American Journal of Analytical Chemistry, 2011, 2, 683-688
doi:10.4236/ajac.2011.26078 Published Online October 2011 (http://www.SciRP.org/journal/ajac)

Effect of Pre-Analytical Conditions on Salivary Nitrite Levels

Rosana Andrea Morelatto[1*], Tomás Enrique Benavidez[2], Ana María Baruzzi[2], Velia Matilde Solís[2], Silvia Adriana López de Blanc[1]
[1]*Department of Oral Pathology, Clinical Stomatology I and II "B", Faculty of Dentistry, National University of Córdoba, Córdoba, Argentina*
[2]*INFIQC-CONICET, Department of Physical Chemistry, Faculty of Chemical Sciences, National University of Córdoba, Córdoba, Argentina*
*E-mail: *rmorelatto@gmail.com*
Received June 29, 2011; *revised July* 29, 2011; *accepted August* 5, 2011

Abstract

The aim of the present paper was to analyze the influence of sampling and storage procedures on nitrite concentration values in the saliva of healthy persons. The samples were obtained and stored under varied conditions, and processed using the Griess method. Results: when the salivary nitrite concentration was measured immediately after collection a significant dependence on the collection time was observed. A mean value of 94 μmol/L (range 3 - 625) was obtained at 8:30 am. This value decreased significantly with time ($p < 0.05$) reaching a value of 68 at 12:30 noon. Concerning the sample storage, a significant increase in the nitrite concentration was observed after 2 hrs, either at 4˚C or at room temperature ($p < 0.05$). In spite of the high variability between individuals the values for each individual showed a marked constancy independent of the sampling day. According to our results, by controlling pre-analytical parameters, principally sampling and storage procedures, reproducibility is improved.

Keywords: Salivary Nitrite, Saliva, Oral Cancer, Storage Conditions

1. Introduction

N-nitroso compounds have been shown to be potent carcinogens in animals [1]; in the human body they can be formed by the interaction of nitrite and a variety of amine precursors, developing infantile methahemoglobinemia and gastric cancer. Nitrite has been extensively studied in relation to carcinogenesis [2]; it is an important factor for gastric nitrozation, and could contribute to the etiology of lung, stomach, esophagus, nasal cavity, bladder and oral cavity cancer, leukemia and Non Hodgkin lymphoma [3]. It has mutagenic effects at cellular levels and acts on p53 gene, closely related to the head and neck squamous cell carcinoma [4].

Several studies have confirmed that the level of salivary nitrite is strictly dependent on salivary nitrate, and thus dependent on the dietary nitrate intake [5-8]. Dietary nitrate derives mainly from vegetables and drinking water [6,7] and in the latter case it could be more hazardous since nitrate in vegetables is counterbalanced by vitamin C and polyphenols that inhibit nitrozation [8]. Ingested nitrate is absorbed from the stomach or intestines and about 25% is secreted in saliva by an anion transport system [5]. As a result, nitrate concentrations in saliva are approximately 10 to 20 times higher than those found in plasma [5]. In addition, it has been estimated that 70% of the orally ingested nitrate is reduced to nitrite by mouth microorganisms, mainly on the posterior surface of the tongue [9,10].

The assay of saliva is an increasing area of research with implications in basic and clinical purposes. Recently, saliva has provided a substantial tool investigation of disease processes and disorders. Although this biological fluid is easy to collect and manipulate, special attention must be paid to minimize variation in specimen integrity [11].

Many authors have investigated the concentration of nitrite in saliva considering the advantages of obtaining this biological fluid and its possible role in the etiology and diagnostic relevance of nitro compounds. A common feature of in vivo studies is the large spread in results, both between individuals and for each individual in rela-

Copyright © 2011 SciRes.

tion to sampling days and time [12]. The changing composition of saliva, dependent on several internal and external factors, as well as on the complex chemistry of nitro-compounds limits the validity of the results for clinical purposes.

In spite of the importance of testing nitro-compounds in saliva, the literature on appropriate sample collection and storage is scarce. The technical variables used for sampling storage in the literature differ substantially. Xu *et al.* [13] and Bojiç *et al.* [14] kept the samples at –20˚C for a maximum of one week and one month until analysis, respectively. On the other hand, Mirvish *et al.* [15] and Yu *et al.* [16], assume that the saliva should be stored at room temperature or 4˚C for up to one day, and at –15˚C for less than a month before analysis. Since there are marked differences in storage conditions in the literature, the comparison of results becomes doubtful.

