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The impact of saliva collection methods on measured salivary biomarker levels

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ABSTRACT

Saliva diagnostics have become increasingly popular due to their non-invasive nature and patient-friendly collection process. Various collection methods are available, yet these are not always well standardized for either quantitative or qualitative analysis. In line, the objective of this study was to evaluate if measured levels of various biomarkers in the saliva of healthy individuals were affected by three distinct saliva collection methods: 1) unstimulated saliva, 2) chew stimulated saliva, and 3) oral rinse.

Saliva samples from 30 healthy individuals were obtained by the three collection methods. Then, the levels of various salivary biomarkers such as proteins and ions were determined. It was found that levels of various biomarkers obtained from unstimulated saliva were comparable to those in chew stimulated saliva. The levels of potassium, sodium, and amylase activity differed significantly among the three collection methods. Levels of all biomarkers measured using the oral rinse method significantly differed from those obtained from unstimulated and chew-stimulated saliva.

In conclusion, both unstimulated and chew-stimulated saliva provided comparable levels for a diverse group of biomarkers. However, the results obtained from the oral rinse method significantly differed from those of unstimulated and chew-stimulated saliva, due to the diluted nature of the saliva extract.

1. Introduction

Saliva diagnostics have gained increasing popularity due to their non-invasive nature, patient-friendly collection process, and the fact that sample collection does not require trained professionals [1–6]. On one hand, this unique biological fluid contains compounds secreted by the salivary glands, exhibiting multiple functions in the oral cavity to serve the maintenance of oral health and to support the digestion of food [7–9]. On the other hand, salivary compounds can originate from the oral microbiome but also tissues and organs throughout the body entering saliva through acinar cells, gingival crevicular fluid and wounds [10–15]. These salivary components can be designated as biomarkers with the potential to reflect the status of various pathologies, beyond the borders of oral health [16–23], making saliva a promising alternative to blood for monitoring health and disease status as well as determining the effectiveness of drug treatments [24]. Additionally, the convenience of saliva as a readily available and easily collectible sample in public settings makes it a versatile tool that extends beyond clinical applications. For instance, in the screening and monitoring of alcohol and drug abuse [25].

While saliva research has predominantly focused on the development of technologies for biomarker analysis [2,4,26,27], the influence of different saliva sampling methods remains largely unexplored. Collecting a reliable saliva sample can be challenging, as the variability in results can depend on various factors, including the type of saliva collected (whole saliva or gland-specific), circadian rhythm [28] and the presence or absence of stimuli [29–35]. Notably, the lack of standardized methodology may introduce variability in outcomes, subsequently affecting the interpretation and future prospects. To exemplify this potential risk of bias the effect of prolonged chew-stimulation on salivary flow in

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healthy individuals was investigated. It was found that salivary flow peaked within the first 2 min of stimulation, and then gradually decreased to the level of the initial unstimulated flow rate at 25 min to reach reached a plateau [36]. Furthermore, others reported similar results for the salivary flow peak, but noted that after 35 min of chew-stimulation, the flow reached a plateau that was significantly higher than the initial unstimulated flow rate [37]. In contrast, Karami-Nogourani and co-workers found that the salivary flow peak occurred within the first minute of chew-stimulation and reached a plateau that was significantly higher than the initial unstimulated flow rate after 6 min [38]. Despite similar objectives and methodologies, the obtained results differed. These findings suggest the necessity of standardized protocols and methods for saliva collection is required to ensure reliability and reproducibility of research findings.

The aim of our study was therefore to assess the impact and potential use of three distinct saliva collection methods: unstimulated saliva, chew-stimulated saliva, and oral rinse on various types of biomarkers. In fact, a diverse range of potential biomarkers and biomarker activity [39–44], including total protein, MUC5B, albumin, electrolytes (ammonium, sodium, potassium, chloride, phosphate), amylase activity, cortisol, and IL6, were assessed in a healthy population. To our knowledge, this is the first comparative study investigating the effect of different sampling methods on a wide range of biomarkers in a comparative manner. In our view, this research will contribute to the development of robust methodologies that ensure accurate and consistent measurement of saliva biomarkers, providing a solid foundation for effective saliva-based diagnostics.

2. Materials and methods

2.1. Study population

The study received approval from the Ethical Institutional Board of the Utrecht University of Applied Sciences for human studies (no.171-000-2022). Participants were recruited passively from university students and volunteers who visited the dental department at the University Teaching Clinique. Prior to participation, all volunteers were informed about the study's purpose, signed an informed consent form, completed a questionary, and underwent an oral health check at the dental department of the university. Exclusion criteria included presence of oral diseases such as periodontitis and gingivitis, use of medication with anticholinergic effects, pregnancy, and smoking as these factors can alter saliva composition [45–50]. A total of 30 participants were included in the study consisting of 17 females and 13 males (age 31 $\pm$ 13 years, BMI 24 $\pm$ 4 kg/m^2). To ensure consistency and reliability of saliva samples, participants were instructed to abstain from tooth brushing, eating, drinking, exercising, and applying lip make-up products for one hour prior to saliva collection. Additionally, Body Mass Index (BMI) calculations were performed for each participant by dividing the weight in kilograms by the square of their height in meters.

