JOSÉ Antonio Santos SOUZA

EFEITO DO TRIMETAFOSFATO DE SÓDIO E DO FLUORETO SOBRE A SOLUBILIDADE DA HIDROXIAPATITA: ESTUDO *IN VITRO*

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Efeito do trimetafosfato de sódio e do fluoreto sobre a solubilidade da hidroxiapatita: Estudo *In vitro*

Dissertação apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção do título de Mestre em Ciência Odontológica, área de concentração Saúde Bucal da Criança.

Orientador: Prof. Alberto Carlos Botazzo Delbem

Coorientador: Prof. Juliano Pelim Pessan

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Dedicatória

Dedico este trabalho

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não se recente do mal;
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Resumo Geral

SOUZA, J. A. S. **Efeito do trimetafosfato de sódio e do fluoreto sobre a solubilidade da hidroxiapatita: Estudo *In vitro*.** Dissertação (Mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2014.

Resumo Geral

O objetivo deste estudo foi avaliar o efeito de diferentes concentrações de trimetafosfato de sódio (TMP) associado ao fluoreto (F) sobre a solubilidade da hidroxiapatita (HA), bem como as propriedades físicas e químicas da HA depois de um ciclo de pH. O pó de HA sintética (1,0 g; n= 6) foi misturado com soluções de TMP nas concentrações de 0; 0,1; 0,2; 0,4; 0,6; 0,8; 1,0; 2,0; 4,0; 6,0; 8,0 e 10%, associadas ao F nas concentrações de 0, 100, 250 e 500 ppm F, durante 2 min. A suspensão foi filtrada e o precitado foi seco por 24 horas a 37°C e moído. Depois do tratamento, as amostras de HA tratada foram submetidas a um ciclo de pH. As amostras de HA ciclada foram caracterizadas por energia dispersiva de raios-X (EDX), espectroscopia de infravermelho (FTIR) e difração de raios-X (DRX). Depois, as concentrações de F solúvel em base e em ácido, cálcio (Ca) e fósforo (P) foram determinadas na HA ciclada, assim como as concentrações de P e F no sobrenadante. Os dados de F, Ca, P e proporção molar Ca/P na HA, e F e P no sobrenadante foram submetidas à Análise de Variância e teste de Student-Newman-Keuls ($p<0,05$). Houve uma redução do tamanho dos cristais de HA com o aumento da concentração de TMP. A HA tratada com 500 ppm F e TMP a 1% produziu uma HA com cristalitos de tamanho maiores. O FTIR mostrou uma redução na intensidade das bandas de fosfatos e carbonato quando comparado à HA sintetizada. As maiores proporções Ca/P foram observadas na HA tratada com TMP entre 0,4% e 1% quando combinado com 250 ppm F, e entre 0,4% e 2% em associação com 500 ppm F ($p<0,05$). Um aumento na concentração de TMP levou a uma redução na incorporação de F solúvel em ácido na estrutura do cristal para todos os grupos, nas duas situações (depois do tratamento e depois do ciclo de pH) ($p<0,05$). Adicionalmente, um aumento na concentração de TMP também proporcionou uma maior adsorção de TMP à HA para as soluções de 0 e 100 ppm F, mas menor para as soluções de 250 e 500 ppm F ($p<0,05$). Os resultados indicam que o mecanismo de ação do TMP está relacionado à adsorção de TMP à

estrutura do cristal de HA e que o F e TMP em combinação podem precipitar uma HA mais cristalina e com uma menor quantidade de impurezas. Na presença do F, houve uma menor adsorção de TMP na estrutura da HA, confirmando a hipótese de que o TMP e o F competem pelo mesmo sítio de ligação na HA.

Palavras-chave: durapatita - polifosfatos - fluoretos.

General Abstract

SOUZA, J. A. S. **Effect of sodium trimetaphosphate and fluoride on hydroxyapatite solubility: An *in vitro* study.** Dissertação (Mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2014.

