

U. PORTO



FACULDADE DE
MEDICINA DENTÁRIA
UNIVERSIDADE DO PORTO

**Artigo de Investigação Médico-Dentário
Mestrado Integrado em Medicina Dentária**

**“OPTIMIZATION OF A COLORIMETRIC METHOD FOR
DETERMINATION OF FLUORIDE IN SALIVA”**

Liliana Sofia Ribeiro Dias

Porto, 2012

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DETERMINAÇÃO DO FLÚOR NA SALIVA”**

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**“OTIMIZAÇÃO DE UM MÉTODO COLORIMÉTRICO PARA A
DETERMINAÇÃO DO FLÚOR NA SALIVA”**

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Abstract

Introduction: Fluoride is an important anion present in saliva. The ion-selective electrode (ISE) method is the most widely used for the determination of fluoride in saliva. The spectrophotometric methods are still seldom used. These methods have the advantage of being easily applicable to reduced samples volumes, an important feature considering the difficulties in collecting human saliva samples. However, the colorimetric methods are susceptible to several interfering substances.

Objective: The present study aimed to optimize a colorimetric method, the SPADNS (trisodium 2 - (parasulfophenylazo) - 1,8-dihydroxy - 3,6 - naphthalenedisulfonate), for the correct and sensitive fluoride determination in saliva.

Methods: Standard solutions were prepared for both methods (ion-selective electrode and colorimetric method) and calibration curves were drawn. Several approaches were carried out to eliminate the presence of interfering substance in the colorimetric method, namely the turbidity: addition of acids, filtration, heating, "saliva digestion" and distillation. Wavelength scans were performed and the absorbance at 570 nm was recorded for each analyses.

Results: In comparison to fluoride-ISE method, the colorimetric method was much less linear and sensitive for fluoride concentrations ranging from 0.01 to 1.00 mg/l. The colorimetric method revealed inappropriate for saliva samples due to saliva intrinsic turbidity. The approaches performed to eliminate the saliva turbidity were not successful or the color complex was altered.

Conclusion: Our study showed that the colorimetric method wasn't accurate for fluoride determination in saliva. Also, the interferences of colorimetric method such as turbidity were not successfully eliminated. The fluoride-ISE remains the most appropriate method for fluoride saliva determination.

Keys – Words: Saliva; Fluoride; Ion-selective electrode method; Spectrophotometric method; SPADNS; Turbidity

Resumo

Introdução: O flúor é um ião importante presente na saliva. O método eléctrodo ião-seletivo (ISE) é o mais amplamente utilizado para a determinação de flúor na saliva. Os métodos espectrofotométricos são, ainda, pouco utilizados. Estes métodos têm grande a vantagem de serem facilmente aplicáveis a volumes reduzidos de amostras, uma característica importante considerando as dificuldades na recolha de saliva humana. No entanto, os métodos colorimétricos são suscetíveis a várias substâncias interferentes.

Objetivo: O presente estudo tem como objetivo otimizar um método colorimétrico, o SPADNS(trisodium 2 - (parasulfophenylazo) – 1,8-dihydroxy - 3,6 – naphthalenedisulfonate), para a determinação correta e sensível do flúor na saliva.

Métodos:Foram preparadas soluções padrão para o método eléctrodo ião-seletivo e para o método colorimétrico. Foram traçadas curvas de calibração para ambos. Decorrente da presença de várias substâncias interferentes no método espectrofotométrico foi necessário realizar diversos procedimentos, com intuito de as eliminar. A turvação da saliva foi o interferente mais difícil de eliminar. Na tentativa de a eliminar foram efetuados procedimentos, como: adição de ácidos, filtração, aquecimento, “digestão da saliva” e destilação. Foram realizados scans do comprimento de onda e a absorvância a 570 nm foi registada para cada análise.

Resultados: Comparando com o método eléctrodo ião-seletivo, o colorimétrico foi menos seletivo e menos linear para concentrações de flúor entre 0.01 e 1,00 mg/l. O método colorimétrico revelou ser inadequado para leitura das amostras de saliva, devido à presença de turbacão, característica intrínseca da saliva. As abordagens realizadas para a eliminar não foram bem sucedidas ou provocaram uma alteraçãodo complexo.

Conclusão: Este estudo demonstrou que o método colorimétrico não consegue determinar com precisão a concentração de flúor na saliva. A eliminação das substâncias interferentes não foi conseguida. O método eléctrodo ião-seletivo continua a ser o método mais apropriado para a determinação de flúor na saliva.

Palavras-chave:Saliva; Método eléctrodo ião-seletivo; Método espectrofotométrico; SPDANS; Turbacão

Introduction

Saliva is an oral fluid secreted by major salivary glands (parotid, submandibular and sublingual), together with minor salivary glands present in the mucosa of the tongue (Von Ebner glands), cheeks, lips, palate and pharynx¹⁻³.

Salivary fluid is an exocrine secretion consisting mainly of water (99.5%), electrolytes, proteins, enzymes, mucins and nitrogenous products¹⁻⁷. All of these components interact with each other and are responsible for functions such as lubrication, protection, buffering action, clearance, maintenance of tooth integrity, antimicrobial activity, taste, speech and digestion^{1, 2, 6}. The average daily flow of whole saliva varies in health between 1 and 1.5 liters^{1, 2}.

The amount and composition of saliva depends on many factors, namely the flow rate, circadian rhythm, type and size of salivary gland, duration and type of the stimulus, diet, drugs, age, gender and physiological status^{1-3, 7}.