2.2. Saliva collection

Saliva samples were collected using three distinct saliva collection methods: unstimulated saliva, chew-stimulated saliva, and oral rinse. To minimize the impact of variations [10,51,52], all samples were collected between 9:00 and 12:00 a.m. The order of collection methods was randomized for each participant. Before each collection, participants rinsed their mouth with water. A 5 min break was introduced between each collection procedure.

Unstimulated saliva was collected as described previously [31]. Participants were instructed to sit comfortably in an upright position and tilt their heads down slightly to pool saliva in the mouth. Every 30 s, saliva was expectorated into a pre-labeled and pre-weighed 50 mL sterile polypropylene tube. Chew-stimulated saliva was collected essentially as described before [37]. Participants were instructed to chew on a piece of parafilm (5 cm x 5 cm x 0.12 mm). Every 30 s, saliva was expectorated into a pre-labeled and pre-weighed 50 mL sterile polypropylene tube. To collect saliva using the oral rinse method, participants were instructed to rinse their mouth principally as described elsewhere [53,54] with 4 mL Millipore of water for 20 s and then expectorate the sample into a pre-labeled and pre-weighted 50 mL sterile polypropylene tube. During collection all samples were kept on ice**.** Subsequently, saliva samples were centrifuged (2,075 x *g* for 5 min at 4 °C) to remove cellular debris. After centrifugation, the supernatant was carefully separated and aliquoted into 1.5 mL Eppendorf tubes and stored at −80 °C until further analysis.

2.3. Salivary flow rate

Immediately following each collection, the tubes were weighed to calculate the salivary flow rate. This was determined by dividing the weight difference between the empty sterile tube and the tube containing the saliva extract by the collection time (post-weight measure – pre-weight measure / collection time). The outcome measure was expressed in mL/min, with the conversion based on the approximation that 1 g of saliva is roughly equivalent to 1 mL of saliva.

2.4. Quantification of salivary total protein content

The total protein content was measured essentially as described earlier using a Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Bleiswijk, The Netherlands), according to manufacturer's instructions [55]. Saliva samples were 1:3 v:v prediluted in PBS. A calibration curve was included using serial dilutions of bovine serum albumin ranging from 0 to 1500 μg/mL. A volume of 20 μL of prediluted samples and standards was added to 180 μL of protein selective detection reagent in 96-well microplates (Greiner Bio-One, Frieckenhausen, Germany). The absorbance was measured at 562 nm with a plate spectrophotometer reader (Multiskan FC, Thermo Scientific, Waltham, MA, USA). Salivary total protein levels were calculated in mg/mL.

2.5. Quantification of salivary α-amylase activity

Salivary α–amylase activity was determined using a colorimetric enzymatic assay, essentially as described earlier [56]. Saliva samples were 1:100 v:v prediluted in Millipore water and 10 μL was added to 90 μL of 2-chloro-4-nitrophenyl-α-D-maltotrioside, an amylase-specific substrate (Sigma-Aldrich, Zwijndrecht, The Netherlands). The rate of formation of the degradation product (2-chloro-4-nitrophenol) was measured photometrically using a plate spectrophotometer reader (Multiskan FC) at 405 nm for 15 min, providing a direct measure of the of α–amylase activity. The enzyme activity (U/mL) was calculated using a reference of known concentration of α-amylase (1 U) measured together with the samples.

2.6. Quantification of salivary cortisol levels

Cortisol levels were measured using the Cortisol Competitive ELISA Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Samples were undiluted. A calibration curve was included of 0 to 3200 pg/mL cortisol. Absorbance was measured at 450 nm using a plate spectrophotometer reader (Multiskan FC). Salivary cortisol levels were calculated using 4 parametric logistic regression and expressed in pg/mL.

2.7. Quantification of salivary IL6 levels

The levels of IL-6 were measured using the IL-6 Human High Sensitivity in vitro ELISA kit (Abcam B.V., Amsterdam, The Netherlands), using the manufacturer's instructions. Samples were analyzed undiluted. A calibration curve was included utilizing a series of IL-6

standards ranging from 0 to 50 pg/mL. Absorbance was measured at 450 nm using a plate spectrophotometer reader (Multiskan FC). Salivary IL6 levels were calculated using 4 parametric logistic regression and expressed in pg/mL.

2.8. Quantification of salivary MUC5B levels

MUC5B levels were measured by ELISA essentially as described earlier [57]. Briefly, samples were diluted (1:800 v:v) in coating buffer (0.1 M $NaHCO_3$, pH 9.6). A calibration curve was included using standard of MUC5B in concentration ranging from of 0 to 6 μg/mL. Samples and standards were then coated onto NUNC MaxiSorp™ High protein-binding 96-well ELISA plates (Thermo Fisher Scientific) and incubated overnight at 4 °C. The levels of MUC5B were then measured using F2, a MUC5B specific antibody [57]. The absorbance was measured at 492 nm using a plate spectrophotometer reader (Multiskan FC). MUC5B levels were calculated using 4 parametric logistic regression in mg/mL.

