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COLORIMETRIC ASSAY OF SALIVARY NITRITE CONTENT

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Abstract. Salivary nitrate/nitrite concentration has often been used as a biomarker of human exposure to nitrate. A spectrophotometric method for estimation of nitrite in saliva is described. Nitrite (NO_2^-) is determined through formation of a reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized compound with 1-Naphthylamine. The method is suitable for the estimation of 0.25-10 μg/ml. The preservation procedure and deproteinization of saliva sample remove possible errors.
Key words: nitrite in human saliva, spectrophotometric method

Rezumat. Concentraţia de nitrit în salivă este adesea utilizată ca biomarker al expunerii la nitraţi. Lucrarea prezintă o metodă spectrofotometrică simplă, cu reproductibilitate bună pentru determinarea concentraţiei de nitrit în salivă în domeniul 0,25-10 μg/mL. Prin prezervarea şi deproteinizarea probelor de salivă se obţine stabilizarea concentraţiei de nitrit înlăturând erorile de determinare.
Cuvinte cheie: nitrit în salivă, metoda spectrofotometrică

INTRODUCTION

Nitrate exposure has been considered a risk factor at least in three different conditions:

- methemoglobinemia in formula fed babies where drinking water with high nitrate levels is used (1);
- the development of neural tube defects in babies whose mother are similarly exposed during pregnancy;
- formation of carcinogenic N-nitroso compounds.

In most of these cases, nitrite the main metabolite byproduct of nitrate, is involved in these pathological changes(2,3). It is known that nitrate is chemically inert under human physiological condition while nitrite is a reactive compound (4,5).

Salivary nitrate and nitrite analysis is commonly used for studies of body nitrate intake and endogenous synthesis (6-9).

After gastrointestinal resorption and recirculation of nitrate taken up by food and/or drinking water, its reduction into nitrite occurs in the oral cavity and it has been estimated that about 70% of ingested nitrite is formed in this way (10,11). The amount of nitrate converted into nitrite depends on the activity of salivary nitrate reductase up to about 20% (12). Saliva contains microorganisms which reduce nitrate to nitrite and which can also utilize nitrite as substrate (13-15).

In this work a spectrophotometric method for estimation of salivary nitrite is presented.

1. **Method**

a) Collection and deproteinization of samples

To prevent further reduction of nitrate and nitrite after saliva sampling, a preservation procedure of saliva was used. About 5 mL of whole saliva sample were collected in glass tubes containing 0.5 mL of 1 N sodium hydroxide. Sodium hydroxide is added as stabilizer because nitrite is unstable in acid solution.

0.2 mL of 0.5M $ZnSO_4$ were added to an aliquot of 3 mL saliva, followed by mixing. The mixture was subsequently centrifuged for 10 min. at 3000 rpm.

Treatment with $ZnSO_4$ removes proteins and other substances which could inhibit chromogen formation from nitrite. This step is of particular importance in the case of saliva and, if it is omitted, nitrite concentration may be underestimated by as much as 80%.

b) Principle. Nitrite (NO_2^-) is determined through formation of reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized compound with 1-naphthylamine:

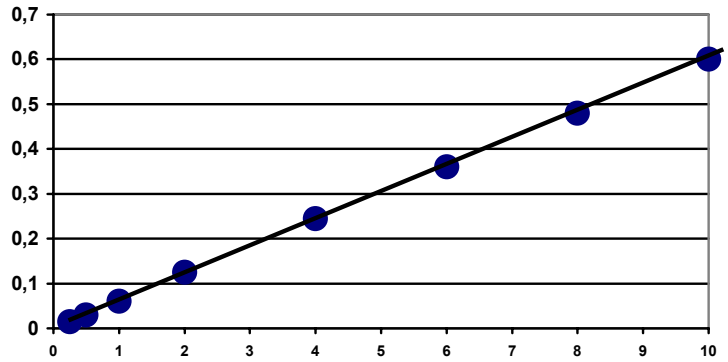
2. **Apparatus**. Spectrophotometer for use at 520 nm providing a light path of 1 cm.
3. **Color development and measurement**

- **Procedure.**Deproteinized samples (2mL) were put in glass tubes and diluted to 10 mL with distilled water. 0.4 mL 4-Aminobenzen sulfonic acid solution (1.6 g % in glacial acetic acid 30%) were added. The tubes were placed in ice for 15 min. Then, 0.4 mL of 1-naphthylamine solution (0.5 g % in glacial acetic acid 30%) were added. 20 min. after adding of this last solution to samples, the absorbance at 520 nm (A_{520}) was measured at room temperature.
- **Preparation of nitrite standard curve.** A series of standards was prepared by accurately pipeting of calculated volumes of standard nitrite solutions containing 0-10 μg NO_2^-, and diluted to 10 mL with distilled water in glass tubes. The general procedure was used followed by plotting absorbance versus nitrite concentration. Absorbance against distilled water at 520 nm was measured as blank value. The relationship between A_{520} and nitrite concentrations was linear (fig.1). The correlation coefficient of calibration curve was r = 0.99 and the linear regression equation between the concentrations of nitrite and absorbance was perfectly suited the Lambert – Beer's low.

