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Impact of different pre-analytical protocols on total protein concentration in human saliva

Impacto de diferentes protocolos pré-analíticos na concentração de proteínas totais em saliva humana

Impacto de diferentes protocolos preanalíticos en la concentración de proteínas totales en saliva humana

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ABSTRACT

Objective: To evaluate and compare the protein concentrations in whole saliva samples subjected to different pre-analytical processing protocols. **Methods:** Whole saliva was collected from 10 participants aged 23 to 65 years (mean age: 31.10 ± 4.059), and salivary flow rate was calculated (mL/min). Samples were aliquoted and assigned to six distinct groups (G1–G6), each reflecting a unique pre-analytical protocol combining the following combination of pre-analytical factors, including centrifugation, use of protease inhibitors, temperature, and storage duration. Total protein concentration was measured in duplicate using the bicinchoninic acid (BCA) method. Statistical significance was set at $p < 0.05$. **Results:** A significant negative correlation was observed between salivary flow rate and total protein concentration in G1 ($r = -0.794$; $p = 0.020$), G4 ($r = -0.690$; $p = 0.027$), and G5 ($r = -0.626$; $p = 0.05$). In G3, total protein concentration was significantly positively correlated with age (rho = 0.762; $p = 0.010$). No statistically significant differences were found in mean total protein concentrations across groups ($F(5,45) = 1.132$; $p = 0.358$). Similarly, no differences were found in flow rate–normalized protein concentrations ($F(5,45) = 2.068$; $p = 0.087$). **Conclusion:** Methodological variations in saliva sample processing did not significantly affect total protein concentrations, suggesting robustness of this biomarker to pre-analytical conditions.

Keywords: Saliva, Total protein, Protease inhibitors, Salivary flow.

RESUMO

Objetivo: Avaliar e comparar a concentração proteica de amostras de saliva total submetidas a alterações metodológicas no preparo pré-analítico. **Métodos:** Saliva total de 10 participantes de 23 a 65 anos (31.10±4.059) e o fluxo salivar foi calculado (mL/min). As amostras foram fracionadas e divididas em seis diferentes grupos. Cada grupo (G1-G6) correspondeu a um diferente tipo de preparo pré-analítico, combinando os seguintes fatores: centrifugação, uso de inibidor, temperatura e tempo de conservação. A concentração de proteínas totais foi avaliada pelo método do ácido bicinconínico em duplicata, e os dados analisados estatisticamente ($p<0.05$). **Resultados:** A concentração média de proteínas totais demonstrou uma correlação negativa significativa com o fluxo salivar em G1 ($r= -0,794$; $p= 0,020$), G4 ($r= -0,690$; $p= 0,027$) e G5 ($r= -0,626$; $p= 0,05$). Proteínas totais e idade demonstraram correlação significativa em G3 (rho= 0,762; $p= 0,01$). A concentração de proteínas totais média não diferiu de forma significante entre grupos, $F(5,45)= 1,132$, ($p= 0,358$). Igualmente não foram observadas diferenças ao comparar as médias de proteínas totais normalizadas com base no fluxo salivar, $F(5,45) = 2,068$, ($p= 0,087$). **Conclusão:** Os diferentes protocolos no

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tratamento das amostras de saliva não demonstraram alterações quantitativas significantes nas concentrações de proteínas totais neste fluido.

Palavras-chave: Saliva, Proteínas totais, Inibidores de protease, Fluxo salivar.