2.9. Quantification of salivary albumin levels

Albumin levels were measured by a sandwich ELISA as previously described [58]. Briefly, 100 uL of diluted (1:8000 v:v in PBS + 0.1 % Tween20) target-specific antibody (Rabbit anti-human albumin, cat# A0001 DAKO) was coated on high binding 96-well ELISA plates (Thermo Fisher Scientific) and incubated overnight at 4 °C. 100 μL of samples were 1:1000 v:v diluted in PBS + 0.1 % Tween-20 and added to the microplates. A standard was included. Absorbance was measured at 492 nm using a plate spectrophotometer reader (Multiskan FC), and salivary albumin levels were calculated using 4 parametric logistic regression in mg/mL.

2.10. Quantification of salivary electrolyte levels

The electrolyte composition (cations and anions) of the samples was determined using a Capillary Electrophoresis system (CAPEL-205 Lumex Instruments Canada, Mission, BC, Canada). The samples were diluted in Millipore water (1:10 v:v) and the concentration was measured using cation standards contained with ammonium, potassium, sodium, magnesium, strontium, barium, and calcium and anion standards of chloride, nitrite, sulphate, nitrate, fluoride, and phosphate. The analysis involved the use of a cation background electrolyte buffer (BGE) solution, which consisted of 20 mM benzimidazole, 5 mM tartaric acid, and 2 mM 18-Crown-6, identified by kit number 0,300,001,550 according to the instructions of the manufacturer. For the anion analysis, an anion BGE buffer solution was employed, which contained 10 mM chromium (VI) oxide, 30 mM diethanolamine (DEA), and 2 mM hexadecyltrimethylammonium hydroxide (CTA-OH), identified by kit number 0300001523.The quantification of electrolyte levels in saliva was carried out through the external calibration method, with data analysis performed using the ELFORUN-205 software (Envico, Zoeterwoude, The Netherlands). Electrolyte levels were calculated in millimolar (mM).

2.11. Data analysis

The statistical analysis was conducted using IBM SPSS 28.0 software (IBM, Armonk, NY, U.S.A.). Non-parametric tests were used, and measures of central tendency were expressed as medians with interquartile ranges. To compare quantitative data, the Friedman's test was used to evaluate the overall group differences while the Wilcoxon's test was used for pairwise comparisons between the different sampling methods. The Mann-Whitney *U* test was used to compare independent measures specifically between females and males and the Spearman's rank test was used to assess correlations. A significance level of 0.05 was used to determine statistical significance.

3. Results

3.1. Effect of sampling method on salivary flow rate

Table 1 displays the findings of salivary flow rate in both unstimulated and chew-stimulated saliva. As, by oral rinse, the sample volume, and inherently, the saliva volume varied tremendously by an unknown dilution factor, it was not possible to calculate the flow rate using this method. Among the total population, significant differences were observed between the salivary flow rates of unstimulated and chew-stimulated saliva ($p < 0.001$). Unstimulated saliva exhibited a lower flow rate (0.28 mL/min) compared to chew-stimulated saliva (1.4 mL/min). Although unstimulated and chew-stimulated saliva samples collected from male subjects showed higher salivary flow rates than those collected from female subjects, these differences were not statistically significant ($p = 0.169$ and 0.425) (table 1).

3.2. Comparison of measured salivary biomarker levels using different sampling methods

The results of the biomarker analysis from the different sampling methods (unstimulated, chew-stimulated, and oral rinse) are summarized in Table 2. The data indicate that the overall p-value for all outcome measures is significant ($p < 0.05$). Additionally, the pairwise comparison between the different methods revealed that levels of various salivary biomarkers, including ammonium, calcium, chloride, phosphate, total protein content, MUC5B, albumin, IL-6, and cortisol are comparable between unstimulated and chew-stimulated saliva. In contrast, the levels of potassium, sodium, and amylase activity demonstrated an apparent reverse pattern, with chew-stimulated saliva exhibiting significantly higher ($p < 0.05$) levels compared to unstimulated saliva. In general terms, comparing the levels of the various biomarkers obtained from oral rinse with unstimulated or chew-stimulated saliva, the results for all biomarkers were found to be significantly different ($p < 0.05$).

3.3. Correlation between salivary flow rate and the various biomarkers

The impact of the salivary flow rate on the various biomarkers is shown in Table 3. The data suggests that potassium, sodium, calcium, and amylase activity, are influenced by the salivary flow rate. In particular, sodium exhibits a pronounced dependence on salivary flow, with a strong correlation ($r = 0.539$, $p < 0.001$). All other biomarkers, including ammonium, chloride, phosphate, total protein, MUC5B, albumin, IL6, and cortisol did not significantly correlate to flow rate.

4. Discussion

This study aimed to explore the influence of various saliva sampling methods on the measured levels of saliva biomarkers in the samples. For

Table 1
Salivary flow rate (mL/min.) of saliva samples collected under unstimulated and chew stimulated conditions.

	Total population		
	Median (IQR)		P-value
Unstimulated saliva	0.28 (0.18–0.63)		(a) < 0.001
Chew stimulated saliva	1.4 (0.80–2.4)		
	Comparison based on sex		
	Female	**Male**	
	Median (IQR)	Median (IQR)	P-value
Unstimulated saliva	0.26 (0.15–0.51)	0.41 (0.20–1.2)	(b) 0.169
Chew stimulated saliva	1.4 (0.72–2.1)	1.8 (0.92–2.5)	(b) 0.425

(a) Wilcoxon signed-rank Test; IQR = interquartile range;
(b) Mann-Whitney U Test; IQR = interquartile range.
$P < 0.05$ is considered significant.

