-pairs test, but no significant difference ($P < .05$ indicated a significant difference) was detected in the following comparisons: (A) morning vs. daytime; (B) daytime vs. evening; (C) morning + daytime vs. daytime + evening; (D) day-1 vs. day-3; (E) day-3 vs. day-5; (F) day-1 + day-3 vs. day-3 + day-5.

with respect to sampling time (e.g., pyruvate concentration between daytime and evening ($P < .05$, Steel-Dwass test)). Stimulated saliva also showed 11 significant differences with respect to sampling time. However, these values were not significant after FDR correction. Unstimulated saliva showed no significant differences among sampling days. Stimulated saliva showed seven significant differences with respect to sampling days ($P < .05$); however, no differences remained after correction.

## 4. Discussion

In this study, salivary metabolites in unstimulated and stimulated saliva were analyzed using metabolomic analysis, and the influence of differences in the sampling time and day on salivary metabolites were compared. Previous studies have reported intra-day variations in several metabolites, i.e., salivary secretion rate increases from the time of waking up, peaks in the evening, and gradual decreases [21,36]. A similar trend was observed in our study, although the results were not statically significant (Table 1, Fig. 1).

In the current study, a significant difference was observed between the morning and evening concentrations for unstimulated saliva (Fig. 4b). For stimulated saliva, a significant difference was observed between the morning and daytime and between the daytime and evening concentrations (Fig. 4d). Among the various quantified metabolites, Asn and 2-hydroxyglutarate (at the center of the loading plots, Fig. 2) showed smaller changes compared to the other metabolites (at the periphery of the loading plots, Fig. 2). Taken together, the salivary metabolite concentrations showed homogeneous changes. For example, urinary metabolites are generally normalized by creatinine to eliminate individual variation in overall urinary concentrations. However, our data indicate that such normalization (i.e., dividing absolute concentrations of quantified metabolites by the concentration of a single metabolite) is insufficient to eliminate unexpected bias derived from the sampling time; thus, more sophisticated normalization methods are necessary. Statistically significant intra-day differences were not observed in the metabolomic profiles. Despite the lack of statistically significant differences based on PCA data, differences in PC values of stimulated saliva were larger than those observed in PC values of unstimulated saliva (Fig. 2). Numerous similarly large molecules are present in saliva, including amylase, mucin, albumin, and statins [35]. Notably, albumin is a large protein that binds to many compounds. It is reported that the concentrations of salivary components that are actively secreted from the salivary glands are maintained at high concentration in stimulated saliva. Salivary proteins are those originating in the oral cavity, such as gingival crevicular fluid, and those secreted from the serum-derived salivary glands. However, the concentration of salivary proteins that are not actively transported will tend to decrease in stimulated saliva compared with unstimulated saliva [9]. Salivary albumin is present as a component of gingival crevicular fluid; the albumin level during stimulated salivation is known to be low compared with that during unstimulated salivation [37].

Salivary hormones, such as cortisol, calcium, inorganic phosphoric acid, amylase, and chromogranin A reportedly show intra-day variations [38,39]. For example, the concentrations of salivary alpha-amylase, cortisol and dehydroepiandrosterone showed larger change just after the awakening compared to those in the salivary samples collected during daytime [39]. Large individual differences in diurnal variation of salivary cortisol depend on health condition and stress levels [40–42]. Salivary melatonin is known as a marker of diurnal variation. The concentration of salivary melatonin showed drastic change in the morning and afternoon in response to exercise-induced heartbeat changes [43]. These salivary component change may affect salivary metabolites.

In addition to diurnal change, various other factors may affect salivary metabolites. Previous studies have shown that the concentration patterns of amino acids can be affected by stress [44], cortisol levels can be affected by both age and congenital heart disease [45], and several immunological reaction-related metabolites can be affected by sex [46–48]. Thus, before using salivary secretion analysis clinically for the diagnosis of specific diseases, such effects should be carefully investigated using a larger cohort with various subjects.

The present study has several limitations. Only young and healthy subjects were included in the analysis, few of which were women. We previously observed sex-dependent differences in salivary metabolomic profiles [49]. Therefore, a large cohort comprising diverse subjects is needed to obtain more reliable results. We analyzed the metabolites identified using standard compounds in our library [35]. Non-targeted analyses are expected to detect a larger variety of metabolites using fragment-based annotation with tandem MS or matched $m/z$ and migration times computationally predicted based on molecular structures