Fluoride is an ion available in human body in low concentrations, essentially in the dental structures and in the bone skeleton. Small amounts of fluoride are vital for the human organism, however in larger amounts it's toxic. The bioavailability of fluoride is dependent upon various factors such as fluoride administration, fluoride formulation and salivary secretion rate. Interestingly, two decades ago it has been postulated that site-specific aspects of salivary fluoride clearance may have important implications for the site-specificity of oral diseases^{5, 8-10}. Salivary fluoride has a cariostatic effect, which results from the ability to modify the physiochemical properties of the teeth and to adversely affect oral microorganism. Additionally, fluoride ions interfere with bacterial adhesion, aggregation and metabolism^{8, 11, 12}, reduce acid production in dental plaque and demineralization, enhancing the rate of remineralization of enamel^{8, 11}.

Numerous methodologies have been applied for the measurement of fluoride in various samples. These include potentiometry with fluoride ion-selective electrodes (ISE), atomic absorption spectrometry, inductively coupled plasma emission spectrometry, molecular emission cavity spectrometry, UV-VIS spectrophotometry, fluorimetry, ion and gas chromatography, electrochemical methods and electrophoresis^{13, 14}. In water samples, various methods of fluoride analysis have been used. Currently, the colorimetric and fluoride ion-selective electrode methods are the most common employed. In saliva samples, there are still few methods used; the most common one is the ISE¹⁵.

The potentiometric method (ISE) is based on measurement of a potential electrode. This method measures the potential difference between an indicator electrode and a reference

electrode. The reference electrode has a constant potential, and the potential indicator electrode is changing with the concentration of certain ions¹³. ISE have been widely used for determining fluoride since Frant and Ross first constructed a fluoride-ISE¹⁶. The fluoride electrode is a potentiometric sensor selective for this ion, which has as key element, a single crystal of lanthanum fluoride (LaF_3) that has been doped with europium, across which a potential is established for fluoride solutions of different concentrations^{10, 16, 17}. The fluoride-ISE is widely used due to its advantages, such as sensitiveness, simplicity, selectivity, portability, efficiency and analysis time. Moreover, the analysis and preparation of the aqueous matrices is quite simple. In addition, it allows the measurement of a wide range of fluoride concentrations, even in the presence of other ions, which are generally in the public water supply and in biological systems^{9, 18}.

An alternative method to fluoride-ISE is the spectrophotometric method based in Zr-SPADNS (trisodium 2 - (parasulfophenylazo) – 1,8-dihydroxy - 3,6 – naphthalenedisulfonate) complex¹⁵. Most colorimetric methods for the determination of fluoride are based on the bleaching of colored complexes of these metals with organic dyes when fluoride is added. The colorimetric method, SPADNS, is based on the reaction between fluoride and a dark red zirconium, forming a colorless complex anion. This method results in a bleaching of the red color in a proportional amount to the fluoride concentration. The color is determined photometrically using spectrophotometer reading at 570 nm wavelength^{16, 19, 20}. This method has advantages such as: low cost, rapid color development and not specific equipment requirement²¹. In addition, this method is easily integrated in hospital automated diagnostic analyzers and applicable to reduced sample volumes, an important feature to human saliva samples and is easily.

The present study aimed to compare the colorimetric SPADNS method and the commonly used fluoride-ISE for the saliva fluoride determination. The colorimetric SPADNS method revealed inappropriate for saliva samples due to saliva intrinsic turbidity. Several approaches were tested to remove saliva turbidity unsuccessfully.

Materials and Methods

Ten students from the 5th year of Master degree of Faculty of Dental Medicine of Porto University were invited to voluntarily participate in the present study. The ethics committee of the same faculty approved the consent form and research protocol. Whole saliva was collected from all participants in a quiet room over 30 minutes. When the saliva volume was insufficient for the required measurements, a pool of saliva samples was done.

Fluoride-ISE method

For the detection of fluoride by the potentiometric method it was prepared a stock solution of sodium fluoride 10 mg/l from a solution of 1000 mg/l F⁻ (Certipur Merck[®], Germany). The solution TISAB (Total Ionic Strength Adjustment Buffer) was prepared by dissolving in distilled water in a final volume of 1000 ml: 114 ml glacial acetic acid, 116 g NaCl and 0.60 g sodium nitrate dehydrate. Standard solutions of fluoride with 40% of TISAB were prepared in triplicated with distilled water in volumetric flasks and the following concentration: 0.01 mg/l, 0.025 mg/l, 0.05 mg/l, 0.1 mg/l, 0.25 mg/l, 0.5 mg/l and 1 mg/l. For the construction of a calibration curve the solutions were vigorously shaken and the potential in mV were obtained through the immersion of the fluoride-ISE (Orion 9609BN) in each solution.

Saliva samples for fluoride-ISE determination were prepared in volumetric flasks as follows: 50% of saliva, 40% of TISAB and 10% of distilled water. Fluoride-ISE potential was obtained for each sample and the fluoride concentration calculated through the calibration curve.

Saliva filtration. A saliva sample was split in two, and one part was submitted to a filtration with filters with 0.45 µm of membrane (Acrodisc Syringe Filter – Supor Membrane). Afterwards the samples were prepared and measured for fluoride as previously mentioned.

Colorimetric SPADNS method

For the detection of fluoride by the spectrophotometric method it was prepared the Zirconyl – SPADNS complex. A solution of 1.916 g/l of SPADNS (Sigma-Aldrich[®], Spain) and 5.320g/l of zirconyl chloride octahydrate (ZrOCl₂.8H₂O) (Sigma-Aldrich[®], Spain) were prepared in distilled water. Afterwards, 350 ml of concentrated chloride acid (Suprapur Merck[®],

Germany) was added to 25 ml of zirconyl solution and diluted in a final volume of 500 ml of distilled water. Equal volumes of SPADNS solution and zirconyl acid solutions were mixed (Zr-SPADNS). In addition, it was prepared a stock solution of sodium fluoride of 10 mg/l from a solution of 1000 mg/l F⁻. Standard solutions of fluoride containing 20% of Zr-SPADNS and one drop of sodium arsenite solution (NaAsO₂)(Fluka Analytical[®], Spain) were prepared in triplicated with distilled water in volumetric flasks and the following concentration: 0.01 mg/l, 0.025 mg/l, 0.05 mg/l, 0.10mg/l, 0.25mg/l, 0.50 mg/l and 1.00 mg/l. The calibration curve was drawn by the measurements of the absorbance of standard solutions in an UV/VI Spectrophotometer (UNICAM UV/Visible Spectrometer UV2-100 v4.14) at 570 nm against the blank solution (without fluoride). The samples were read in cuvettes of 1 cm quartz cells.

