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Article

Salivary calcium and phosphate stability in different time and temperature storage

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The non-invasive collection and inexpensive nature of saliva has made it an attractive sample for use for diagnosis and research on several diseases. Storage circumstances may affect salivary component concentrations. The objective was to analyze calcium and phosphate stability in saliva samples stored at different conditions. Saliva of healthy people was stored and analyzed by spectrophotometry under different time and temperature conditions in order to evaluate calcium and phosphate stability. Calcium concentration was measured by Arsenazo III reaction at 600nm and phosphate by an acid-molybdate method at 650nm. Using Lin's concordance correlation coefficient (k), we observed very good agreement ($k>0.8$) for all samples frozen at -20 °C up to 50 days. Thaw/refreezing cycles can compromise phosphate stability even though there is good agreement ($0.61<k<0.8$). Because of higher variability for refrigerated samples, they are not the best storage method, although calcium and phosphate levels could be considered stable when the samples were stored at 4 °C for 7 days. Our results revealed that under different conditions, calcium and phosphate levels are stable in saliva samples, and that freezing at -20°C is the storage condition of choice, allowing to accumulate a higher number of samples before analysis, making it suitable for routine and research assays.

Uniterms: Saliva/study/analysis. Saliva/storage/analysis. Saliva/calcium/stability/spectrophotometry. Saliva/phosphorus/stability/spectrophotometry.

INTRODUCTION

Saliva is a complex biological fluid composed of secretion of three pairs of major salivary glands (parotid, submandibular and sublingual glands) and multiple minor glands including labial, buccal, lingual, and palatal tissues. Its final product is made up of water, macromolecule proteins (e.g., amylase, lysozyme, carbonic anhydrase, and immunoglobulin A), and various electrolytes including sodium, potassium, calcium, magnesium, bicarbonate, and phosphate (Newkirk *et al.*, 2000; Humphrey, Williamson, 2001; Prasanthi, Kannan, Patil *et al.*, 2014). Calcium and phosphate work together as an antisolubility factor and modulate the process of tooth demineralization and remineralization (Humphrey, Williamson, 2001) in order to prevent caries and dental erosion (Lussi, Jaeggi, 2008). As these ions are found to be decreased in the saliva of patients with active carious lesions (Preethi, Reshma, Anand, 2010; Fiyaz *et al.*, 2013; Hegde *et al.*, 2014; Prasanthi *et al.*, 2014), these biochemical parameters play an important role in determining individual caries and other tooth demineralization susceptibility (Kaur, Kwatra, Kamboj, 2012).

Many local and systemic diseases can affect salivary glands, as well as drugs, hormone and radiation therapy. Saliva-based diagnostics are emerging and offer a promising clinical strategy, characterizing the association between salivary analytes and a particular disease (De Almeida Pdel *et al.*, 2008; Zhang *et al.*, 2012). Furthermore, saliva has an easy and non-invasive collection nature, ready availability, which makes it a very attractive, safe, and inexpensive diagnostic tool (Herr *et al.*, 2007; Schipper, Silletti, Vingerhoeds, 2007; Emekli-Alturfan *et al.*, 2013). Several studies (Table I) analyzed changes to saliva components (including calcium and phosphate) in various diseases/conditions using different

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protocols (immediately or previously frozen analysis) but some of them do not mention the time elapsed between saliva collection and laboratory analysis, neither temperature storage. However, these circumstances may affect several salivary component concentrations; thus, there has to be available information on the use of saliva in both research and laboratorial/clinical practice. There are few studies that have evaluated the stability of saliva components after collection. Therefore, in the present study, a stability analysis was carried out by determining calcium and phosphate in samples stored under different conditions.