RESUMEN

Objetivo: Evaluar y comparar la concentración proteica en muestras de saliva total sometidas a variaciones metodológicas en el procesamiento preanalítico. **Métodos:** Se recolectó saliva total de 10 participantes de 23 a 65 años (edad promedio: 31,10 ± 4,059) y se calculó el flujo salival (mL/min). Las muestras se fraccionaron y dividieron en seis grupos distintos (G1–G6), cada uno correspondiente a un protocolo preanalítico diferente que combinaba los siguientes factores: centrifugación, uso de inhibidor de proteasas, temperatura y tiempo de almacenamiento. La concentración de proteínas totales se cuantificó por duplicado mediante el método del ácido bicinconínico (BCA), con significancia estadística establecida en $p < 0,05$. **Resultados:** La concentración media de proteínas totales mostró una correlación negativa significativa con el flujo salival en G1 ($r = -0,794$; $p = 0,020$), G4 ($r = -0,690$; $p = 0,027$) y G5 ($r = -0,626$; $p = 0,05$). Las proteínas totales y la edad presentaron una correlación significativa en G3 ($rho = 0,762$; $p = 0,01$). No se observaron diferencias estadísticamente significativas en la concentración media de proteínas totales entre los grupos ($F(5,45) = 1,132$; $p = 0,358$). Tampoco hubo diferencias al comparar las medias de proteínas totales normalizadas según el flujo salival ($F(5,45) = 2,068$; $p = 0,087$). **Conclusión:** Las variaciones en los protocolos de procesamiento de muestras de saliva no alteraron significativamente las concentraciones de proteínas totales en este fluido.

Palabras clave: Saliva, Proteína total, Inhibidores de proteasa, Flujo salival.

INTRODUCTION

The combination of emerging biotechnologies has expanded the range of diagnostic possibilities from the oral cavity to the entire physiological system, as most compounds found in the bloodstream can also be detected in saliva at proportional concentrations. In this way, saliva can reflect the body's physiological state, making it a promising matrix for monitoring both oral and systemic health (DURUK G & LALOGLU E, 2022; PAPPA E, et al., 2020). Moreover, compared to blood or urine, saliva collection offers several important advantages: it is non-invasive, painless, easily accepted by patients, and demonstrates good reproducibility (BHAMIDIMARRI PM, et al., 2024).

Over the past decades, salivary diagnostic methods have been applied to screen for and monitor systemic diseases, including endocrine, nutritional, and metabolic disorders (GONZÁLEZ-HERNÁNDEZ JM, et al., 2019; ABDUL NS, et al., 2022), as well as oral conditions such as dental caries and periodontal disease (DURUK G & LALOGLU E, 2022; VELJOVIĆ T, et al., 2020). However, for saliva to become a reliable alternative matrix to plasma, a strong correlation between compound concentrations in both fluids must be established (VELJOVIĆ T, et al., 2020). Additionally, ensuring the stability of salivary samples before and after collection is essential, as instability can significantly affect analytical results (THOMADAKI H, et al., 2011; GONZÁLEZ-HERNÁNDEZ JM, et al., 2019; KHURSHID Z, et al., 2021).

Among the main challenges in preserving saliva samples are their complex composition and high endogenous proteolytic activity (HELMERHORST EJ & OPPENHEIM FG, 2007; KHURSHID Z, et al., 2021). As a result, careful handling and appropriate storage conditions are required to maintain the integrity of sample components (CROUCH DJ, 2005).

Other factors can compromise salivary analysis, such as blood contamination (CHIAPPIN S, et al., 2007) and the influence of circadian rhythm, which may alter salivary flow at specific times of day and, consequently, affect the expression levels of salivary compounds (Hoek GH, et al., 2002). Preserving the integrity of the salivary proteome is another critical aspect for diagnostic applications (PAPPA E, et al., 2020). Variables such as collection method, storage temperature, the use of protease inhibitors, and the nutritional status of the individual directly influence peptide abundance and biomarker expression (LIU G, et al., 2010; JONG EP, et al., 2011; KHURSHID Z, et al., 2021).

In this context, the present study hypothesizes that methodological variations in the pre-analytical processing of saliva samples—such as the use of protease inhibitors, centrifugation, and differences in storage time and temperature—do not lead to statistically significant changes in total protein concentrations. If confirmed, this would support the analytical reliability of saliva as a diagnostic fluid, even under diverse handling protocols. Accordingly, the aim of this study was to assess the protein composition of saliva by evaluating total protein concentrations across different pre-analytical conditions.