Saliva samples for fluoride colorimetric determination were prepared as follows: 80% of saliva, 20% of Zr-SPADNS complex and a one drop of NaAsO₂. The measurements of saliva samples absorbance were out of range, so it was performed a wavelength scan of samples ranging from 400 to 600 nm.

Approaches for saliva turbidity removal

Acids addition. To 2.5 ml of saliva samples prepared as previously reported for SPADNS method, 1 ml of nitric acid 65% (p.a. plus Fluka[®] SA, Germany) or chloric acid 30% (Suprapur Merck[®], Germany) were added. A wavelength scan was made to show the action of each of the added acids. In addition, the samples where it was added nitric acid were heated in a water bath at 100°C for 30 minutes. The absorbance of the samples was read at wavelength of 570 nm.

Filtration. Saliva was filtered through 0.45 µm membrane filters. Subsequently, the saliva samples were prepared as previously reported for SPADNS method. Another attempt was made in order to remove the turbidity through the filtration of the saliva samples and the addition of chloric acid 30%. A wavelength scan was also performed. The absorbance was read at wavelength of 570 nm.

“Saliva digestion”. Ten ml of saliva were digested with 5 ml of hydrogen peroxide 30% and 2 ml of nitric acid 65% for 12 hours at 170°C in a reactor (Teflon vessels). Fluoride was added to the reactor in order to obtain the following concentrations: 0 µg/ml, 0.29 µg/ml and 0.56 µg/ml. Subsequently, the samples were dried at 120°C. Afterwards, 5 ml of Zr-SPADNS complex and a one drop of NaAsO₂ were added to the samples. A wavelength scan was made and the

absorbance at 570 nm was recorded.

Saliva distillation. Hundred ml of saliva were distilled for 1 hour, at approximately 100°C. From the recovered volume, approximately 10 ml, 5 ml were prepared as previously described for SPADNS method. A wavelength scan was performed and the absorbance at 570 nm was recorded.

Results Analyses

Data analyses of calibration curves were performed using linear regression of GraphPad Prism[®] software package version 5.0d.

Results

Fluoride-ISE method

A quantitative analyses of fluoride was performed with calibration curves obtained with standard solutions. A calibration curve was constructed by plotting the obtained electrode potential (mV) versus the $\log_{10} [F^-]$ of the standard solution (Fig.1).

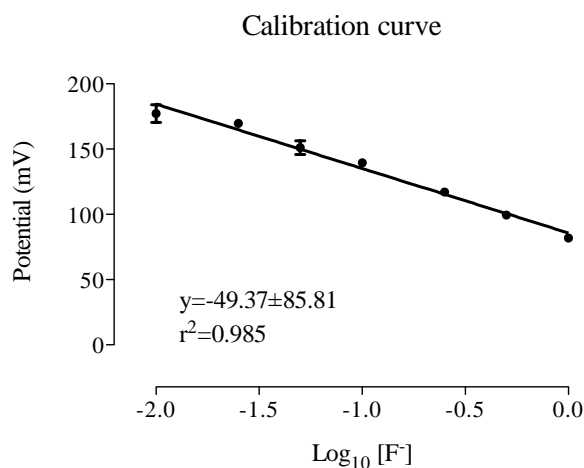


Fig. 1 - Calibration curve for fluoride determination by fluoride-ISE method using fluoride standard solutions ranging from 0.010 to 1.000 mg/l. Dots represent means and error bars represent standard deviation. Linear regression was calculated using GraphPad Prism[®] software.

The calibration curve of Fig. 1 was used to calculate the fluoride concentration of the saliva samples. The Table I shows the final fluoride concentration in saliva samples ranging from 0.0226 to 0.2045 mg/l.

Table I - Fluoride concentrations of different human saliva samples obtained by fluoride-ISE method.

Saliva samples	$[F^-]$ mg/l
A	0.2045
B	0.0611
C	0.0278
D	0.1815
E	0.0322
F	0.0226

In Table II, it is represented the potentials of saliva samples before and after filtration process with 0.45 μm filters. The filtered sample had practically the same potential value than the non-filtered sample.

Table II - The potentials values obtained by fluoride-ISE method the saliva samples before and after filtration process.

Saliva samples	mV
Before filtration	107.9
After filtration	108.0

Colorimetric SPADNS method

A calibration curve was constructed by plotting the obtained spectrophotometer absorbance versus the fluoride concentration in mg/l of the standard solutions (Fig.2).

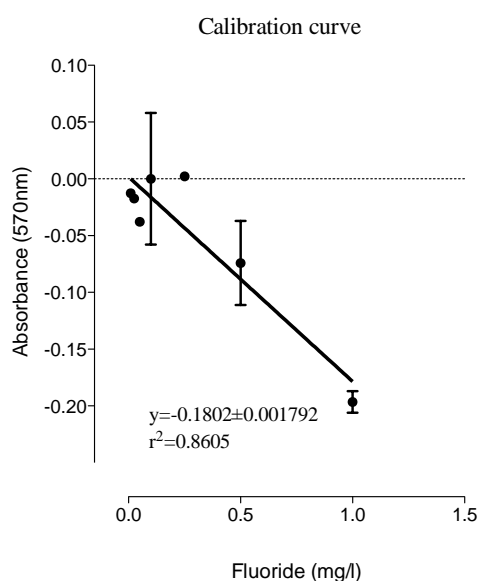


Fig. 2 - Calibration curve for fluoride determination by SPADNS method using fluoride standard solutions ranging from 0.010 to 1.000 mg/l. Dots represent means and error bars represent standard deviation. Linear regression was calculated using GraphPad Prism® software.