General Abstract

The aim of this study was to evaluate the effect of different concentrations of sodium trimetaphosphate (TMP) associated with fluoride (F) on hydroxyapatite (HA) solubility, as well as in chemical and physical properties of HA after a pH-cycle. Synthetic HA powder (1.0 g; n= 6) was mixed with solutions of TMP at 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0 and 10%, associated with fluoride at 0, 100, 250 and 500 ppm F, during 2 min. The suspension was filtered and the precipitates were dried for 24 h at 37°C and grinded. After the treatment, HA samples were submitted to a pH-cycle. Post-cycled HA samples were analyzed by energy-dispersive X-ray spectroscopy (EDX), infrared spectroscopy (FTIR) and X-ray diffraction (XRD). Afterwards, the concentration of alkali and acid soluble F, Ca and P were determined in post-cycled HA, as well as P and F in the supernatant. The data of F, Ca, P, Ca/P ratio of HA, and F and P in the supernatant were submitted to ANOVA and Student-Newman-Keuls' test ($p<0.05$). A reduction of the size of the HA crystallites was seen with increasing TMP concentrations in the solutions. HA treated with 500 ppm F and 1% TMP produced an HA with crystallites of larger size. The FTIR showed a reduction in the bands corresponding to phosphates and to carbonate for all groups when compared to the HA synthetized. The highest Ca/P ratios were observed for HA treated with TMP concentrations between 0.4% and 0.8% when combined with 250 ppm F, and between 0.4% and 2% in association with 500 ppm F ($p<0.05$). The increase of TMP led a reduced acid-soluble F incorporation in HA for all groups, both immediately after treatment and after the pH cycle ($p<0.05$). Additionally, the increase in TMP concentrations led to higher P adsorption to HA for the 0 and 100 ppm F solutions, but lower for the 250 and 500 ppm F solutions ($p<0.05$). The results indicate that the mechanism of action of TMP seems to be related with the TMP adsorption to crystal structural of the HA and that F and TMP in combination can precipitate a HA more crystalline and with a smaller amount of impurities. In the presence of F, there was a lower TMP adsorption on

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Keywords: durapatite - polyphosphates - fluorides.

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LISTA DE ABREVIATURAS

ANOVA=	Análise de Variância
°C=	Graus Celsius
Ca=	Cálcio
F=	Fluoreto
FHA=	Flúor-hidroxiapatita
g=	Grama
g=	Gravidade
h=	Hora
HA=	Hidroxiapatita
HCl=	Ácido clorídrico
HNO_3=	Ácido Nítrico
KOH=	Hidróxido de Potássio
L=	Litro
n=	Número de amostra
NaOH=	Hidróxido de sódio
P=	Fósforo
pH=	Potencial de Hidrogênio
s=	Segundo
SD=	Desvio padrão
TISAB=	Tampão ajustador de força iônica total
min=	Minuto
mg=	Miligrama
mL=	Mililitro
mmHg=	Milímetro de mercúrio

$mol\ L^{-1}$= Molaridade

NH_4OH= Hidróxido de amônio

nm= Nanômetro

mV= Milivolt

se= erro padrão da média

TMP= Trimetafosfato de sódio

μ= Micro

cm= Centímetro

FTIR= Espectroscopia no infravermelho transformada de Fourier

EDX= Energia dispersiva de raios-X

XRD= Difração de raios-X

H_2O= Água

OH^-= Íon hidroxila

HPO_4^{2-}= Íon fosfato monohidrogenado

PO_4^{3-}= Íon fosfato

CO_3^-= Íon carbonato

ppm= Partes por milhão

ν_1= Estiramento simétrico

ν_2= Vibração angular

ν_3= Estiramento assimétrico

ν_4= Vibração angular

KBr= Brometo de Potássio

a.u.= Unidade Aleatória

Sumário

Sumário

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EFFECT OF SODIUM TRIMETAPHOSPHATE AND FLUORIDE ON HYDROXYAPATITE SOLUBILITY: AN *IN VITRO* STUDY