The purpose of this work was to evaluate the influence of saliva collection, sampling and storage on nitrite concentration in order to obtain better reproducibility conditions. Special attention was paid to the effects of: day time of collection, temperature of storage, and the time elapsed until the concentration of nitrite was determined in the saliva samples. Intra- and inter-individual variability was also studied.

2. Material and Methods

2.1. Subjects

This study was performed in 13 healthy (10 females and 3 males) non-smoking volunteers, from 26 to 53 years old. Exclusion criteria used were: high alcohol intake, consumption of food-vitamin supplements, pregnancy and use of antibiotics or anti-inflammatories. Informed consent of the nature of the study was signed by all the volunteers. They were also instructed not to eat food rich in nitrate and nitrite such as beets, carrots, green beans, spinach, and collard greens, hot dogs, cured ham, bacon, bologna and salami, during the previous 24 hrs and not to eat at all at least 8 hrs before the test; although they were allowed to brush their teeth.

2.2. General Procedures

Volumes of 3 mL of unstimulated whole saliva were collected; individuals spat directly into a sterile plastic tube containing 60 μL of 1 M NaOH solution in order to stop further reduction of nitrate and to destroy vitamin C in the incubates (20). For the deproteinization of the samples, 3 mL of 0.15 M $ZnSO_4$ were added to the collected sample and then it was centrifuged for 15 min at 9000 g (28). The supernatant was used for the determination of nitrite concentration using the Griess method, the one most frequently used for this metabolite analysis in biological fluids. Proteins were removed with the purpose of reducing turbidity, which might interfere with the absorbance lecture [9].

2.2.1. Effect of Day Time of Collection

The dependence of nitrite concentration with the time of collection was evaluated in full duplicate samples from 13 subjects at the following day times: 8:30 a.m., 10:30 a.m. and 12:30 noon. Volunteers had fasted and did not eat anything until completion of the trial. After collection, samples were immediately processed.

2.2.2. Effect of Storage Conditions on the Nitrite Concentration

A sample taken from each of the 9 volunteers was divided into 5 aliquots, one was processed immediately after collection at 8:30 a.m., and four were analyzed throughout the subsequent 4 hours under different conditions of storage.

To analyze the influence of the time of storage, another set of samples was taken from the same 9 voluntaries, each one was divided into 4 aliquots; one was analyzed immediately and the others were frozen and stored at –20˚C, then defrozen and processed to determine the nitrite concentration, after 7, 14 and 21 days.

2.2.3. Individual Variation over Time

To evaluate individual variation, salivary nitrite under fasting conditions was determined in 10 subjects. Each one was sampled once per month over a period of three months, and the saliva was processed immediately after collection at 8:30 a.m.

2.3. Experimental

The concentration of nitrite in saliva samples and in standard solutions prepared daily was analyzed measuring absorbance with a UV-1700 Shimadzu spectrophotometer at 540 nm (1 nm resolution, 0.005 absorbance accuracy). A detection limit of 6 μmol/L and a linear behavior up to 200 μmol/L were obtained from the calibration curve. Samples with higher NO^{2-} concentrations were diluted. NO^{2-} was not detectable in the blank solutions that contained only NaOH and $ZnSO_4$. The analyzed solution contained 200 μL of the sample, 400 μL of water and 400 μL of the Griess reagent. The precision of the Griess method is very high, not only when used for standard solutions, but also with saliva samples. Standard deviation in the absorbance values corresponding to duplicates of each sample is ±0.005, indicating high reproducibility of the values.

Copyright © 2011 SciRes.

2.4. Statistical Methods

One-way analysis of variance with a randomized block design was used to evaluate the effect of day time of collection; the subjects were considered as blocks to reduce the biological differences between them. The paired t test was used to evaluate the effect of temperature and time after collection.

Nitrite variability was assessed by means of a mixed linear model, with REML estimations of variance components. Best linear unbiased predictors (BLUP) of volunteer effects were derived from the fitted mixed linear model. Day of collection was regarded a fixed effect and the volunteer effect was considered as random.