Although the calibration curve was not linear, with a low correlation coefficient (r^2) of 0.8605, the saliva samples were also read by this method. Table III shows the result obtained in each saliva sample analyzed. The results that were obtained by the spectrophotometer were “under range” and “over range”, which are out of the detection limit.

Table III - The absorbance value and concentration of fluoride present in each saliva sample analyzed.

Saliva samples	Absorbance	[F ⁻] mg/l
A	1.170	Over range
B	1.266	Over range
C	1.360	Over range
D	0.624	Under range

To understand these results a wavelength scan (ranging from 400 to 600 nm) was performed to a saliva sample (Fig. 3A), to a saliva sample with addition of the Zr-SPADNS complex (Fig. 3B), to a blank solution (Fig. 3C) and to a standard solution of fluoride (Fig. 3D). In the Fig. 3B it is noticeable the presence of a band, either in the zone of 440-450 nm, either in the zone of 550-600 nm. The wavelength used by SPADNS method, 570 nm, is within zone of 550-600 nm. This scan revealed that the turbidity, which is a feature of the saliva, may be an interfering factor. In Fig. 3C it is represented a wavelength scan of the blank solution, fluoride free. In Fig. 3D it is represented a wavelength scan of the standard solution of the 0.50 mg/l F⁻. In these figures can be observed the Zr-SPADNS complex behavior from 400 to 600 nm. In the blank solution (Fig. 3C) and standard solution (Fig. 3D) the two bands observed in the regions of 400 to 450 nm and of 550 to 600 nm from the saliva samples were absent. The absorbance value of the standard solution at 570 nm was 0.113 (Fig. 3D).

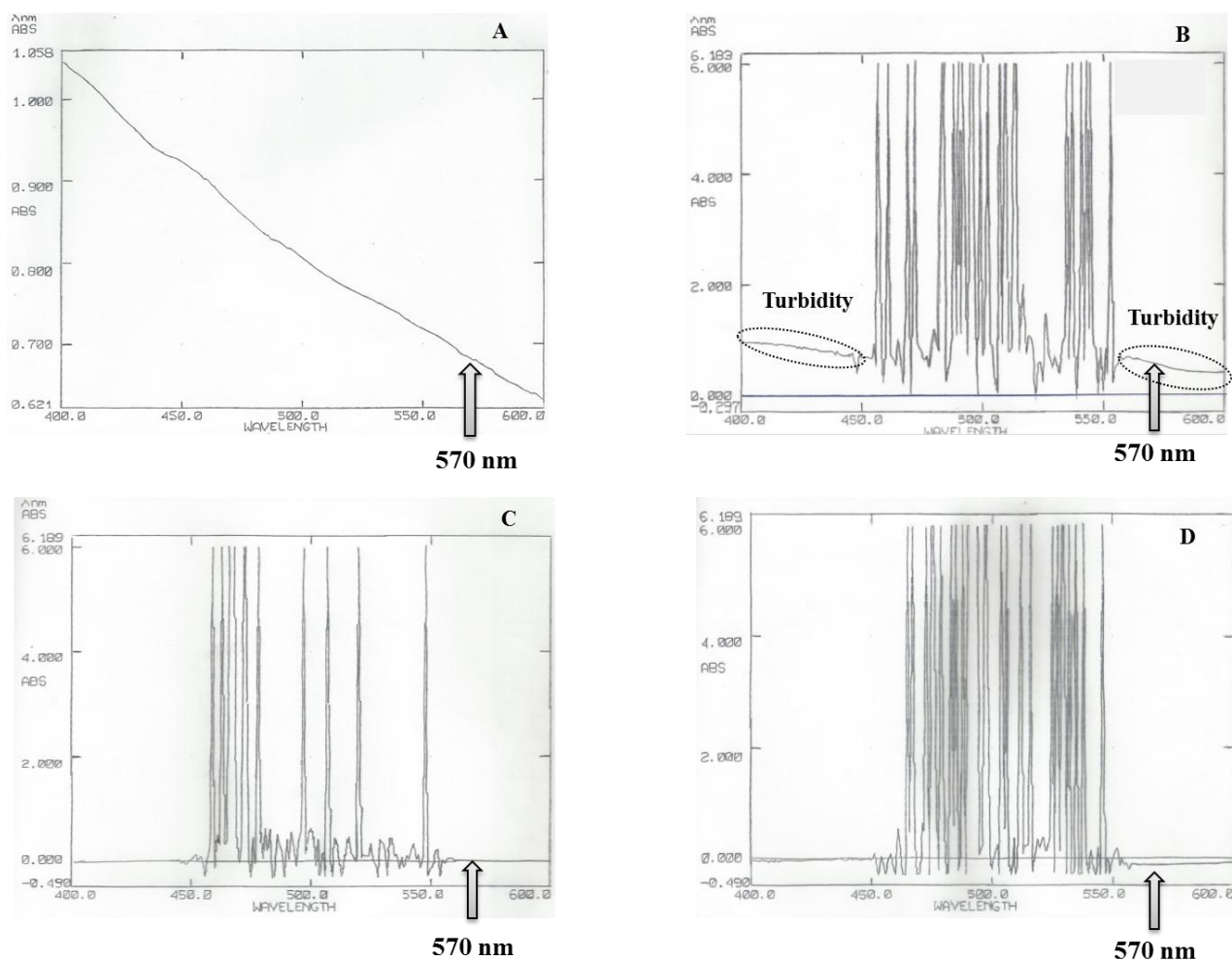


Fig. 3 – Wavelength scans (400 to 600 nm) of a saliva sample (A), a saliva sample prepared according to the SPADNS method (B), a blank solution (C) and a standard solution (D). It's observed in Fig. A and B the presence of turbidity in the region of 570 nm and in Fig. C and D the absence of turbidity in the region of 570 nm.