METHODS

This experimental and analytical study was submitted to and approved by the Research Ethics Committee for Human Studies at the Federal University of Ceará (Approval Number: 1.225.703 and CAAE 48471021.2.1001.5054). Participants were recruited and enrolled in the study after signing a written informed consent form, in accordance with the guidelines and regulatory standards of the Brazilian National Health Council (Resolution No. 466/2012).

Eligible participants were healthy adults of both sexes, aged over 20 years, and not using medications that could alter salivary flow and/or composition (e.g., tricyclic antidepressants, antihistamines, antiemetics, bronchodilators). Individuals who had undergone surgical procedures in the craniofacial region with potential impact on salivary flow, as well as those with ulcerative lesions in the oral cavity, were excluded. The final sample consisted of 10 participants—six females and four males—aged between 23 and 65 years (mean age: 31.10 ± 4.06). Recruitment took place in May 2015.

Clinical Saliva Collection Protocol

Saliva samples were collected at the Integrated Clinic of the School of Dentistry, Federal University of Ceará (UFC), in Fortaleza, Brazil. Each participant first underwent an anamnesis to gather information about their general health status and any factors that might influence salivary patterns. Unstimulated whole saliva was collected from each participant between 8:00 and 10:00 a.m. to minimize circadian variability. Participants were asked to fast for at least one hour prior to collection and to perform their usual oral hygiene routine one hour beforehand.

Before sampling, participants rested for 10 minutes. They were then instructed to tilt their heads slightly forward, allowing saliva to accumulate and drain passively over the lower lip into a graduated plastic cup. The total volume collected over 10 minutes was measured in milliliters, with foam and debris excluded. Salivary flow rate was calculated by dividing the total volume by the collection time (mL/min) (FONTELES CS, et al., 2009). Following collection, each saliva sample was aliquoted into six Eppendorf® tubes, each containing 0.5 mL of saliva. For groups requiring the addition of protease inhibitor, 5 µL per mL of the following solution was added: Protease Inhibitor Cocktail (Product Reference: P2714 - 1BTL, Sigma Aldrich, Saint Louis, MO, USA). Samples were then processed according to the experimental conditions outlined in **Table 1**.

Table 1 - Experimental groups and the respective pre-analytical protocols applied to saliva samples, according to centrifugation, addition of enzyme inhibitor, and storage conditions.

Groups	Centrifugation (1200 G, 4^{0}C, 10 minutes)	Enzyme Inhibitor	Temperature and Storage Time
G1	Yes (soon after saliva collection)	No	Room temperature (24h) + -80°C (29 days)
G2	Yes (soon after saliva collection)	No	-80°C (30 days)
G3	Yes (soon after saliva collection)	Yes, during saliva collection	-80°C (30 days)
G4	Yes (soon after saliva collection)	Yes, during analysis	-80°C (30 days)
G5	Yes (30 days after saliva collection)	No	-80°C (30 days)
G6	Yes (30 days after saliva collection)	Yes, during analysis	-80°C (30 days)

All groups used protease inhibitor at a ratio of 5 µL/mL, when applicable. Centrifugation: 1200 G for 10 minutes at 4°C.

Source: Bomfim AGM, et al., 2025.

Quantification of Total Salivary Proteins Using the Bicinchoninic Acid (BCA) Method

Total protein concentrations in the saliva aliquots used for electrophoresis were determined using the bicinchoninic acid (BCA) method, with a standard curve generated from bovine serum albumin (BSA). The procedure was performed according to the manufacturer's instructions. Samples were homogenized, and absorbance was measured at 595 nm using a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). Protein concentrations were calculated based on the BSA standard curve.

Statistical Analysis

Data were collected and organized using Microsoft Excel® version 2019 and analyzed using IBM SPSS Statistics version 25.0 for Mac OS X. Descriptive statistics (mean, median, and standard deviation) and frequency distributions were reported. The Shapiro-Wilk test was used to assess the normality of the data. Pearson and Spearman correlation coefficients were calculated to evaluate correlations between variables. Comparisons between groups were performed using repeated measures ANOVA. A significance level of 5% ($p < 0.05$) was adopted for all statistical tests.