F test was used for comparing variances.

Statistical analysis was done using InfoStat/Professional version 1.1 statistical software package (Faculty of Agronomy, National University of Córdoba).

3. Results

A mean value of 94 µmol/L (range 3 - 625) was obtained for the salivary nitrite concentration. This value corresponds to fasting levels of 115 samples taken from 13 volunteers, determined immediately after collection, at 8:30 a.m.

3.1. Effect of the Day Time of Collection

In this experiment a mean concentration of 93 µmol/L at 8:30 a.m. in 13 volunteers was found. A significantly lower value ($p < 0.05$) was obtained when the sample as collected at 10:30 and 12:30 h (**Figure 1**). At both times the values were not statistically different, reaching a mean concentration 68 µmol/L at noon. These results were analyzed using one-way ANOVA with a randomized block design. Although the average value is plotted in **Figure 1**, the statistical analysis considers the variation between individuals.

3.2. Effect of Storage on the Nitrite Concentration

Table 1 shows the results for saliva collected at 8:30 a.m. from nine volunteers that were processed according to the conditions stated previously in material and methods section. Paired t test indicated that up to 2 hrs, both at 4˚C and at room temperature, there are no significant differences, although a slight decrease at room tempera-

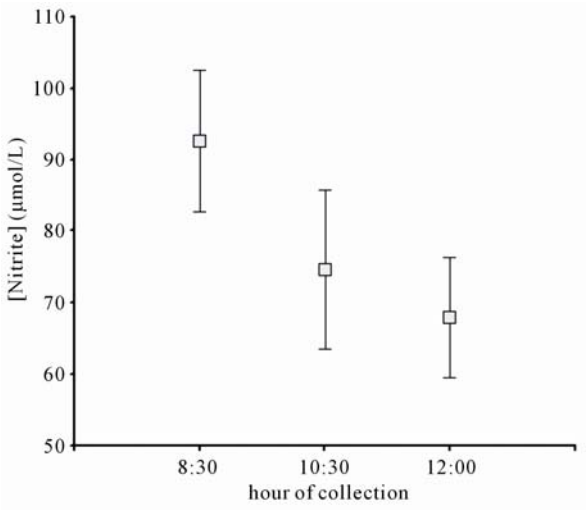

Figure 1. Effect of the specimen collection time on the Salivary nitrite concentration, n = 13

Table 1. Salivary nitrite concentration (µmol/L) under different storage conditions, n = 9.

Subject	Immediately (mean ± SD)[1,2]	2 h - 4˚C (mean ± SD)	2 h - room T (mean ± SD)	4 h - 4˚C (mean ± SD)[1]	4 h - room T (mean ± SD)[2]
1	58 ± 33	47 ± 16	37 ± 9	41 ± 13	50 ± 27
2	93 ± 3	87 ± 5	88 ± 2	139 ± 2	98 ± 5
3	100 ± 46	60 ± 34	68 ± 42	62 ± 48	73 ± 57
4	79 ± 5	84 ± 9	86 ± 10	134 ± 8	87 ± 4
5	93 ± 5	121 ± 5	99 ± 4	194 ± 8	137 ± 14
6	444 ± 51	647 ± 28	569 ± 94	813 ± 50	554 ± 20
7	87 ± 19	98 ± 6	76 ± 1	167 ± 6	101 ± 6
8	198 ± 54	155 ± 67	144 ± 25	185 ± 110	187 ± 108
9	118 ± 57	65 ± 37	78 ± 14	51 ± 36	73 ± 39

[1,2]($p < 0.05$). These p-values, obtained with the paired t test, are relative to the results immediately after specimen collection.

Copyright © 2011 SciRes.

ture was found. Between 2 hrs and 4 hrs the values increased significantly in all the samples ($p < 0.05$). **Table 1** also shows that there were strong inter-individual differences, not only in salivary nitrite concentration, but also in the standard deviation values (SD); for example: subject 2 has a much lower variability in his values than the subject 3; in addition these SD are quite constant with storage time. This assessment was confirmed performing F test to compare the inter-individual variances with respect to the variances in each sample with storage time ($p > 0.05$). This constancy in the variability of the concentration in the samples, corresponding to the same saliva measured after several hours at room temperature or at 4˚C, indicates that the stability of the values is quite dependent of the sample itself.