MATERIAL AND METHODS

Saliva Collection and Preparation

The protocol was approved by the Human Research Ethics Committee (approval number 146/2011) and all patients have signed an informed consent form. About 10 mL of unstimulated whole saliva was collected between 8 AM and 10 AM from 7 young healthy people by expectoration in the absence of chewing movements and placed into a sterile container. The samples were immediately centrifuged for 5 minutes at 282 g at 25 °C. After centrifugation, the saliva supernatant was separated into aliquots placed in microtubes, stored and analyzed under three different conditions as shown in Table II. In order to evaluate storage stability, all samples were evaluated with predefined times and storage methods.

Afterwards, aliquots of each sample were kept at 4 °C for 7 days and another was kept at -20 °C during 50 days. In 24, 48 and 72 hours, the frozen aliquots were submitted to 1, 2 and 3 thaw/refreezing cycles respectively, in order to determine their influence on stability.

Sample Analysis

Both total calcium and phosphate were determined by a Cintra 6 UV-Visible spectrophotometer (GBC, Australia) using commercial kits*. Calcium concentration was measured by Arsenazo III reaction at 600 nm and phosphate by an acid-molybdate method at 650nm, according to the manufacturer's instructions, always in triplicate. In every analysis, one standard sample (Wiener Lab, Argentina) was used to ensure method accuracy. All readings were taken once by a single technician. Method precision was demonstrated by Relative Standard Deviation (RSD) values obtained within 10 for determinations of phosphates and calcium, respectively For calcium determination in frozen samples, there were variations above 20% for RSD; in these cases, a new

TABLE I - Salivary Calcium and Phosphate studies in different conditions and time storages. NA: Non-available

Disease/condition	Storage Conditions	Time to analysis	Reference
Smoking and periodontal status	Room temperature	Immediately	Kolte, Kolte, Laddha, 2012
Dry mouth in menopause	-20°C	Latter	Agha-Hosseini *et al*., 2012
Phosphate/Calcium containing chewing gum	NA	Immediately	Santhosh *et al*., 2012
Dental erosion	-80°C	NA	Wang *et al*., 2011
			Jager *et al*., 2011
Dental caries	-20°C	NA	Cornejo *et al*., 2008
Unstimulated salivary flow	4°C	1 hour	Wu *et al*., 2008
Chronic kidney disease	NA	NA	Savica *et al*., 2008

TABLE II - Aliquots´ analysis time and storage method

Storage method	Conditions	Periods
R1, R3 and R7	Refrigeration (4 ºC)	1, 3 and 7 days, respectively
C1, C2 and C3	Thaw/refreezing cycles	One cycle every 24 h (total of 3 cycles), respectively
F7, F14, F28 and F50	Freezing (-20 ºC)	7, 14, 28 and 50 days, respectively

T0 (immediately analysis), R1, R3, R7 (1, 3 and 7 days, respectively), C1, C2, C3 (One cycle every 24 hours, respectively, total of 3 cycles), F7, F14, F28 and F50 (7, 14, 28 and 50 days, respectively).

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duplicate was analyzed to obtain at least a triplicate with 10% for RSD. Statistical analyses were performed with the aid of the software Stata, ver. 11.2 (©StataCorp) and Microsoft Excel, Ver. 9.0 (© Microsoft Office Professional; Microsoft Corp.). Data were expressed in concentration means ($mg.dL^{-1}$) and Lin's Concordance correlation coefficients.

RESULTS

Salivary calcium and phosphate concentration in each sample were measured by calculating the mean of triplicate analysis. The levels of calcium and phosphates obtained under different storage conditions and at different times are shown in Tables III and IV.

Stability was evaluated by comparing the results found under different storage conditions and at different times with the value at t=0, by determining Lin's concordance correlation coefficient, as shown in Table V and Figure 1.