RESULTS

Descriptive Statistics and Normality

Descriptive statistics for the variables analyzed in this study are presented in **Table 2**.

Correlations Between Groups

A positive correlation was observed between duplicates (first and second aliquots analyzed) for total protein concentration in groups G1 ($r = 0.845$, $p = 0.020$), G2 ($r = 0.769$, $p = 0.009$), G3 ($r = 0.774$, $p = 0.009$), G5 ($r = 0.836$, $p = 0.003$), and G6 ($r = 0.980$, $p < 0.001$). However, no significant correlation was found between the duplicate measurements in group G4 ($r = 0.113$, $p = 0.756$).

Mean total protein concentrations showed a significant negative correlation with salivary flow rate in G1 ($r = −0.794$, $p = 0.020$), G4 ($r = −0.690$, $p = 0.027$), and G5 ($r = −0.626$, $p = 0.050$). In contrast, these correlations were not statistically significant in G2 ($r = −0.568$, $p = 0.087$), G3 ($r = −0.476$, $p = 0.164$), and G6 ($r = −0.528$, $p = 0.117$). Additionally, a positive correlation between total protein concentration and age was observed only in group G3 (rho = 0.762, $p = 0.010$).

Assessment of Data Normality

The assessment of data distribution was carried out using the Shapiro-Wilk test, a statistical method widely recognized for its sensitivity and accuracy, particularly in small to moderate sample sizes. This test was applied to all variables under investigation in order to determine whether they conformed to a normal distribution, which is an important assumption for the appropriate use of many parametric statistical analyses. The findings revealed that, for the majority of the variables, the data followed a distribution consistent with the assumptions of normality. Such a distribution indicates that the observed values for most variables are symmetrically distributed around the mean and that there are no significant skewness or kurtosis issues. This is an important prerequisite for many of the statistical tests typically applied in clinical research, as it ensures the validity of parametric procedures, such as analysis of variance (ANOVA) and Pearson correlation coefficients, among others.

However, despite the overall trend toward normality, specific variables exhibited statistically significant deviations from this expected pattern. The variables that did not conform to a normal distribution included age ($p = 0.000$), salivary flow ($p = 0.012$), the first replicate of mean total protein concentrations in group G5 ($p = 0.014$), as well as the overall total protein concentrations in groups G5 ($p = 0.045$) and G6 ($p = 0.048$). These p-values, all below the conventional threshold of 0.05, indicate that the distributions of these variables differ significantly from normality, suggesting the presence of skewed data or outliers that may impact the reliability of parametric tests if not appropriately addressed. The identification of non-normal variables is crucial, as it alerts the researcher to the potential need for data transformation, alternative statistical methods (such as non-

parametric tests), or further investigation into the underlying reasons for these deviations. Overall, with the exception of these few variables, the dataset displayed characteristics supportive of a normal distribution. This provides confidence in the robustness of the applied methodologies and suggests that, for the majority of the sample, statistical inferences based on parametric techniques remain valid and reliable. Nonetheless, careful attention should be given to the variables that did not meet the normality assumption to ensure that the conclusions drawn from the data are methodologically sound.

Group Comparisons

The comparative analysis of groups subjected to distinct saliva treatment protocols revealed no statistically significant differences in the mean concentrations of total salivary proteins (p = 0.358). Likewise, when evaluating the mean normalized total protein concentrations, the results remained statistically non-significant (p = 0.087), indicating that the methodological variations in saliva processing did not substantially influence the protein outcomes. These findings suggest that the different treatment protocols applied across groups were comparable in terms of their ability to preserve or detect total protein content in saliva samples. Therefore, the choice of protocol does not appear to bias or alter the analytical results related to protein concentration, reinforcing the reliability and consistency of the adopted procedures (**Table 3**).

Table 2 – Descriptive statistics of patients' demographic data and saliva samples.