Table 2
Concentrations of different biomarkers measured in saliva samples collected under unstimulated, chew-stimulated, and oral rinse conditions.

Biomarker type	Biomarker	Unstimulated saliva	Chew stimulated saliva	Oral rinse	P-value			Overall p-value
		Median (IQR)	Median (IQR)	Median (IQR)	1 vs. 2*	1 vs. 3*	2 vs. 3*	
ELECTROLYTES	Ammonium (mM)	0.931 (0.554–1.45)	1.12 (0.377–1.98)	0.283 (0.155–0.421)	0.371	<0.001	<0.001	<0.001
	Potassium (mM)	4.60 (3.94–6.09)	5.68 (5.12–6.63)	1.10 (0.844–1.53)	<0.001	<0.001	<0.001	<0.001
	Sodium (mM)	0.866 (0.609–1.23)	2.04 (1.31–3.22)	0.317 (0.250–0.522)	<0.001	<0.001	<0.001	<0.001
	Calcium (mM)	0.284 (0.237–0.349)	0.289 (0.242–0.342)	0.0674 (0.0524–0.0811)	0.773	<0.001	<0.001	<0.001
	Chloride (mM)	3.92 (2.91–4.77)	4.57 (3.64–6.15)	1.38 (1.10–1.69)	0.133	<0.001	<0.001	<0.001
	Phosphate (mM)	2.12 (1.54–3.31)	1.55 (1.37–2.43)	0.605 (0.484–1.05)	0.267	<0.001	<0.001	<0.001
PROTEINS	Amylase activity (U/mL)	33.0 (14.5–71.0)	54.0 (27.5–98.7)	0.522 (0.0490–2.44)	0.001	<0.001	<0.001	<0.001
	Total protein content (mg/mL)	1.25 (0.900–1.53)	1.25 (1.00–1.53)	0.128 (0.0600–0.228)	0.665	<0.001	<0.001	<0.001
	MUC5B (mg/mL)	17.9 (10.1–24.6)	18.1 (8.90–28.7)	9.53 (4.82–14.4)	0.140	0.010	<0.001	<0.001
	Albumin (mg/mL)	0.0410 (0.0235–0.0505)	0.0350 (0.0245–0.0705)	0.0185 (0.0105–0.0230)	0.977	<0.001	<0.001	<0.001
CYTOKINE	IL6 (pg/mL)	1.22 (0.542–1.74)	1.51 (0.975–2.38)	0.660 (0.000–0.990)	0.256	<0.001	<0.001	<0.001
HORMONE	Cortisol (pg/mL)	111 (47.0–206)	89.5 (45.8–203)	32.0 (25.5–45.3)	1.00	0.002	0.002	<0.001

Wilcoxon signed-rank Test for pairwise comparison of the different methods; IQR = interquartile range.
Friedman's Test to estimate the overall group differences.
$P < 0.05$ is considered significant.
*1 = unstimulated saliva; 2 = chew stimulated saliva; 3 = oral rinse.

Table 3
Correlation between salivary flow rate and the different biomarkers.

Biomarker	r	p-value
Ammonium (mM)	−0.116	0.384
Potassium (mM)	0.260	0.048
Sodium (mM)	0.539	<0.001
Calcium (mM)	−0.379	0.003
Chloride (mM)	0.274	0.106
Phosphate (mM)	−0.172	0.316
Amylase activity (U/mL)	0.260	0.048
Total protein content (mg/mL)	−0.126	0.347
MUC5B (mg/mL)	−0.302	0.055
Albumin (mg/mL)	−0.239	0.133
IL6 (pg/mL)	0.096	0.472
Cortisol (pg/mL)	−0.214	0.316

Spearman's rank Test;
$P < 0.05$ is considered significant.

this, three distinct saliva collection methods were selected including two widely utilized methods *i.e.,* unstimulated, and chew-stimulated saliva and the oral rinse, which can be considered as a relative novel oral sampling method [53,54]. By comparing the results obtained from these different sampling methods, we attempted to gain insights into any variations in the measured levels of salivary biomarkers that were inherently induced by the selected sampling methods. Additionally, we aimed to evaluate the potential applicability of these diverse sampling methods in saliva research.

The results of the current study show that the salivary flow of unstimulated (0.28 mL/min) and chew-stimulated (1.4 mL/min) saliva significantly differed from each other ($p < 0.05$). These findings are consistent with previously reported for healthy individuals [10,59,60]. The difference in salivary secretion between unstimulated and chew-stimulated saliva can be explained by the effect of mechanical stimulation. The parotid glands are strongly affected by mechanical stimuli, leading to an increase in salivary secretion [24]. Moreover, the results of this study provide clear evidence that the levels of a diverse range of salivary biomarkers, including ammonium, calcium, chloride, phosphate, total protein, MUC5B, albumin, IL6, and cortisol, remain unaffected by chew-stimulation, resulting in comparable levels between unstimulated and chew-stimulated saliva ($p > 0.05$).