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Abstract

This study evaluated the effect of different concentrations of sodium trimetaphosphate (TMP) associated with fluoride (F) on hydroxyapatite (HA). Synthetic HA powder (1.0 g; n=6) was suspended in solutions containing TMP varying at 0-10% associated with fluoride at 0, 100, 250 and 500 ppm F, during 2 min. The precipitates were filtered (24 h at 37°C), ground and submitted to a pH-cycle. Samples were analyzed by energy-dispersive X-ray spectroscopy (EDX), infrared spectroscopy (FTIR) and X-ray diffraction (XRD). The concentration of F, Ca and P were determined in HA and P and F in the supernatants. Data were submitted to ANOVA, followed by Student-Newman-Keuls' test ($p<0.05$). A reduction of the size of the HA crystallites was seen with increasing TMP concentrations. The FTIR showed alterations in the bands corresponding to phosphates and to carbonate for all groups when compared to the HA synthetized. Highest Ca/P ratios were observed for HA treated with TMP concentrations between 0.4% and 0.8% when combined with 250 ppm F, and between 0.4% and 2% in association with 500 ppm F ($p<0.05$). The increase of TMP led a reduced acid-soluble F incorporation in HA for all groups ($p<0.05$). The increase in TMP concentrations led to higher P adsorption to HA for the 0 and 100 ppm F solutions ($p<0.05$). Thus, the mechanism of action of TMP seems to be related with the TMP adsorption on enamel surface by binding to HA and that F and TMP in combination can precipitate a HA more crystalline and less soluble.

Keywords: hydroxyapatite - polyphosphates - fluoride.

Introduction

In human tooth enamel, hydroxyapatite (HA - $Ca_{10}(PO_4)_6(OH)_2$), crystals are arranged into highly organized prisms to form the main unit. In the oral cavity, tooth enamel can be damaged by the local cariogenic bacteria in biofilm (caries) or non-bacterially derived erosive challenges (such as acidic beverages) [1]. The maintenance of HA in dental structures by decreasing its dissolution can be achieved by the use of fluoride (F)-containing products, such as mouthrinses, toothpastes, fluoride varnishes, gels and restoratives materials [2].

Several studies have suggested that TMP reduces the demineralization process, and that an ideal TMP/F ratio allows an enhancement of the effects of F-containing products [3-7]. When TMP and F are co-administered, the adsorption of TMP on the enamel surface can change the selective permeability and facilitate the diffusion of ions Ca and F [6] into the enamel [8]. However, the mechanism of action of the TMP has not been completely clarified.

Calcium phosphates have been used as biomaterials and have been considered as tissue engineering scaffolds because their similarity to the mineral phase of hard tissue in the body. HA is a calcium phosphate widely used due its unique properties as biodegradation and bioactivity [9]. These properties added to its high capacity to adsorption and/or absorption molecules may provide an excellent support for prolonged action of anticancer drugs for the treatment of bone tumors. Moreover, the chemical and structural features of HA allow its use in medical field as biocompatible material in implants and prosthesis. In dentistry, HA has been used to avoid bone loss after the restoration or extraction of a tooth, for example. In addition, titanium implants coated with HA have been used to replace the root [10]. It has been demonstrated that TMP and F in combination may improve the crystallinity of the HA [11]. Thus, an appropriate TMP:F molar ratio would be important to obtain a HA crystal with higher crystallinity and less solubility.

Nonetheless, the studies assessing the effects of F and TMP in the dynamics of dental caries and erosion cited above have not considered the direct interactions between F and TMP with dental enamel, which would be helpful to provide new insights on the mechanisms of action of TMP. Thus, the aim of this study was to evaluate the effect of low-F solutions associated with TMP at varying concentrations on chemical and physical properties of HA after pH-cycle. The F concentrations studied are those present in products of home and commercial use, such as

mouthrinses (100 and 250 ppm F) and dentifrices (500 ppm F). The study's null hypothesis was that the chemical and physical properties of HA would not be influenced by the presence of TMP and F, either alone or in combination.