4. **Calculation.** The sample nitrite content was estimated using calibration curve.
5. **Precision of method.** The precision of method was established using four stabilized and deproteinized saliva samples having nitrite concentrations of 2.14, 0.88, 1.14 and 0.73 μg/mL. The five parallel determinations for each sample have been performed in the same day.

The standard deviation ranged between ± 0.008 and ± 0.011 and the coefficient of variation (CV%) between 0.38 and 0.45%, what demonstrates a good precision (table1).

$y = 0.0017 + 0.06\ x \qquad r = 0.99$



µg NO_2^- / ml

Fig. 1 Nitrit standard curve

Table 1. Precision of method

Samples	1st result	2 result	3 result	4 result	5 result	$\bar{x} \pm SD$	CV%
1	2.14	2.15	2.14	2.15	2.13	2.14 ± 0.008	0.38
2	0.88	0.86	0.87	0.87	0.86	0.86 ± 0.008	0.96
3	1.14	1.14	1.12	1.13	1.13	1.13 ± 0.007	0.62
4	0.77	0.76	0.76	0.76	0.76	0.75 ± 0.011	1.45

6. **Stability of nitrite in saliva samples.** Aiming verification of nitrite stability three samples of both fresh saliva and collected saliva in tube containing 0.5 mL of 1 M NaOH solution after 1h, 2h, 4h have been analyzed. Parallel determinations for the same concentrations of 1.55, 0.75 and 0.95 µg NO_2^-/mL were performed. The nitrite concentrations decreased at 2 and 4 h in fresh saliva. In stabilized saliva samples the variation was between 0.64 and 1.33% (table 2).

The lowest level of quantitation was 0.25 µg nitrite/mL saliva.

Table 2. Stability of nitrite in saliva samples

Saliva sample	Time (h)	Sample 1	Variation of nitrite conc. %	Sample 2	Variation of nitrite conc. %	Sample 3	Variation of nitrite conc. %
Fresh saliva	1	1.55	-	0.75	-	0.95	-
	2	1.1	29.03	0.66	12.00	0.75	16.84
	4	0.81	47.74	0.39	48.00	0.48	49.74
Saliva preserved with sodium hidroxide	1	1.55	-	0.75	-	0.95	-
	2	1.54	0.64	0.74	1.33	0.95	-
	4	1.54	0.64	0.75	-	0.95	-

7. **Application of method.** The method was applied in an epidemiological study in Moldova territory where there are areas with high nitrate concentrations (35÷22 mg/L) in drinking water supplies especially ground water (16,17).

The sample consisted of 38 children (6-16 years old) and delivery nitrate content as biomarker has been assessed (table 3).

Table 3. Levels of nitrite concentrations in saliva of the children (μg/mL)

Age (years)	n	Min.	Max.	Mean ± standard deviation	Coeficient % of variation
16	5	0.48	1.81	1.17 ± 0.55	47.00
12	7	0.66	3.13	1.18 ± 0.87	73.72
11	5	0.46	1.33	0.83 ± 0.38	45.78
9	6	0.46	2.00	0.9 ± 0.57	63.33
8	4	0.88	2.18	1.26 ± 0.62	49.20
7	8	0.95	2.48	1.78 ± 0.88	49.43
6	3	1.1	2.14	1.47 ± 0.58	38.77
TOTAL	38	0.46	3.13	1.19 ± 0.59	49.28

As table 3 data show, mean nitrate salivary concentrations for all group was 1.19 ranging from 0.9 up to 1.78 μg/mL. These values seems unrelated to subject's age, as the coefficient of variation indicates.

CONCLUSIONS

- The estimation of salivary nitrite by this spectrophotometric method is simple and doesn't require a particular endowment.
- This method is sensitive, having a good precision in the assay range (0.25-10 μg/mL saliva).
- Collection of saliva samples is relatively easy and noninvasive.
- The nitrite content in saliva collected with alkali remains stable for at least 4 hours at room temperature.
- This method could be used in environmental epidemiology due to its performance parameters.

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