To evaluate the effect of several days of storage on the three remaining aliquots frozen at –20˚C, the analysis was repeated after 7, 14 and 21 days and the results were compared with the sample processed immediately. Most of the cases showed a significant decrease in the nitrite values during the first week ($p = 0.001$), while maintaining similar values at the end of the second and third week (**Figure 2**). These results were analyzed using one-way ANOVA with a randomized block design.

3.3. Effect of the Day of Collection

Table 2 shows the results of applying a mixed linear model; they indicate that the inter-individual variability is high, inducing large difference between volunteers as shown by the best linear unbiased predictors (BLUPs) of volunteer effects. Even though the day of collection effect was significant, the variance within subject expressed as percentage of total variance was only 20%.

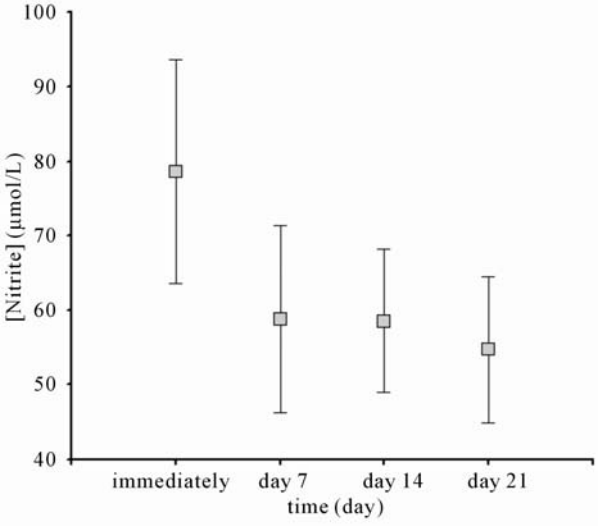

Figure 2. Effect of the storage at –20˚C on salivary nitrite concentration, n = 10.

Table 2. Fitted Mixed Linear Model for salivary nitrite concentration in ten subjects.

Model Term	Estimate	SE	t-value	p-value
Intercept	278.0	86.6	3.2	0.002
Day of collection	271.5	95.4	–2.8	0.006
Volunteer effect				
Subject 1	–36.80			
Subject 2	–10.20			
Subject 3	–53.90			
Subject 4	11.70			
Subject 5	–37.02			
Subject 6	–39.50			
Subject 7	26.90			
Subject 8	145.90			
Subject 9	28.20			
Subject 10	–35.15			

Log Likelihood −346.9; $R^2 = 0.54$.

4. Discussion

In the present study we analyze the variations of salivary nitrite in healthy people before breakfast during the morning and those related to the storage conditions, in order to obtain reliable sampling for further clinical studies.

According to the results the values of salivary nitrite obtained at 8:30 a.m. were higher than those in the saliva collected two or four hours later. We therefore decided to unify 8:30 a.m. as the hour of collection of the samples in all the experiments to avoid variations due to the circadian rhythm.

Some authors also observed variation in salivary nitrate and nitrite concentrations during the day; they reported enhanced values after meals with high nitrate amounts and these levels were sustained for at least 5 hrs post food ingestion, effect probably related with the diet [8]. It is not possible to compare our findings with these results considering that the conditions for collection of the samples are completely different, and that the high variability in the values is strongly dependent on the nitrite dietary intake.

Another important result was the strong influence of the storage conditions on salivary nitrite values. In this sense, the samples should be either processed immediately after collection, or kept at 4˚C and analyzed before the elapsed time reaches two hours. These results are

Copyright © 2011 SciRes.

also not coincident with those of Tanaka [10], who affirms that the concentration of the anionic components remains stable over a period of 48 hrs in the refrigerator and for 4 hrs at room temperature.

In addition to the effects of day time of collection and storage of the samples on the salivary nitrite concentration, we also found some interesting facts concerning the variation between and within individuals. We obtain a mean concentration of 94 μmol/L and a wide range of salivary nitrite level, between 3 and 625 μmol/L, results which are in agreement with those found in the literature [5,17-19]. In spite of the high spread of these results between individuals, it is important to remark that the intra-individual variability is quite dependent on the subject (**Table 1**).