Notably, however, chew stimulation showed a significant increase on the salivary levels of potassium and sodium ($p < 0.05$). In particular, sodium showed a significant dependence on salivary flow ($r = 0.539$, $p < 0.001$). A possible explanation for this is that chew stimulation leads to an increase in watery saliva production. The increased saliva volume within the gland duct system accelerates the flow of saliva through the ducts, influencing the salivation process [24]. In the duct cells, an exchange of sodium and potassium takes place during secretion, where sodium is reabsorbed while potassium is excreted [61]. This ion exchange is facilitated by Na^+/K^+ pumps which require ATP [62]. While an increased concentration of sodium ions in saliva may serve as a possible stimulus for the activity of the Na^+/K^+ pump, in turn a higher salivary flow rate will limit the Na^+/K^+ exchange resulting in relative higher sodium levels reaching the oral cavity [63]. This explanation also supports the observed positive correlation between sodium levels and salivary flow rate.

Additionally, chew stimulation also showed a significant increase on the salivary α-amylase activity ($p < 0.05$). This finding was consistent with a previous study that investigated the effect of chewing on salivary flow rate and amylase activity [64]. Chewing activates oral mechanoreceptors [65] and stimulates autonomic nerve function, leading to the secretion of salivary α-amylase primarily in the parotid glands, and to a lesser extent, in the submandibular glands [10,66].

Comparison of the three distinct methods for saliva collection (unstimulated, chew-stimulated, and oral rinse) revealed that the levels of various biomarkers in unstimulated saliva are comparable to those obtained by chew-stimulated saliva ($p > 0.05$). Both unstimulated and chew-stimulated saliva originate from crude saliva extracts, which is not diluted with any in *vitro fluid*, *e.g.,* the oral rinse. Therefore, both methods are suitable for quantitative analysis, providing reliable and accurate outcomes that can assist in identifying patterns and predicting results, as well as qualitative analysis, which is valuable in exploring new insights and screening for salivary biomarkers [67]. However, the benefit of quantitative analysis over qualitative analysis is that the outcomes obtained can be directly compared to results from blood tests which can be useful *e.g.,* in clinical studies.

The outcomes of the oral rinse method were remarkably different, as all measured biomarkers showed significantly lower levels compared to those observed in both unstimulated and chew-stimulated saliva ($p < 0.05$). The reason for these differences lies in the fact that the results from the oral rinse method were obtained from a diluted saliva extract (a mixture of the rinsing solution and collected saliva) with an unknown dilution factor. Consequently, the levels of measured biomarkers are variable and much lower than those obtained from unstimulated and chew-stimulated saliva. Therefore, comparing the oral rinse method with unstimulated and chew-stimulated saliva, in this stage, is not a

justified comparison. To enable a meaningful comparison between the oral rinse method and other methods, it is necessary to account for differences in saliva volume. This can *e.g.,* be achieved by using an internal standard, similar to how it is done in urine analysis where creatinine serves as an internal standard [68]. The utilization of an internal standard in saliva research has not been previously applied or investigated, and further studies are needed to assess its feasibility and effectiveness. Despite its limitations in terms of providing accurate quantitative data, the oral rinse method remains a valuable tool for qualitative analysis, allowing for the exploration of the oral composition through screening. According to previous research, the composition of saliva can vary based on its location within the oral cavity. For example, Assy et al. reported that the concentrations of MUC5B were significantly higher in the anterior tongue as compared to the interior palate [69]. In addition, Barrett et al. observed the expression of lower molecular weight keratins, including 4 and 13, in the buccal regions [70]. So, although the oral rise method seems to be suitable primarily for qualitative analysis, we feel it tempting to suggest that the oral rinse method may have an advantage over unstimulated and chew-stimulated saliva by sampling the full intra-oral cavity.

5. Conclusion

In conclusion, the levels of various biomarkers, including electrolytes, proteins, and other components, were found to be comparable in both unstimulated and chew-stimulated saliva. Consequently, the choice of sampling method becomes inconsequential for these biomarkers. Furthermore, this suggests that results obtained from different saliva studies where these two methods have been applied, can be compared to each other.

Both unstimulated and chew-stimulated saliva were found to be suitable and useful for quantitative as well as qualitative analysis.

On the other hand, the use of the oral rinse method seems to have limitations arising from the diluted nature of the saliva extract. Nevertheless, the oral rinse method has potential for use in qualitative research, providing a rapid screening tool to detect the presence or absence of specific saliva biomarkers and allowing for exploration of new insights. To adapt this method for quantitative saliva research, optimization measures seem necessary, such as accounting for volume differences through the use of an internal standard.