Approaches for saliva turbidity removal

Acids addition

Fig. 4 shows a wavelength scan of the effect in the turbidity of the chloric acid (A) and nitric acid (B) addition. With the addition of the chloric acid (A), there was a diminution of the turbidity, mainly in zone of 550-570 nm, with an absorbance value at 570 nm of -0.206. The addition of nitric acid (B) caused a more significative shortage of the turbidity, but, at the same time, there was an alteration in the Zr-SPADNS complex, visible in the wavelength zone of 460 to 550 nm. In this sample the absorbance value at 570 nm was < -3.0.

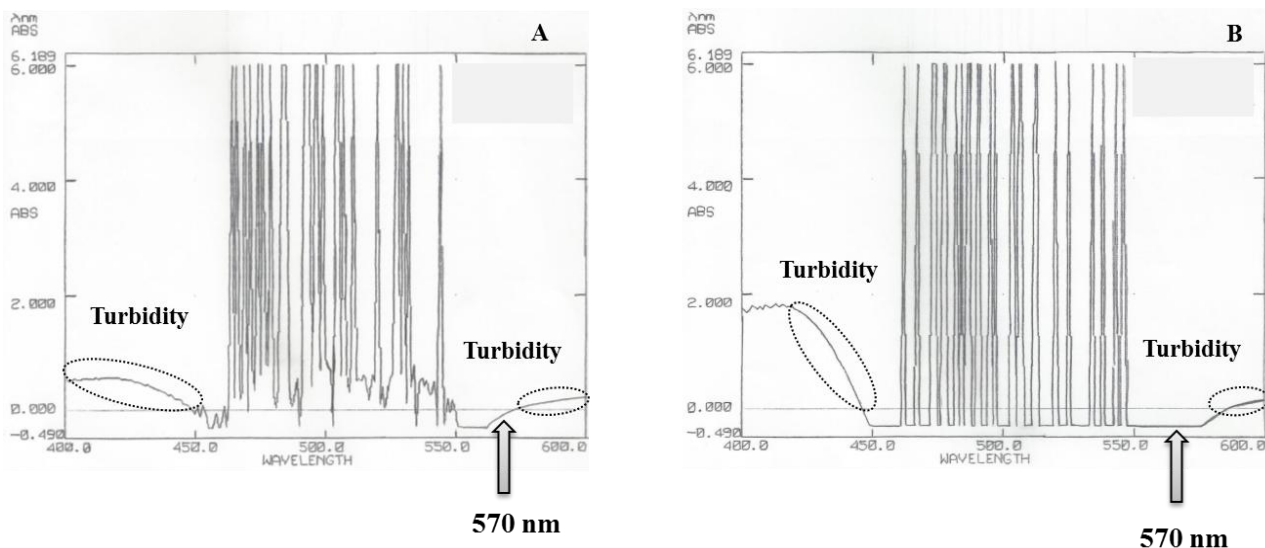


Fig. 4 – A wavelength scan (400 to 600 nm) of the effect the addition of the chloric acid (A) and of the nitric acid (B) on the turbidity. Diminution of turbidity in region of 550-600 nm.

Apart from the addition of the nitric acid to the samples of saliva, it was also carried out the heating of these samples. In Table IV, it is represented the values of absorbance of each saliva sample analyzed, without heating (sample A) and with heating (sample B). There wasn't any differences between the heated sample and the one that wasn't heated. The absorbance for both was < - 0.3.

Table IV – The absorbance value of each sample, without heating (A) and with heating (B)

Saliva samples	Absorbance
A	< - 3.0
B	< - 3.0

Filtration

Filtration slightly reduces the turbidity of saliva (Fig. 5A). This reduction is more visible in the 550-600 nm zone (reading zone), than in the 400-450 nm zone. The absorbance value at 570 nm was 0.263. In Fig. 5B it is represented a wavelength scan of the effect of filtering the samples of saliva and the addition of chloric acid. This procedure eliminated the turbidity, but, at the same time, there was an alteration in the Zr-SPADNS complex, visible in the wavelength ranging from 460 to 550 nm. The absorbance value at 570 nm was < -3.0 .

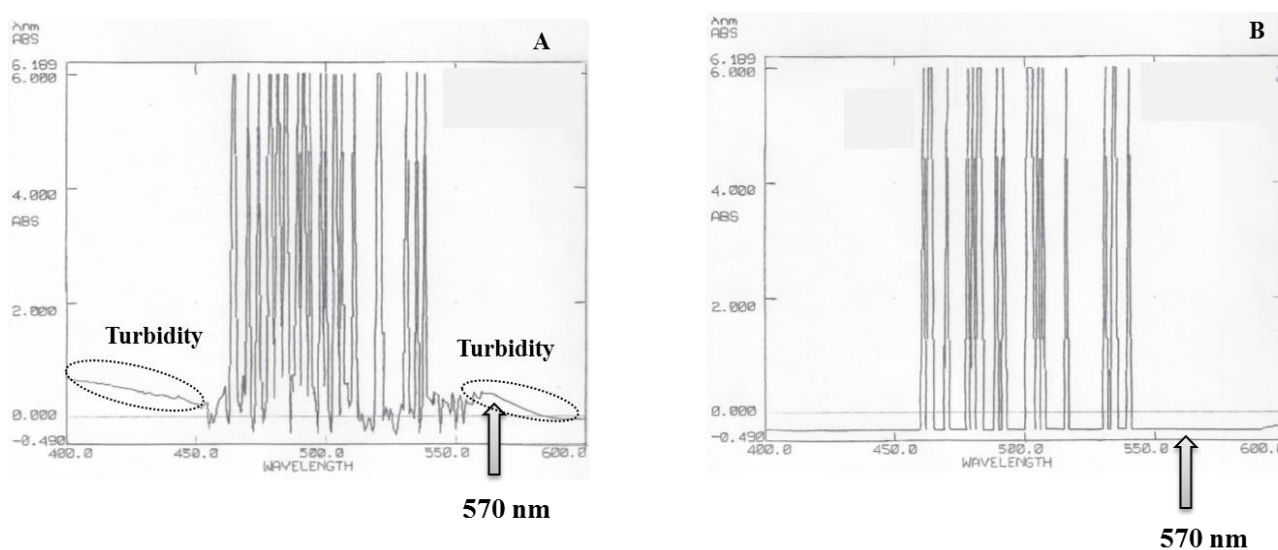


Fig. 5 – A wavelength scan (400 to 600 nm) of the effect of filtering on the turbidity (A) and of the effect of filtering and chloric acid addition on the turbidity (B). Presence of turbidity in the region of 570 nm in Fig. 5A, but absence of turbidity in region of 570 nm in Fig. 5B.