It should be noted, on the other hand, that the fasting values in the monthly collected samples of the same individual immediately processed, remained quite constant, despite the high spread of the values among different subjects.

Measuring nitrite in saliva is of particular importance due to its contribution to the formation of N-nitrosamines, many of which are carcinogenic. Cancer induced by N-nitro compounds formed in the stomach could be prevented by reducing the levels of salivary nitrite according to the studies referred to above. This purpose could be achieved by reducing nitrate in drinking water and diet, improving dental hygiene, and using antiseptic mouthwashes and toothpastes [20]. Some authors point out that improper storage of samples and bacterial contamination may increase nitrite levels in saliva [10]. As the technical variables for storage conditions found in the literature differ significantly, comparison of results is not possible.

Nevertheless in this paper we have found out that by controlling some pre-analytical parameters, such as collection, sampling and storage procedures, reproducibility is improved and some valid clinical information can be obtained.

5. Conclusions

Although the reproducibility of results is shown to be affected by many parameters, variation can be greatly reduced if the following conditions are carefully obeyed.

1) The time of collection of the sample should be the same in all the individuals.

2) Samples should be immediately analyzed or before two hours after collection. Storage of samples produces variation in the nitrite concentration even when they are kept at 4˚C.

The saliva matrix is an upcoming area of research for basic and clinical application purposes, with considerable potential for growth and progress. Although the conditions to stabilize nitrite ion concentrations in the samples are difficult to achieve, the relative constancy in individual salivary nitrite levels when pre-analytical conditions are kept the same, would allow more reliable cohort studies to evaluate their clinical relevance.

6. Acknowledgements

The authors acknowledge Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) for financial support. R. Morelatto thanks Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT) for the fellowship granted.

7. References

[1] P. N. Magee, "The Experimental Basis for the Role of Nitroso Compounds in Human Cancer," *Cancer Surveys*, Vol. 8, 1989, pp. 207-239.

[2] P. E. Hartman, "Review: Putative Mutagens and Carcinogens in Foods. I. Nitrate/Nitrite Ingestion and Gastric Cancer Mortality," *Environmental Mutagenesis*, Vol. 5, No. 1, 1983, pp. 111-121. doi:10.1002/em.2860050112

[3] S. S. Mirvish, "Role of N-Nitroso Compounds (NOC) and N-Nitrosation in Etiology of Gastric, Esophageal, Nasopharyngeal and Bladder Cancer and Contribution to Cancer of Known Exposures to NOC," *Cancer Letters*, Vol. 93, No. 1, June 1995, pp. 17-48. doi:10.1016/0304-3835(95)03786-V

[4] M. Rogers, T. Vaughan, S. Davis and D. Thomas, "Consumption of Nitrate, Nitrite and Nitrosodimethylamine and the Risk of Upper Aerodigestive Tract Cancer," *Cancer Epidemiology, Biomarkers & Prevention*, Vol. 4, No. 1, January 1995, pp. 29-36.

[5] B. Spiegelhalder, G. Einsenbrand and R. Preussman, "Influence of Dietary Nitrate on Nitrite Content of Human Saliva: Possible Relevance *in Vivo* Formation of N-Nitroso Compounds," *Food and Cosmetics Toxicology*, Vol. 14, No.6, 1976, pp. 545-548. doi:10.1016/S0015-6264(76)80005-3

[6] C. L. Walters, R. J. Hart and P. L. Smith, "Analysis of Total N-Nitroso Compounds as a Group by Denitrosation to Nitric Oxide, with Detection Using a Chemiluminescence Analyzer," IARC Scientific Publication, 1983, pp. 295-308.

[7] D. Forman, S. Al-Dabbagh and R. Doll, "Nitrates, Nitrites and Gastric Cancer in Great Britain," *Nature*, Vol. 313, February 1985, pp. 620-625. doi:10.1038/313620a0

[8] A. S. Pannala, A. R. Mani and J. P. Spencer, "The Effect of Dietary Nitrate on Salivary, Plasma, and Urinary Nitrate Metabolism in Humans," *Free Radical Biology and Medicine*, Vol. 34, No. 5, March 2003, pp. 576-584.