CRediT authorship contribution statement

H. Al Habobe: Conceptualization, Methodology, Software, Data curation, Visualization, Investigation, Validation, Formal analysis, Project administration, Writing - original draft, Writing - review & editing. **E.B. Haverkort:** Conceptualization, Writing - review & editing. **K. Nazmi:** Methodology. **R.H.H. Pieters:** Conceptualization, Writing - review & editing. **F.J. Bikker:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] C.K. Yeh, N.J. Christodoulides, P.N. Floriano, C.S. Miller, J.L. Ebersole, S. E. Weigum, J. McDevitt, S.W. Redding, Current development of saliva/oral fluid-based diagnostics, Tex. Dent. J. 127 (7) (2010) 651–661.
[2] T. Pfaffe, J. Cooper-White, P. Beyerlein, K. Kostner, C. Punyadeera, Diagnostic potential of saliva: current state and future applications, Clin. Chem. 57 (5) (2011) 675–687.
[3] M. Navazesh, Methods for collecting saliva, Ann. N. Y. Acad. Sci. 694 (1993) 72–77.
[4] M. Gröschl, H. Köhler, H.G. Topf, T. Rupprecht, M. Rauh, Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs, J. Pharm. Biomed. Anal. 47 (3) (2008) 478–486.
[5] F.G. Bellagambi, T. Lomonaco, P. Salvo, F. Vivaldi, M. Hangouët, S. Ghimenti, A. Errachid, Saliva sampling: Methods and devices. An overview, TrAC Trends Anal. Chem. 124 (2020), 115781.
[6] D. Malamud, Saliva as a diagnostic fluid, Dent. Clin. N. Am. 55 (1) (2011) 159–178.
[7] A.V. Amerongen, E.C. Veerman, Saliva–the defender of the oral cavity, Oral Dis. 8 (1) (2002) 12–22.
[8] M. Faruque, M. Wanschers, A.J. Ligtenberg, M.L. Laine, F.J. Bikker, A review on the role of salivary MUC5B in oral health, J. Oral Biosci. 64 (4) (2022) 392–399.
[9] M. Waasdorp, B.P. Krom, F.J. Bikker, P.P.M. van Zuijlen, F.B. Niessen, S. Gibbs, The Bigger Picture: Why Oral Mucosa Heals Better Than Skin, Biomolecules 11 (8) (2021) 1165.
[10] S.P. Humphrey, R.T. Williamson, A review of saliva: normal composition, flow, and function, J. Prosthet. Dent. 85 (2) (2001) 162–169.
[11] E. Roussa, Channels and transporters in salivary glands, Cell Tissue Res. 343 (2) (2011) 263–287.
[12] L. Eliasson, A. Carlén, An update on minor salivary gland secretions, Eur. J. Oral Sci. 118 (5) (2010) 435–442.
[13] P.D.V. de Almeida, A.M. Gregio, M.A. Machado, A.A. De Lima, L.R. Azevedo, Saliva composition and functions: a comprehensive review, J Contemp Dent Pract 9 (3) (2008) 72–80.
[14] D.P. Lima, D.G. Diniz, S.A.S. Moimaz, D.H. Sumida, A.C. Okamoto, Saliva: reflection of the body, Int. J. Infect. Dis. 14 (3) (2010) e184–e188.
[15] E. Hyvärinen, B. Kashyap, A.M. Kullaa, Oral Sources of Salivary Metabolites, Metabolites 13 (4) (2023) 498.
[16] R.G. Schipper, E. Silletti, M.H. Vingerhoeds, Saliva as research material: biochemical, physicochemical and practical aspects, Arch. Oral Biol. 52 (12) (2007) 1114–1135.
[17] C.F. Streckfus, L.R. Bigler, Saliva as a diagnostic fluid, Oral Dis. 8 (2) (2002) 69–76.
[18] M. Greabu, M. Battino, M. Mohora, A. Totan, A. Didilescu, T. Spinu, R. Radulescu, Saliva–a diagnostic window to the body, both in health and in disease, J. Med. Life 2 (2) (2009) 124–132.
[19] S. Amer, M.U.N.I. Yousuf, P.Q. Siddqiui, J.U.N.A. Alam, Salivary glucose concentrations in patients with diabetes mellitus–a minimally invasive technique for monitoring blood glucose levels, Pak J Pharm Sci 14 (1) (2001) 33–37.
[20] J.A. Loo, W. Yan, P. Ramachandran, D.T. Wong, Comparative human salivary and plasma proteomes, J. Dent. Res. 89 (10) (2010) 1016–1023.
[21] C.Z. Zhang, X.Q. Cheng, J.Y. Li, P. Zhang, P. Yi, X. Xu, X.D. Zhou, Saliva in the diagnosis of diseases, Int. J. Oral Sci. 8 (3) (2016) 133–137.
[22] A. Segal, D.T. Wong, Salivary diagnostics: enhancing disease detection and making medicine better. European journal of dental education: official journal of the Association for Dental Education, Europe 12 Suppl 1(Suppl 1) (2008) 22–29.
[23] Y.H. Lee, D.T. Wong, Saliva: an emerging biofluid for early detection of diseases, Am. J. Dent. 22 (4) (2009) 241.
[24] J.K. Aps, L.C. Martens, Review: The physiology of saliva and transfer of drugs into saliva, Forensic Sci. Int. 150 (2–3) (2005) 119–131.
[25] N. Waszkiewicz, S. Chojnowska, A. Zalewska, K. Zwierz, A. Szulc, S.D. Szajda, Salivary hexosaminidase in smoking alcoholics with bad periodontal and dental states, Drug Alcohol Depend. 129 (1–2) (2013) 33–40.
[26] D.R. Walt, T.M. Blicharz, R.B. Hayman, D.M. Rissin, M. Bowden, W.L. Siqueira, E. J. Helmerhorst, N. Grand-Pierre, F.G. Oppenheim, J.S. Bhatia, F.F. Little, J. S. Brody, Microsensor arrays for saliva diagnostics, Ann. N. Y. Acad. Sci. 1098 (1) (2007) 389–400.
[27] C. Holm-Hansen, G. Tong, C. Davis, W.R. Abrams, D. Malamud, Comparison of oral fluid collectors for use in a rapid point-of-care diagnostic device, Clin. Diagn. Lab. Immunol. 11 (5) (2004) 909–912.
[28] C. Dawes, Circadian rhythms in human salivary flow rate and composition, J. Physiol. 220 (3) (1972) 529–545.
[29] M. Navazesh, S.K. Kumar, University of Southern California School of Dentistry, Measuring salivary flow: challenges and opportunities, J. American Dental Assoc. (1939), 139 Suppl (2008) 35S–40S.
[30] D.J. Crouch, Oral fluid collection: the neglected variable in oral fluid testing, Forensic Sci. Int. 150 (2–3) (2005) 165–173.
[31] M. Navazesh, C.M. Christensen, A comparison of whole mouth resting and stimulated salivary measurement procedures, J. Dent. Res. 61 (10) (1982) 1158–1162.
[32] H. Flink, A. Tegelberg, F. Lagerlöf, Influence of the time of measurement of unstimulated human whole saliva on the diagnosis of hyposalivation, Arch. Oral Biol. 50 (6) (2005) 553–559.
[33] M. Gröschl, M. Rauh, Influence of commercial collection devices for saliva on the reliability of salivary steroids analysis, Steroids 71 (13–14) (2006) 1097–1100.
[34] C. Fenoll-Palomares, J.V. Muñoz Montagud, V. Sanchiz, B. Herreros, V. Hernández, M. Mínguez, A. Benages, Unstimulated salivary flow rate, pH and buffer capacity of saliva in healthy volunteers, Rev. Esp. Enferm. Dig. 96 (11) (2004) 773–783.