**Table 2**
Comparison of unstimulated and stimulated saliva (μM).

| | Unstimulated | | | Stimulated | | | Wilcoxon signed-rank test | |
|---|---|---|---|---|---|---|---|---|
| Metabolite | Median | Min | Max | Median | Min | Max | P-value | Q-value |
| Gly | 52.99 | 5.52 | 361.84 | 77.75 | 14.91 | 285.86 | 0.000474 | 0.001238** |
| Ala | 22.20 | 2.26 | 140.43 | 33.35 | 6.53 | 128.27 | 0.000006 | 0.000018*** |
| 2AB | 1.21 | 0.58 | 4.44 | 1.19 | 0.54 | 2.78 | 0.967458 | 0.967458 |
| Ser | 10.19 | 1.87 | 80.57 | 12.53 | 3.22 | 83.06 | 0.001985 | 0.004911** |
| Pro | 35.62 | 2.00 | 375.37 | 56.08 | 9.11 | 278.88 | 0.000282 | 0.000781*** |
| Thr | 3.07 | 0.47 | 20.08 | 3.23 | 0.96 | 16.95 | 0.947955 | 0.967458 |
| Ile | 2.23 | 0.16 | 36.80 | 1.69 | 0.40 | 24.26 | 0.159683 | 0.214431 |
| Leu | 6.22 | 0.86 | 62.11 | 4.41 | 0.90 | 38.62 | 0.037716 | 0.061125 |
| Ornithine | 12.44 | 1.17 | 63.15 | 17.85 | 3.98 | 57.86 | 0.003252 | 0.007643** |
| Asp | 13.57 | 1.97 | 47.14 | 16.72 | 5.75 | 52.23 | 0.000001 | 0.000004*** |
| Gln | 10.18 | 1.05 | 162.21 | 11.66 | 2.89 | 165.33 | 0.030183 | 0.050664 |
| Lys | 23.54 | 2.09 | 153.65 | 36.16 | 4.71 | 124.16 | 0.000000 | 0.000000*** |
| Glu | 19.29 | 2.08 | 151.28 | 28.24 | 8.48 | 116.25 | 0.000000 | 0.000000*** |
| His | 6.30 | 1.49 | 65.95 | 6.76 | 1.55 | 39.23 | 0.775200 | 0.828055 |
| Phe | 10.77 | 0.82 | 72.97 | 8.98 | 1.08 | 42.96 | 0.009027 | 0.017679* |
| Arg | 16.36 | 2.11 | 89.23 | 24.97 | 7.34 | 75.75 | 0.000000 | 0.000001 *** |
| Citrulline | 10.82 | 0.47 | 98.62 | 20.71 | 1.87 | 89.33 | 0.000000 | 0.000000*** |
| Tyr | 12.68 | 2.05 | 101.00 | 13.20 | 2.56 | 73.58 | 0.439863 | 0.516839 |
| Glycolate | 16.48 | 10.57 | 29.79 | 15.85 | 10.19 | 23.63 | 0.006320 | 0.012915* |
| Pyruvate | 15.72 | 3.52 | 89.12 | 26.47 | 4.69 | 85.62 | 0.000000 | 0.000001*** |
| Lactate | 74.09 | 14.39 | 512.93 | 98.73 | 9.25 | 712.61 | 0.000000 | 0.000000*** |
| 3-Hydroxybutyrate | 3.36 | 0.95 | 17.72 | 3.51 | 1.20 | 35.06 | 0.000000 | 0.000000*** |
| Fumarate | 0.73 | 0.26 | 2.69 | 0.97 | 0.32 | 2.97 | 0.000000 | 0.000000*** |
| Succinate | 21.10 | 2.21 | 170.86 | 20.18 | 2.74 | 88.41 | 0.237843 | 0.294174 |
| Malate | 3.46 | 1.02 | 11.96 | 5.49 | 2.22 | 17.78 | 0.000000 | 0.000000*** |
| 2-Oxoglutarate | 2.59 | 0.81 | 11.37 | 6.65 | 1.32 | 17.81 | 0.000000 | 0.000000 *** |
| Glycerophosphate | 6.87 | 1.80 | 20.31 | 7.20 | 2.79 | 16.95 | 0.021089 | 0.036710* |
| cis-Aconitate | 0.22 | 0.09 | 0.60 | 0.32 | 0.15 | 0.85 | 0.000000 | 0.000000*** |
| Citrate | 11.34 | 0.18 | 36.73 | 17.53 | 0.50 | 63.53 | 0.000000 | 0.000000 *** |
| Val | 6.28 | 0.00 | 58.90 | 5.48 | 1.39 | 31.31 | 0.070926 | 0.104173 |
| GABA | 1.15 | 0.00 | 8.96 | 1.01 | 0.17 | 4.48 | 0.825634 | 0.862329 |
| beta-Ala | 1.15 | 0.00 | 2.97 | 0.88 | 0.00 | 3.51 | 0.016728 | 0.030239* |
| Trp | 0.60 | 0.00 | 4.60 | 0.54 | 0.00 | 3.13 | 0.039040 | 0.061163 |
| 2-Oxoisopentanoate | 0.63 | 0.00 | 3.74 | 0.85 | 0.00 | 3.40 | 0.000000 | 0.000001*** |
| Malonate | 0.92 | 0.00 | 2.81 | 0.81 | 0.00 | 2.03 | 0.079605 | 0.113376 |
| Met | 0.72 | 0.00 | 6.68 | 0.84 | 0.00 | 6.62 | 0.365148 | 0.440050 |
| Ala-Ala | 0.56 | 0.00 | 3.42 | 0.65 | 0.00 | 2.79 | 0.064784 | 0.098220 |
| 6-Phosphogluconate | 0.32 | 0.00 | 1.69 | 0.38 | 0.00 | 1.74 | 0.004593 | 0.010280* |
| Glu-Glu | 0.18 | 0.00 | 1.66 | 0.25 | 0.00 | 1.97 | 0.105978 | 0.146499 |
| Gly-Gly | 0.38 | 0.00 | 5.35 | 0.46 | 0.00 | 6.39 | 0.519292 | 0.581112 |
| Gluconate | 0.00 | 0.00 | 3.51 | 0.00 | 0.00 | 3.53 | 0.742442 | 0.811506 |
| Isocitrate | 0.00 | 0.00 | 1.75 | 0.00 | 0.00 | 1.74 | 0.000001 | 0.000003*** |
| Cysteine sulfinate | 0.00 | 0.00 | 0.78 | 0.00 | 0.00 | 0.79 | 0.466622 | 0.534908 |
| Asn | 0.00 | 0.00 | 1.80 | 0.00 | 0.00 | 0.37 | 0.005922 | 0.012651* |
| Gly-Leu | 0.00 | 0.00 | 0.63 | 0.00 | 0.00 | 0.24 | 0.233953 | 0.294174 |
| Leu-Leu-Tyr | 0.00 | 0.00 | 0.09 | 0.00 | 0.00 | 0.00 | 0.014266 | 0.026820* |
| 2-Hydroxyglutarate | 0.00 | 0.00 | 0.18 | 0.00 | 0.00 | 0.15 | 0.181449 | 0.236892 |