Copyright © 2011 SciRes.

doi:10.1016/S0891-5849(02)01353-9

[9] P. J. R. Phizackerley and A. S. Al-Dabbagh, "The Estimation of Nitrate and Nitrite in Saliva and Urine," *Analytical Biochemistry*, Vol. 131, No. 1, May 1983, pp. 242-245. doi:10.1016/0003-2697(83)90161-6

[10] Y. Tanaka, N. Naruishi, H. Fukuya, J.Sakata, K. Saito and S. Wakida, "Simultaneous Determination of Nitrite, Nitrate, Thiocyanate and Uric Acid in Human Saliva by Capillary Zone Electrophoresis and Its Application to the Study of Daily Variations," *Journal of Chromatography*, *A*, Vol. 1051, No. 1-2, July 2004, pp. 193-197.

[11] H. Li, C. Duncan and J. Townend, "Nitrate-Reducing Bacteria on Rat Tongues," *Applied and Environmental Microbiology*, Vol. 63, No. 3, March 1997, pp. 924-930.

[12] S. Chiappin, G. Antonelli, R. Gatti and E. F. De Palo, "Saliva Specimen: A New Laboratory Tool for Diagnostic and Basic Investigation," *Clinica Chimica Acta*, Vol. 383, No. 1-2, April 2007, pp. 30-40. doi:10.1016/j.cca.2007.04.011

[13] J. Xu, X. Xu and W. Verstraete, "Quantitative Measurement of the Nitrate Reductase Activity in the Human Oral Cavity," *Food and Chemical Toxicology*, Vol. 39, No. 4, 2001, pp. 393-400. doi:10.1016/S0278-6915(00)00150-2

[14] D. Bojiç, V. Aleksandar, L. J. Bojiç and J. M. Peroviç, "The Effects of Dietary Nitrate, Ph and Temperature on Nitrate Reduction in the Human Oral Cavity," *Physics*, *Chemistry and Technology*, Vol. 3, No. 1, May 2004, pp. 53-60.

[15] S. S. Mirvish, K. J. Reimers and B. Kutler, "Nitrate and Nitrite Concentrations in Human Saliva for Men and Women at Different Ages and Times of the Day and Their Consistency over Time," *European Journal of Cancer Prevention*, Vol. 9, No. 5, October 2000, pp. 335-342. doi:10.1097/00008469-200010000-00008

[16] B. S. Yu ,P. Chen, L. H. Ni and S. Z. Yao, "Simultaneous Determination of Nitrate and Nitrite in Saliva and Food-Stuffs by Non-Suppressed Ion Chromatography with Bulk Acoustic Wave Detector," *Analitycal Sciences*, Vol. 17, No. 4, April 2001, pp. 495-498. doi:10.2116/analsci.17.495

[17] V. Mori and M. Bertotti, "Amperometric Detection with Microelectrodes in FIA: Studies on the Conversion of Nitrate to Nitrite in Human Saliva," *Analytical Letters*, Vol. 32, No. 1, 1999, pp. 25-37. doi:10.1080/00032719908542596

[18] P. M. J. Bos, P. A. Van Den Brandt, M. Bedel and T. H. Ockhuizen, "The Reproducibility of the Conversion of Nitrate and Nitrite in Human Saliva after a Nitrate Load," *Food and Chemical Toxicology*, Vol. 26, No. 2, 1988, pp. 93-97. doi:10.1016/0278-6915(88)90104-4

[19] A. F. Badawi, G. Hosny, M. El-Hadary and M. H. Mostaf, "Salivary Nitrate, Nitrite and Nitrate Reductase Activity in Relation to Risk of Oral Cancer in Egypt," *Disease Markers*, Vol. 14, No. 2, January 1998, pp. 91-97.

[20] J. Van Maanen, D. Pachen, M. Dallinga and J. Kleinjans, "Formation of Nitrosamines during Consumption of Nitrate and Amine-Rich Foods, and the Influence of the Use of Mouthwashes," *Cancer Detection and Prevention*, Vol. 22, No. 3, 1998, pp. 204-212. doi:10.1046/j.1525-1500.1998.0oa26.x

Copyright © 2011 SciRes.