[35] C. Golatowski, M.G. Salazar, V.M. Dhople, E. Hammer, T. Kocher, N. Jehmlich, U. Völker, Comparative evaluation of saliva collection methods for proteome analysis, Clin. Chim. Acta; Int. J. Clin. Chem. 419 (2013) 42–46.
[36] K.E. Polland, F. Higgins, R. Orchardson, Salivary flow rate and pH during prolonged gum chewing in humans, J. Oral Rehabil. 30 (9) (2003) 861–865.
[37] C. Dawes, K. Kubieniec, The effects of prolonged gum chewing on salivary flow rate and composition, Arch. Oral Biol. 49 (8) (2004) 665–669.
[38] M. Karami-Nogourani, R. Kowsari-Isfahan, M. Hosseini-Beheshti, The effect of chewing gum's flavor on salivary flow rate and pH, Dental Research Journal 8 (Suppl 1) (2011) S71–S75.
[39] M. Falchi, J.S. Moustafa, P. Takousis, et al., Low copy number of the salivary amylase gene predisposes to obesity, Nat Genet. 46 (5) (2014) 492–497.
[40] H. Viljakainen, J.C. Andersson-Assarsson, M. Armenio, et al., Low copy number of the AMY1 locus is associated with early-onset female obesity in Finland, PLoS One 10 (7) (2015) e0131883.
[41] P.B. Vaziri, M.O.H.S.E.N. Vahedi, S.H. Abdollahzadeh, H.R. Abdolsamadi, M. Hajilooi, S.H. Kasraee, Evaluation of salivary albumin in diabetic patients, Iran. J. Public Health 38 (3) (2009) 54–59.
[42] E.S. Frenkel, K. Ribbeck, Salivary mucins in host defense and disease prevention, J. Oral Microbiol. 7 (2015) 29759.
[43] M. Anwar, B.F. Alam, S. Ali, S.F. Tariq, K. Aali, E. Abrar, D.H. Alotaibi, et al., Evaluation of Salivary Mucin, Amylase, Protein Profile, and Periodontal Parameters among Hypertensive and Diabetic Patients, Appl. Sci. 12 (15) (2022) 7407.
[44] S. Cozma, L.C. Dima-Cozma, C.M. Ghiciuc, V. Pasquali, A. Saponaro, F. R. Patacchioli, Salivary cortisol and α-amylase: subclinical indicators of stress as cardiometabolic risk, Braz. J. Med. Biol. Res. 50 (2017).
[45] P. Axelsson, J. Paulander, J. Lindhe, Relationship between smoking and dental status in 35-, 50-, 65-, and 75-year-old individuals, J. Clin. Periodontol. 25 (4) (1998) 297–305.
[46] M. Edgar, C. Dawes, D.O. Saliva, Saliva and oral health an essential overview for the health professional fourth edition, 2012.
[47] W.M. Thomson, J.M. Chalmers, A.J. Spencer, G.D. Slade, Medication and dry mouth: findings from a cohort study of older people, J. Public Health Dent. 60 (1) (2000) 12–20.
[48] J. Guggenheimer, P.A. Moore, Xerostomia: etiology, recognition and treatment, J. American Dental Assoc. (1939), 134(1) (2003) 61–119.
[49] I.V. Grigoriev, L.V. Nikolaeva, I.D. Artamonov, Protein content of human saliva in various psycho-emotional states, Biochemistry. Biokhimiia 68 (4) (2003) 405–406.
[50] P.V. Rao, A.P. Reddy, X. Lu, S. Dasari, A. Krishnaprasad, E. Biggs, S.R. Nagalla, Proteomic identification of salivary biomarkers of type-2 diabetes, J. Proteome Res. 8 (1) (2009) 239–245.
[51] C.P. Bots, H.S. Brand, E.C.I. Veerman, B.M. Van Amerongen, A.N. Amerongen, Preferences and saliva stimulation of eight different chewing gums, Int. Dent. J. 54 (3) (2004) 143–148.
[52] K.D. White, Salivation: the significance of imagery in its voluntary control, Psychophysiology 15 (3) (1978) 196–203.