Note: *P < .05, **P < .01, and ***P < .001 (FDR-corrected *P*-value).
The median, min, and max values were calculated using all sample times.

[50,51]. In addition, single instrument-based metabolomics limit the observation of detected metabolites and each method can detect metabolites showing different chemical features. Recent studies utilized nuclear magnetic resonance spectroscopy and liquid chromatography-MS and gas chromatography-MS [52–54]. Thus, our conclusions are limited within the observation range allowed by our measurement methods.

## 5. Conclusions

There was no significant difference in salivary secretion volume in both inter- and intra-day comparisons. In contrast, the metabolite concentration patterns in unstimulated and stimulated saliva showed significant intra-day variation; although inter-day variation was also observed, the difference was not significant. Taken together, our results indicate that sampling time (e.g., morning) is an important factor to ensure reproducibility in the quantification of salivary metabolites. Our results will contribute to the development of appropriate protocols for saliva sample collection in order to ensure reproducibility in the clinical use of salivary metabolomics for the detection of oral cavity diseases, especially diseases resulting in change in salivary secretion volume. However, to obtain more generalizable results covering a diverse range of metabolites, combinational analysis using various analytical methods is necessary.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.030.

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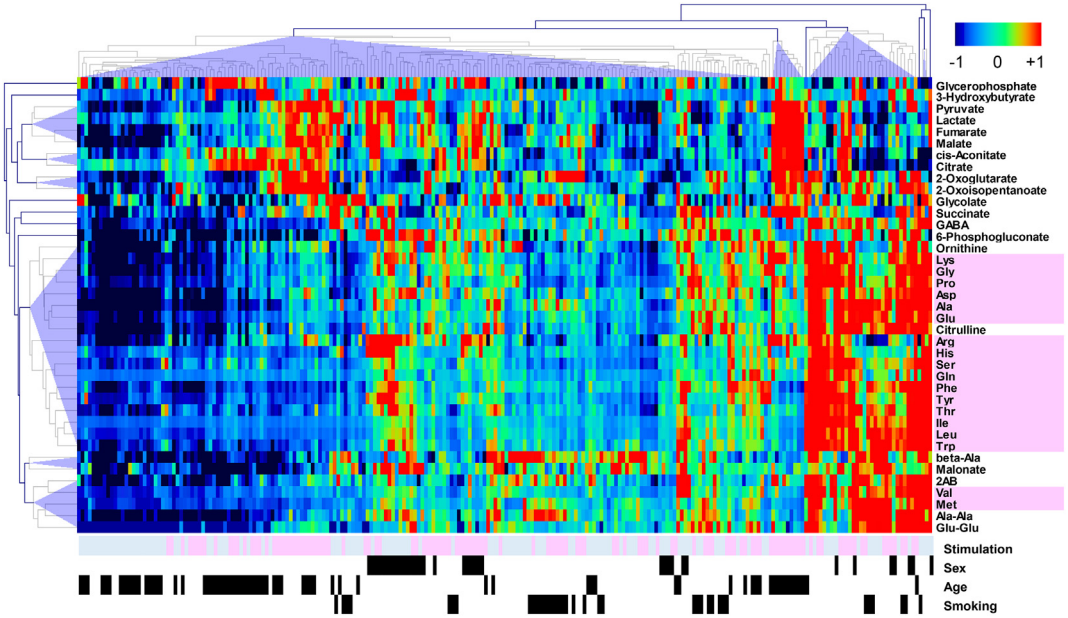