“Saliva digestion”

In Table V it is represented the absorbance value and concentration of fluoride present in each saliva sample analyzed after saliva sample digestion. The results obtained in the spectrophotometer were “over range”, which means that it failed to read correctly the saliva samples, since they were out of the detection limit. Fig. 6 shows a scan of the effect of this procedure on the turbidity of the saliva sample (sample 3). There was a significative shortage of the turbidity with this laboratory procedure.

Table V – Absorbance value of each saliva sample analyzed after saliva sample “digestion”

Saliva samples	Absorbance
1	Over range
2	Over range
3	Over range

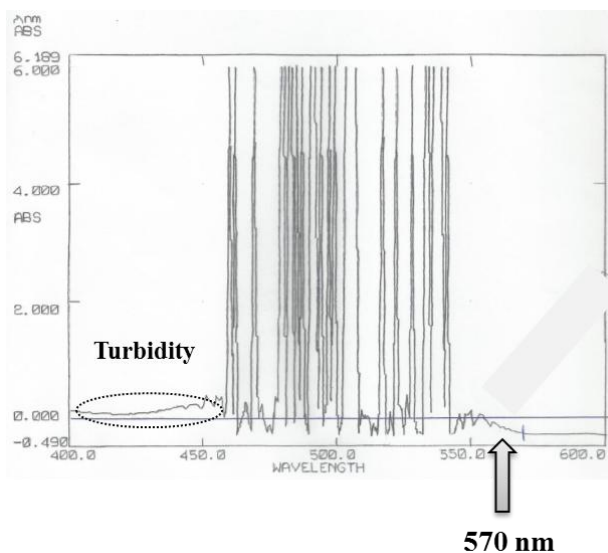


Fig. 6 – A wavelength scan (400 to 600 nm) of the effect of “saliva digestion” on the turbidity. Absence of turbidity in the region of 570 nm.

Saliva distillation

Fig. 7 shows a scan of the effect of distillation on the turbidity of the saliva. The distillation of the sample didn’t alters the saliva turbidity. The absorbance value at 570 nm was 0.675.

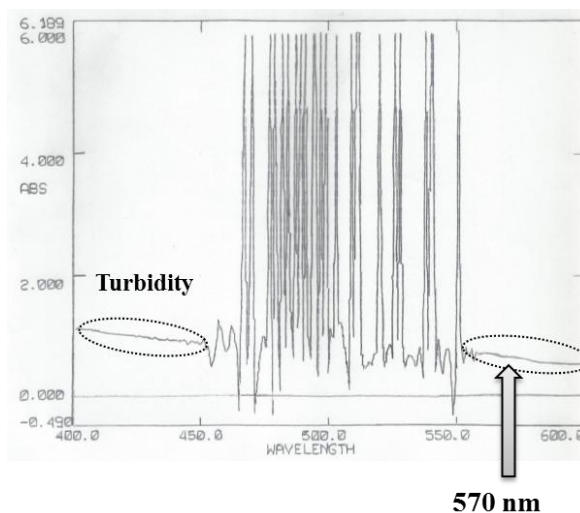


Fig. 7 - A wavelength scan (400 to 600 nm) of the effect of distillation on the turbidity of the saliva. Presence of turbidity in the region of 570 nm.

In Fig. 8 it is represented a summary at the absorbances values at 570 nm obtained by the several approaches attempt for turbidity removal in the colorimetric SPADNS method. In this figure it is noticeable that absorbance value of the saliva + Zr-SPADNS complex was greater (1.306). Contrary, the absorbance values of the nitric acid, saliva heating and filtration + chloric addition were lower (-3.0).

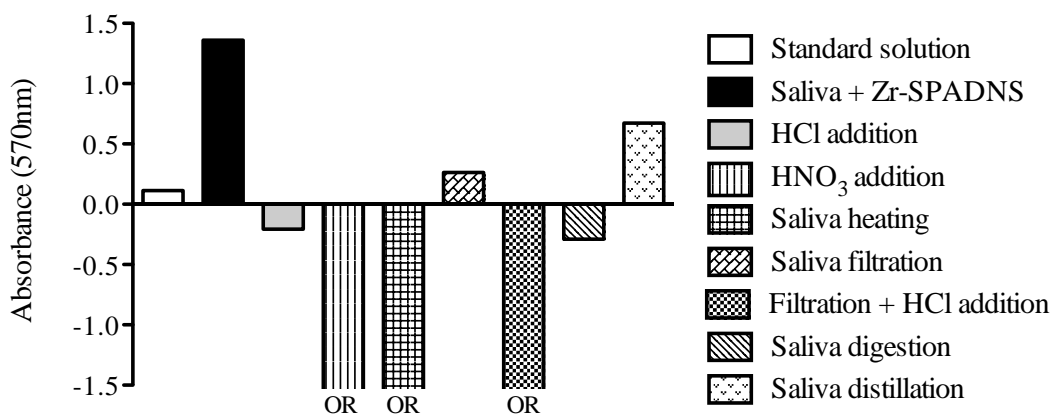


Fig. 8 – Graph with the absorbance values at 570 nm of the several solutions used in the optimization SPADNS method. The highest value was of the saliva + Zr-SAPDNS complex. The lowest value of absorbance were of the nitric acid, saliva heating and filtration + chloric addition, which were outside detection limit (OR= out of).