[53] P. Katsiki, K. Nazmi, B.G. Loos, M.L. Laine, K. Schaap, E. Hepdenizli, F.J. Bikker, H. S. Brand, E.C.I. Veerman, E.A. Nicu, Comparing periodontitis biomarkers in saliva, oral rinse and gingival crevicular fluid: A pilot study, J. Clin. Periodontol. 48 (9) (2021) 1250–1259.
[54] X. Fan, B.A. Peters, D. Min, J. Ahn, R.B. Hayes, Comparison of the oral microbiome in mouthwash and whole saliva samples, PLoS One 13 (4) (2018) e0194729.
[55] F.J. Bikker, G.G. Nascimento, K. Nazmi, A. Silbereisen, G.N. Belibasakis, W. E. Kaman, R. Lopez, N. Bostanci, Salivary Total Protease Activity Based on a Broad-Spectrum Fluorescence Resonance Energy Transfer Approach to Monitor Induction and Resolution of Gingival Inflammation, Mol. Diagn. Ther. 23 (5) (2019) 667–676.
[56] P. Morquecho-Campos, F.J. Bikker, K. Nazmi, K. de Graaf, M.L. Laine, S. Boesveldt, Impact of food odors signaling specific taste qualities and macronutrient content on saliva secretion and composition, Appetite 143 (2019), 104399.
[57] E.C. Veerman, J.G. Bolscher, B.J. Appelmelk, E. Bloemena, T.K. van den Berg, A. V. Nieuw Amerongen, A monoclonal antibody directed against high M(r) salivary mucins recognizes the SO3-3Gal beta 1–3GlcNAc moiety of sulfo-Lewis(a): a histochemical survey of human and rat tissue, Glycobiology 7 (1) (1997) 37–43.
[58] G.H. Hoek, H.S. Brand, E.C. Veerman, A.V. Amerongen, Toothbrushing affects the protein composition of whole saliva, Eur. J. Oral Sci. 110 (6) (2002) 480–481.
[59] G. Iorgulescu, Saliva between normal and pathological. Important factors in determining systemic and oral health, J. Med. Life 2 (3) (2009) 303–307.
[60] L.M. Sreebny, Saliva in health and disease: an appraisal and update, Int. Dent. J. 50 (3) (2000) 140–161.
[61] J. Sneyd, E. Crampin, D. Yule, Multiscale modelling of saliva secretion, Math. Biosci. 257 (2014) 69–79.
[62] A.S. Pivovarov, F. Calahorro, R.J. Walker, Na+/K+-pump and neurotransmitter membrane receptors, Invertebrate Neuroscience: IN 19 (1) (2018) 1.
[63] S. Sundelacruz, M. Levin, D.L. Kaplan, Role of membrane potential in the regulation of cell proliferation and differentiation, Stem Cell Rev. Rep. 5 (3) (2009) 231–246.
[64] D.A. Mackie, R.M. Pangborn, Mastication and its influence on human salivary flow and alpha-amylase secretion, Physiol. Behav. 47 (3) (1990) 593–595.
[65] G.B. Proctor, The physiology of salivary secretion, Periodontology 2000, 70(1) (2016) 11–25.
[66] L.C. Schenkels, E.C. Veerman, A.V. Nieuw Amerongen, Biochemical composition of human saliva in relation to other mucosal fluids, Critical Reviews in Oral Biology and Medicine: an Official Publication of the American Association of Oral Biologists 6 (2) (1995) 161–175.
[67] D. Harvey, Modern Analytical Chemistry, 1st edition, James M. Smith, USA, 2000.
[68] C.E. West, B.N. Rhodes, Determination of Urinary Creatinine in Washington State Residents via Liquid Chromatography/Tandem Mass Spectrometry, International Journal of Analytical Chemistry 2014 (2014), 247316.
[69] Z. Assy, D.H.J. Jager, H.S. Brand, F.J. Bikker, Salivary film thickness and MUC5B levels at various intra-oral surfaces, Clin. Oral Invest. 27 (2) (2023) 859–869.
[70] A.W. Barrett, S. Selvarajah, S. Franey, K.A. Wills, B.K.B. Berkovitz, Interspecies variations in oral epithelial cytokeratin expression, The Journal of Anatomy 193 (2) (1998) 185–193.