**Fig. 4.** Heatmap for visualization of salivary metabolite concentrations. Absolute metabolite concentrations were converted to *Z*-scores for each metabolite and were colored based on a blue-green-red colour scheme. "Stimulation" is either unstimulated (light blue) or stimulated (pink) saliva. "Sex" is either female (black) or male (white). "Age" is either ≥30 (black) or < 30 (white) years. "Smoking" indicates subjects with smoking habits (black). Among metabolites, amino acids were colored in pink. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

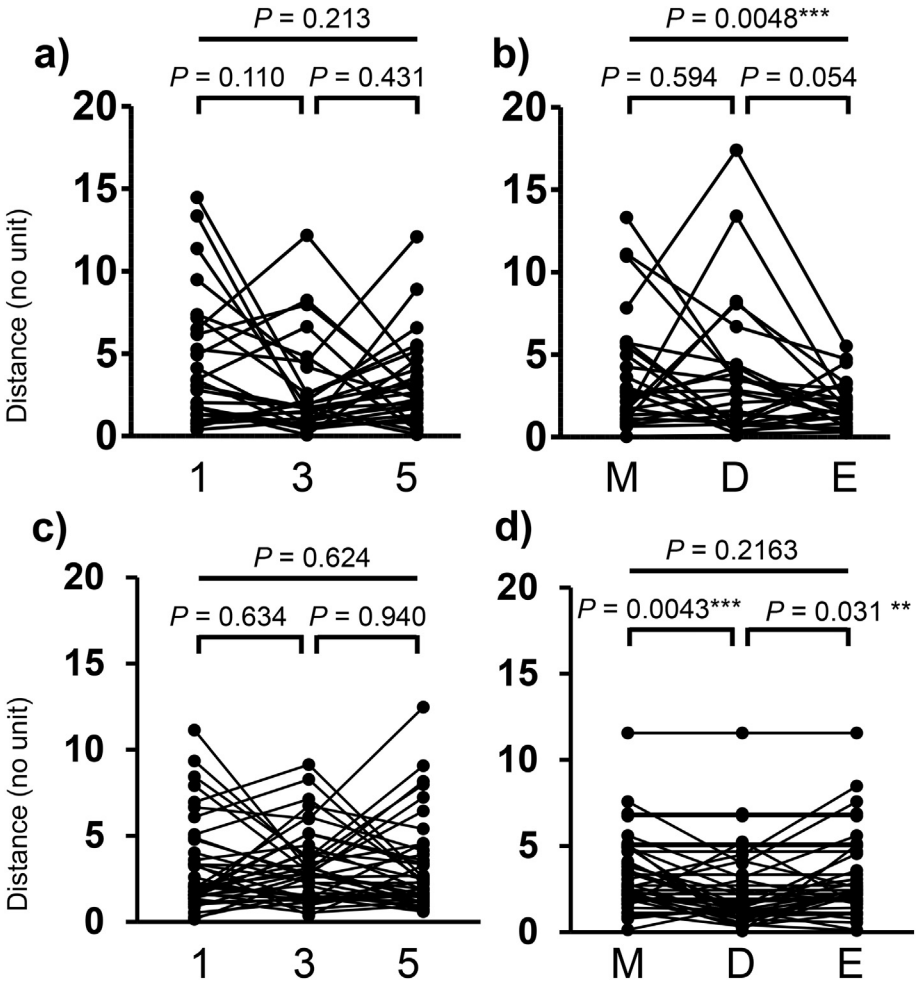


**Fig. 5.** The Euclidean distance of each point was calculated to assess the influence of the sampling time and day. (A) Unstimulated saliva sampling days; (B) unstimulated saliva sampling time-points; (C) stimulated saliva sampling days; (D) stimulated saliva sampling time-points. The labels 1, 3, 5 on the x-axis of panels (A) and (C) indicate day 1, day 3, and day 5, respectively; the labels M, D, and E on the x-axis of panels (B) and (D) indicate sample collection times in the morning, daytime, and evening, respectively. Comparisons of the distances between points on the score plots were analyzed using the Wilcoxon matched-pairs signed-rank test. $^{**}P < .01$ and $^{***}P < .001$.

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