DISCUSSION

In this assay, all concentrations were compatible with results found in other research studies where calcium (Kavanagh, Svehla, 1998; Rode *et al.*, 2001; Kiss *et al.*, 2010; Singh *et al.*, 2012) and phosphate (Kavanagh, Svehla, 1998; Rode *et al.*, 2001) were analyzed in control groups. Therefore, the commercial kits in use were able to detect these salivary elements, although the manufacturer's indications were only for serum and urine. Calcium-arsenazo III methodology uses a monoreagent liquid where saliva samples are added and immediately read by the spectrophotometer, thus making it an easy and fast detection system, while the phosphate/molybdenum blue multiple step method takes about half-hour to complete the reaction and reading. However, in calcium-arsenazo III, a higher coefficient of variation was found when analyzing the same sample in triplicate, which means this system is more likely to have variations in time, temperature, and homogeneity. In the present research, RSD was considered

TABLE III - Salivary calcium concentration ($mg.dL^{-1}$) by sample and storage methods

Samples	Storage methods										
	T0	R1	R3	R7	C1	C2	C3	F7	F14	F28	F50
A1	6.75	5.71	7.95	4.60	6.67	2.75	6.05	4.13	4.00	8.16	5.57
A2	5.39	3.29	4.62	3.11	4.05	4.10	3.66	8.27	3.61	5.71	5.92
A3	4.78	3.35	8.76	4.94	3.81	3.81	8.72	4.86	3.14	5.32	4.69
A4	5.81	2.99	6.15	4.73	4.28	3.45	3.72	4.43	5.18	5.46	5.57
A5	5.39	3.65	6.91	4.54	3.63	8.39	3.86	5.35	3.32	5.46	5.09
A6	4.49	3.50	4.56	4.42	4.03	4.96	4.87	4.17	3.00	5.85	5.75
A7	6.48	4.55	6.10	6.75	6.66	6.88	4.12	9.08	7.81	6.86	5.71

T0 (immediately analysis), R1, R3, R7 (1, 3 and 7 days, respectively), C1, C2, C3 (One cycle every 24 hours, respectively, total of 3 cycles), F7, F14, F28 and F50 (7, 14, 28 and 50 days, respectively).

TABLE IV - Salivary phosphate concentration ($mg.dL^{-1}$) by sample and storage methods

Samples	Storage methods										
	T0	R1	R3	R7	C1	C2	C3	F7	F14	F28	F50
A1	21.49	21.48	16.53	18.01	17.31	16.91	14.81	19.49	15.03	19.92	21.4
A2	9.61	11.5	9.58	10.04	9.95	11.66	10.41	9.78	9.76	10.5	11.76
A3	8.28	10.53	7.65	10.54	9.84	13.03	8.18	10.92	9.43	9.64	9.79
A4	12.4	12.68	11.68	16.8	9.12	10.72	12.31	17.07	13.1	12.03	11.83
A5	9.69	9.84	7.23	10.36	9.27	10.69	7.12	12.74	10.00	10.04	9.71
A6	6.36	7.31	5.49	6.55	9.21	6.10	5.12	6.17	6.49	7.06	5.00
A7	19.11	22.31	14.85	16.27	21.72	17.46	14.05	17.29	21.31	19.97	21.11

T0 (immediately analysis), R1, R3, R7 (1, 3 and 7 days, respectively), C1, C2, C3 (One cycle every 24 hours, respectively, total of 3 cycles), F7, F14, F28 and F50 (7, 14, 28 and 50 days, respectively).

TABLE V - Lin's concordance correlation with T0 coefficient for all storage methods analyzed

Element	Storage methods									
	R1	R3	R7	C1	C2	C3	F7	F14	F28	F50
Ca	0.850	0.841	0.923	0.899	0.865	0.816	0.866	0.929	0.817	0.924
P	0.951	0.832	0.857	0.870	0.815	0.735	0.870	0.860	0.979	0.937

T0 (immediately analysis), R1, R3, R7 (1, 3 and 7 days, respectively), C1, C2, C3 (One cycle every 24 hours, respectively, total of 3 cycles), F7, F14, F28 and F50 (7, 14, 28 and 50 days, respectively).