Discussion

The fluoride analysis in water samples is frequent and several optimized methods are available. However, in saliva the methods for fluoride analysis are scarce. The most common method for fluoride analysis is the fluoride-ISE¹⁵. Although this method is accurate for saliva samples, the fluoride-ISE is not always available in dental laboratories and the requested minimum volumes for analysis are not easy to collect, especially for unstimulated saliva. The fluoride analysis in water samples by the colorimetric SPADNS method is easily performable in all laboratories with a simple spectrophotometer and easily applicable to reduced saliva volumes. Given this, in the present research work, an attempt was made to optimize the colorimetric method, the SPADNS, to analyze saliva fluoride content.

The fluoride-ISE method is designed to sense fluoride ions selectively. A standard reference electrode has a constant potential and the indicator electrode reads the potential established by the fluoride ions across a crystal between a standard solution and the sample solution¹⁵. According to previous studies, the fluoride-ISE method enables the determination of very low concentrations of fluoride (to 10^{-4} mg/l)^{14, 15, 18}. Similarly, in the present study we found a good linearity of this method down to 0.01mg/l of fluoride (Fig. 1).

Under normal conditions, the salivary concentrations of fluoride ranges within narrow limits, despite the influence of circadian rhythm, individual variation and stimulation²². In resting saliva the concentration of fluoride ranges from 0.00380 mg/l to 0.0380 mg/l²³. In stimulated saliva the concentration of fluoride ranges from 0.0152 mg/l to 0.120 mg/l²³. In our study the fluoride concentration in saliva measured by fluoride-ISE were within this reference values. In addition, these salivary fluoride concentrations were within the interval where the ISE method is sensitive and linear, so, salivary fluoride could be detected through the ISE method.

The colorimetric SPADNS method is mainly based on the reaction between fluoride and a red zirconium, forming a colorless complex anion. This method results in the bleaching of the red color in an amount proportional to the fluoride concentration. As the amount of fluoride increases, the resulting color becomes lighter. Color is then determined photometrically using a spectrophotometer in wavelength of 570 nm^{15, 22}.

Our study showed that the SPADNS method was not accurate for fluoride concentrations in saliva. This was revealed by the low correlation coefficient ($r^2 = 0.8605$) of the calibration curve performed with concentrations ranging from 0.01 to 1.00 mg/l of fluoride (Fig. 2). In

addition, according to previous studies, the SPADNS method detection limits for fluoride are rarely better than 0.02 mg/l, which is in agreement with our results^{14,24}.

Nevertheless, the problem of SPADNS method in reduced ranges of saliva fluoride concentration could be overcome by the addition of a known fluoride concentration to saliva samples increasing the reading range. So, the process of optimization continued to evaluate the possible saliva matrix interferences.

According to literature, the colorimetric SPADNS method is susceptible to several interfering substances. High concentrations of alkalinity, aluminum, chloride, turbidity, color, iron, sodium hexamethaphosphate, phosphate and sulfate in the sample will result in an error in the determination of fluoride content, since they interfere with the formation of the complex and consequently with the reading of the absorbance^{13, 15, 25}. Because the effect is neither linear nor algebraically additive, the mathematic compensation is impossible. Whenever one of the substances is present in sufficient quantities to produce an error superior to 0.1 mg/l, and when the samples are colored and/or turbid, a pretreatment of the sample should be carried out^{15, 25}.

The chloride and phosphate ions in a solution produce a positive error when an analysis is made, in concentrations of 7000 mg/l and 16 mg/l, respectively. Even though the concentration of the chloride ion in the saliva is lower than the necessary to induce an error (around 500 mg/l), its presence in this study was masked with the addition of sodium arsenite^{25, 26}. The concentration of phosphate in the saliva ranged from 350 to 380 mg/l, therefore, this ion is an interfering agent^{26, 27}. In this sense, it is important to find a method that allows eliminating or masking the presence of phosphate.

The Al^{3+} , Fe^{3+} and SO_4^{2-} ions produce a negative error when their concentrations are 0.1, 10 and 200 mg/l, respectively. Nevertheless, all these ions were present in the saliva in lower quantities than those mentioned. The iron concentration in this oral fluid, as referred by Chicharro *et al*²⁸, is around 5 mg/l. According to Watanabe *et al*²⁹, the aluminum is non-essential for humans, resulting in a low-level distribution in organs²⁹. The concentration of sulfate in the saliva, as mentioned by Chen *et al*²⁶, is approximately 15 mg/l^{26, 30}. Given this, all these previously referred ions (except phosphate ion) are in the saliva in concentrations that wouldn't be interferent in the colorimetric method.

Concerning alkalinity, the neutralization with chloride or nitric acid is indicated, when it is the only significant interfering agent in the solution. It acts as negative error²⁵. In our study, this procedure was not necessary because the pH of the saliva ranged from 6.2 to 7.4^{1, 3}.

The turbidity produces a positive interference in SPADNS method, and this is noticeable in Fig. 3. In Fig. 3A and 3B it is observed the presence of a band in the zone of 440-600 nm, which correspond to the turbidity of the saliva. This band is absent in wavelength scan of blank solution and of standard solution (Fig. 3C and 3D). The wavelength used by SPADNS method, 570 nm, is within zone of 550-600 nm. In this sense, the turbidity interferes with the reading of saliva samples in the spectrophotometer (Table III). Herein, several approaches were made aiming to eliminate the turbidity of the saliva samples, such as the addition of acids, filtration, heating, “saliva digestion” and distillation.