Variables	Groups	Mean ± Standard Deviation	Median (Min-Max)	Percentile	
				25%	75%
Age (years)		31.10 ± 4.059	25.50 (28-65)	0.240	33.00
Flow rate (mL/min)		4340 ± 13199	2500 (07-1.2)	0.1150	0.8000
TPC*	**G1****	1.2107 ± 25096	9510 (38-2,64)	0.4845	1.9821
	G2	1.2012 ± 23042	1.1073 (.27-2.28)	0.5265	1.8968
	G3	1.4545 ± 24259	1.1483 (66-2.66)	0.7683	2.2789
	G4	1.0994 ± 13301	1.0105 (60-1.94)	0.7310	1.4556
	G5	1.3096 ± 22296	1.0188 (59-2.30)	0.6585	2.0151
	G6	1.3548 ± 30650	1.0458 (34-3.66)	0.6581	1.7946
NTPC*	**G1**	0.3120 ± 04912	0.2750 (14-60)	0.1725	.4450
	G2	0.3660 ± 08168	0.3550 (.09-.84)	0.1200	.5725
	G3	0.4920 ± 13552	0.2400 (.16-1.46)	0.1850	.7700
	G4	0.3690 ± 08428	0.3150 (.11-.88)	0.1725	.5125
	G5	0.4010 ± 08916	0.2700 (.13-.98)	0.1800	.6475
	G6	0.3950 ± 09922	0.2800 (12-1.12)	0.1900	.5250

* TPC, Total protein concentrations were expressed in milligrams (mg), and for NTPC, normalized total protein concentrations values were adjusted according to individual salivary flow rates (mg/min). Data are presented as mean ± standard deviation, median, minimum and maximum values, and 25th and 75th percentiles. ** See **Table 1** for detailed specifications of each experimental group (G1-G6).
Source: Bomfim AGM, et al., 2025.

Table 3 – Comparison of total protein concentration (TPC) and normalized total protein concentration (NTPC) in saliva between experimental groups using repeated measures ANOVA (Sphericity Assumed). Significance level: $p < 0.05$.

Variable	Source	Sum of Squares	Df	Mean Square	F	*P*
TPC	Between Groups	0.801	5	0.160	1.132	0.358
	Error	6.372	45	0.142		
NTPC	Between Groups	0.176	5	0.035	2.068	0.087
	Error	0.768	45	0.017		

Source: Bomfim AGM, et al., 2025.

DISCUSSION

The use of saliva as a diagnostic medium presents several challenges related to sample handling and preservation, primarily due to its complex biochemical composition and high endogenous proteolytic activity (GONZÁLEZ-HERNÁNDEZ JM, et al., 2019). The present work aimed to determine whether factors such as centrifugation, storage temperature, and the use of protease inhibitors influence the analysis of total protein concentration in saliva. The strong positive correlations observed between duplicate measurements in nearly

all experimental groups indicate high reproducibility and internal consistency of the data. This suggests that pre-analytical protocol variations had no substantial impact on total protein concentrations with the exception of group G4, where this consistency was not observed. The apparent stability of salivary proteins under various experimental conditions contrasts with findings by Esser G, et al. (2008), who reported rapid protein degradation within the first 30 minutes following collection of fresh whole saliva in the absence of protease inhibitors. Conversely, our results align with those of Liu G, et al. (2010), who similarly observed no significant effects of protease inhibitor use on salivary protein stability. These authors concluded that the addition of protease inhibitors does not interfere with oral microbial viability or hinder the characterization of salivary protein profiles. Although multiple strategies for saliva handling, storage, and transport have been explored in recent years, there remains a lack of standardized guidelines defining optimal protocols (MORTAZAVI H, et al., 2024).