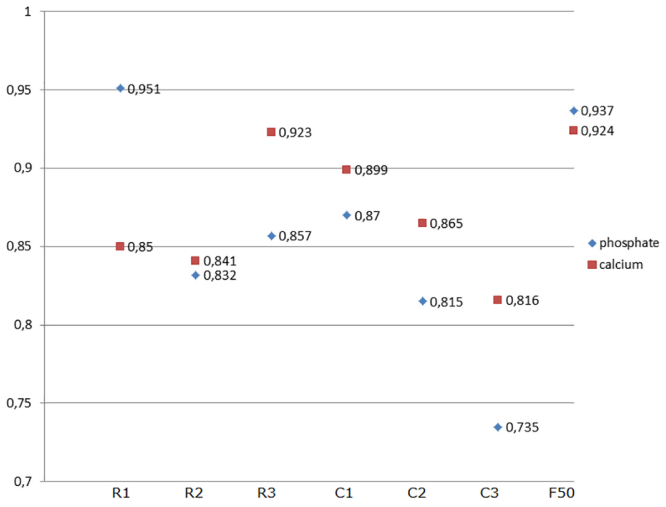

FIGURE 1 - Lin's concordance correlation coefficient of Calcium and Phosphate in saliva sample at refrigeration (R1, R3, and R7), thaw/refreezing cycles (C1, C2, and C3) and 50 days´ freezing (F50), compared to T0.

acceptable at 10% for triplicates. However, for calcium determination in frozen samples, there were variations above 20% for RSD for triplicates; in these cases, a new duplicate was analyzed to obtain at least a triplicate with 10% for RSD. Therefore, frozen samples may be used to determine calcium concentration, and a greater number of replicates may be required to find a result with acceptable accuracy.

Centrifugation was carried out before storage and analysis in order to remove any cellular debris and turbidity that could interfere with spectrophotometric determination. Even with this procedure, in some cases there was a precipitate formed after thawing frozen samples, not broken after rigorous homogenization with vortex before each pipetting. According to Schipper, Silletti and Vingerhoeds (2007), calcium could be part of this precipitate and this could be responsible for the major variability between calcium determination of the same sample in different storage methods, as seen in samples A1 (at T5 and T9), A2 (at T7), A3 (at T2 and T6) and A7 (at T7).

For phosphate determination, the RSD values were lower, within 10%, suggesting less analytical variability. It seems that these elements are distributed more uniformly in the sample even under different storage conditions. One can still say that the method used for phosphate determination is more robust than the method for calcium determination.

The determination of trace elements immediately after collection of the sample is difficult to occur in practice. Thus, samples have to be stored until analysis. In this study, three different conditions (refrigeration, thaw/refreezing cycles and freezing) were evaluated for both elements. When determining Lin's concordance correlation coefficient (k) for each storage method, compared to T0, there was good agreement (when $0.61<k<0.8$) in the second thaw/refreezing cycle when analyzing phosphate, and very good agreement ($k>0.8$) for the other cycles, including the third one and all cycles for calcium determination. The difference found in the second cycle should be most likely due to the method of analysis than to storage condition per se.

For the refrigerated samples, there was greater variability for k values, hence it is not the best storage method, but both calcium and phosphate levels can be considered stable when the samples are stored at 4 °C for 7 days. Frozen samples, especially those analyzed at 50 days, showed very good agreement with T0 and the higher k values for both elements. Although some authors (Schipper *et al.*, 2007) recommended analysis immediately after collection, Czégény *et al.* (2001) evaluated calcium stability in saliva and concluded that this element has good stability when refrigerated and frozen for 20 days.

CONCLUSION

Our results revealed that under different conditions, calcium and phosphate levels are stable in saliva samples for different periods. If saliva samples are required to be stored after centrifugation, as described in this study, the storage condition of choice to determine calcium and phosphates levels is -20°C up to 50 days.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STANDARDS

The authors declare that the protocol was approved by the Ethics Committee in Human Research (approval number 146/2011) and all patients have signed informed consent.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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