The added acids to the saliva samples were the chloride acid and the nitric acid. Chloride acid provoked a diminution of the turbidity in the 550-570 nm zone, though it didn't eliminate it. The nitric acid provoked a more significant reduction of the turbidity at 570 nm, but it was observed an alteration of the Zr-SPADNS complex, revealed by the comparison of wavelength scan with the blank solution. The heat seemed to reduce the turbidity, but did not improve the Zr-SPADNS complex alteration, because there wasn't any differences between the heated sample and the one that wasn't heated and absorbance values obtained (Table IV).

Another procedure that was carried out to try to eliminate the turbidity was the filtration. This procedure provoked a slight decrease of the turbidity, when compared with wavelength scan of a saliva sample (Fig. 3B). In comparison to acids addition, filtration has a worse effect. So, chloride acid was added to previously filtered samples. Nevertheless, and despite the fact that this procedure provoked a complete elimination of the turbidity, an alteration of the Zr-SPADNS complex was observed, revealed by the wavelength scan (Fig. 5B). This happens because the chloride acid is a strong acid, which acidifies the solution and alters the optimum pH for the action of the complex. Patel *et al*¹⁹, in their study, mention that the acidity range of Zr-SPADNS complex is critical¹⁹. Moreover, the absorbance value obtained was quite inferior compared to the one obtained when the chloride acid was added, however it was equal to the value obtained when the nitric acid was added (Fig. 8). The addition of the nitric acid after the filtration wasn't tested in this procedure, for reasons that have been already mentioned.

The “saliva digestion” was performed and it was observed an efficient elimination of the turbidity in the saliva (Fig. 6), however no results were obtained (Table V). One hypothesized that some interference in the method might have occurred.

Lastly, the effect of distillation in the turbidity of the saliva was also tested. The distillation is the reference method to be carried out to eliminate all possible interferents in the

colorimetric method^{15, 20}. However, saliva distillation didn't reduce significantly its turbidity (Fig. 7).

Our results show that among all performed approaches to eliminate the turbidity in the saliva, the addition of chloride acid, the filtration and the "saliva digestion" lead to better results. However they were not enough to allow the determination of fluoride concentrations in our saliva samples. The others procedures had an effect in the turbidity, but they also led to an alteration of Zr-SPADNS complex. The distillation didn't produce any effects in the turbidity of the saliva, contrary to what is described in literature^{15, 20}. The turbidity is an inherent feature of the saliva, which is difficult to eliminate. Its presence in the saliva was one of the biggest difficulties found during the attempt to optimize the colorimetric SPADNS method, since its elimination was not achieved.

Concerning ISE method, according to Bratovcić *et al*³¹ this method is relatively free from interferences, provide a rapid, convenient and non-destructive means to quantify anions and cations³¹. However, Brossoket *et al*¹⁵ referred that this method may be affected by the same substances that affect the colorimetric methods. With the exception of alkalinity, the concentrations of the interfering substances have to be high to result in error in the electrode reading¹⁵. The ions Al^{3+} , Fe^{3+} and SO_4^{2-} , in concentrations of 3, 200 e 5000 mg/l, produce a negative error¹³. Additionally, the cations Ca^{2+} , Mg^{2+} , Cu^{2+} , the anions Cl^- , NO_3^- , PO_3^{4-} and colloids influence low-level F^- analyses²⁴. The anion interferences yield insignificant errors, but the cation interferences, namely Al and Fe, were problematic even after adding TISAB. The TISAB was used in order to control the ion strength of the solution, adjusts of the pH value and releases fluoride from metal complexes²⁴. Most ionic interferences, except the Al ion, which readily form stable complexes with F^- ions, can be removed by using TISAB III²⁴. Rajković *et al*¹⁶ refer that all negative interfering effects are eliminated by usage of TISAB buffer¹⁶.

According to Brossoket *et al*¹⁵, color and turbidity do not interfere with the results of the fluoride-ISE method^{13, 24}. Nevertheless, considering that the turbidity was one of the most difficult interferences to eliminate in the spectrophotometric method, it was tested if the turbidity really didn't have any influence on the ion-selective method. Indeed, saliva filtration didn't seem to interfere with the reading of the fluoride-ISE; these results are in accordance to Brossoket *et al*¹⁵.

For Bratovcić *et al*³¹, the ISE method has possible interferences, such as OH^- and the stirring. Concerning OH^- , this author mentions that the crystal lanthanum-doped with europium has the unique property of apparently being permeable to the fluoride ion and it is virtually

impermeable to other anions or cations³¹. Since ion-selective electrode responds to activity of the analytic, the ionic strength solution, it is extremely important. From the literature it is known that the OH⁻ ions are only interfering ions for fluoride electrode, at pH greater than eight. However, at pH lower than five, the hydrogen ions also interfere. The interference for this fluoride-electrode is pH less than five and higher than eight³¹. The pH of the saliva do not produce interference in this method because its pH varies between 6.2 and 7.4^{1, 3}. The stirring may have a substantial effect on the observed potential. The example referred by the authors (Bratovcić *et al*³¹) is that in a solution of 10⁻³ M NaF, the potential changes from -61.5 mV to -5.5 mV when the solution is submitted to a rapid stirring, it was that this change was less at high concentration³¹.

The saliva is a fluid that exists in the oral cavity, composed by several components which are essential for the maintenance of oral structures. In this way, a deep knowledge of the composition of the saliva is of extreme importance. Indeed, it would be important to carry out more studies with the intention of implementing not only a new method for the determination of fluoride in saliva (more advantageous than the reference method), but also other methods that enables a wider determination of other metals and/or ions. A possible method for that would be the ICP-MS (Inductively Coupled Plasma Mass Spectrometry).

In conclusion, our results were conditioned by saliva's turbidity among others interfering substances. Despite all procedures performed, saliva turbidity elimination was not achieved and consequently the attempt to optimize the SPADNS method for the determination of saliva fluoride was not successful. The fluoride-ISE method for fluoride determination in saliva is faster, more efficient and more reliable than SPADNS method.

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