With respect to temperature, our findings corroborate with previous evidence suggesting that total protein concentrations can be preserved for up to 24 hours at room temperature. Jong EP, et al. (2011) evaluated the effects of freezing and ambient storage on salivary peptide concentrations and found no significant differences between samples frozen immediately at −80°C and those held at 20°C for 24 hours prior to freezing. However, samples stored beyond this 24-hour window exhibited a marked reduction in peptide abundance, indicating a critical period for sample processing and transport without analytical compromise. In contrast, our results diverge from those of Xiao H and Wong DT (2012), who reported significant changes in salivary protein expression when comparing samples stored at −80°C versus room temperature in the absence of inhibitors. Notably, the addition of 20% ethanol prior to room temperature storage mitigated these effects, with no statistically significant differences observed. Similarly, Chevalier F, et al. (2007), using one- and two-dimensional electrophoresis to analyze stimulated saliva under different temperature, centrifugation, and inhibitor conditions, found no changes in total protein concentrations for samples stored at room temperature for up to 24 hours. However, reductions in alpha-amylase band intensity were observed after 7 to 30 days of storage.

A negative correlation between salivary flow rate and total protein concentration was identified in groups G1, G4, and G5. This finding is consistent with literature reporting dilution of salivary proteins under conditions of increased secretion. Walsh NP, et al. (2004) observed a decrease in flow rate and a concurrent rise in total protein concentration in dehydrated individuals. Similarly, López ME, et al. (2003), in a study assessing the physical and biochemical properties of saliva in children with diabetes, reported an inverse relationship between salivary flow and total protein levels. Similarly, Panchbhai AS, et al. (2010) found a significant negative correlation between salivary flow and total protein concentration across diabetic cohorts in India.

The positive correlation observed between age and total protein concentration in group G3 is also supported in the literature. Bhuptani D, et al. (2018) investigated variations in total protein concentration and salivary profiles in relation to age, sex, and dental eruption in individuals from Gujarat, India. Their findings demonstrated a positive association between age and total protein concentration, along with sex-related differences. They reported that total protein levels tend to remain stable during adulthood and decline again in older age groups, with no statistically significant differences across age brackets.

The present findings indicate that neither centrifugation (or lack thereof) within 30 days, storage at room temperature for 24 hours, nor the timing of protease inhibitor addition (either before or after 30 days) significantly affected total protein concentrations. These results support the feasibility of using saliva as a robust alternative biological matrix to quantify total protein. Overall, these findings suggest a viable 24-hour window for sample transport without compromising analytical integrity. None of the tested conditions—including variations in centrifugation timing, inhibitor use, and storage time/temperature—negatively impacted the stability of total protein levels. These results reinforce the potential of saliva as an accessible, cost-effective, and analytically viable matrix for both clinical diagnostics and research, particularly in low-resource settings. The lack of necessity for on-site centrifugation and the elimination of immediate addition of expensive reagents further support the applicability of saliva in public health research and field studies. These advantages enhance the appeal of saliva as a practical and scalable diagnostic tool.

Nonetheless, certain limitations must be acknowledged. The small sample size (n = 10) reduces the statistical power of the analyses and limits the generalizability of the findings. Furthermore, this study focused exclusively on the quantification of total protein, without evaluating potential qualitative alterations in protein composition. Important aspects such as protein denaturation or loss of biological function were not assessed. Advanced methodologies—including proteomic analyses, identification of specific proteins, and evaluation of post-translational modifications—are necessary to determine whether the tested pre-analytical conditions affect the structural or functional integrity of the salivary proteome (MORTAZAVI H, et al., 2024).

Future studies should aim to increase sample size and employ qualitative analytical approaches to thoroughly investigate the impact of pre-analytical protocols on the qualitative and quantitative characteristics of the human salivary proteome.

CONCLUSION

In summary, total protein concentration in saliva was found to decrease as salivary flow rate increased. Samples that were centrifuged immediately after collection exhibited comparable total protein profiles, regardless of whether protease inhibitors were added. Similarly, maintaining saliva samples at room temperature for 24 hours prior to freezing at −80 °C did not produce significant differences in total protein concentration. Centrifugation performed only after 30 days of storage also had no effect on total protein levels, nor were differences observed between samples frozen immediately at −80 °C and those stored for 24 hours at room temperature before freezing. These findings underscore the robustness of total salivary protein concentrations under varying pre-analytical conditions and support the analytical stability of saliva as a diagnostic matrix, even when subject to moderate delays in processing and differences in sample handling.

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