Materials and Methods

Synthesis of HA

HA powder was prepared based on the protocol by Qu and Wei's [12]. Briefly, 300 mL of 1 mol L^{-1} calcium nitrate solution ($Ca(NO_3)_2 \cdot H_2O$, Sigma-Aldrich Corp. St. Louis, MO, USA) and 600 mL of 0.3 mol L^{-1} diammonium phosphate solution ($(NH_4)_2HPO_4$, 600 mL, Sigma-Aldrich Corp. St. Louis, MO, USA) were prepared and had their pH raised to 10–12 by adding NH_4OH (29.5%). Afterwards, the diammonium phosphate solution was added slowly to the calcium nitrate solution (2–5 mL/min), under constant agitation at 37°C. The suspension was aged for 7 days at 37°C and the pH was adjusted diary at around 10 in order to allow the growth and a formation of a single crystalline phase. The system remained open in order to precipitate a carbonated HA similar to that present in the dental tissue. Then, the suspension was filtered using a Buchner funnel attached to a vacuum system (–600 mmHg), the precipitates were washed with anhydrous ethanol and with deionized water (250 ml/0.5 g HA) to remove the contaminant ions (NH_4^+ and NO_3^-) [9]. After, the precipitates were dried for 24 h at 70°C and then grinded into a fine powder with the aid of a ball mill (Pulverisette 7, Fritsch, Germany). Six samples of approximately 0.5 g were separated for characterization through of energy-dispersive X-ray spectroscopy (EDX), infrared spectroscopy (FTIR) and X-ray diffraction (XRD) and to perform the F, calcium (Ca) and phosphorus (P) analysis (Anexo A).

Treatment and pH-Cycle

Solutions (100 mL, n=6) containing TMP ($Na_3P_3O_9$, Sigma-Aldrich Co., USA) at 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0, and 10%, associated with 0, 100, 250 and 500 ppm F (NaF, Merck, Darmstadt, Germany) were prepared. At first, the synthetic HA powder (1.0 g) was mixed with each of the prepared solutions during 2 min under constant agitation to 37°C. Afterwards, the precipitate was collected by filtration using a Buchner funnel attached to a vacuum system (–600 mmHg), washed repeatedly with deionized water (250 mL/0.5 g HA) and dried for 24 h at

37°C. The precipitates were then grinded again into a fine powder with a ball mill (Pulverisette 7, Fritsch, Germany). During the treatment of synthetic HA powder, an aliquot of the suspension was collected and centrifuged (Combi – 514R) in order to calculate the P and F adsorption in the HA. After the treatment, HA samples (0.5 g) of each respective group was mixed with deionized water and the pH of the suspensions was slowly reduced to 4.0 using 1 mol L^{-1} nitric acid (HNO_3, Merck, Darmstadt, Germany) under agitation. After 30 min, the pH of each solution was raised to 7.0 by the addition of 1 mol L^{-1} sodium hydroxide (NaOH, Merck, Darmstadt, Germany) (post-cycled HA). Samples of synthetic HA powder (n= 6) were mixed with deionized water (negative control). After completion of this process, the precipitates were separated by filtration, washed with deionized water, dried for 24 h at 37°C and grinded into a fine powder (post-cycled HA) as described above. Post-cycled HA was analyzed for fluoride, calcium and phosphorus concentrations (Anexo B).

Calcium and phosphorus analysis in hydroxyapatite

For calcium (Ca) and phosphorous (P) determination, 5 mg of HA powder was weighed into pre-weighed micro-centrifuge tubes and 2.0 mL of 1 mol L^{-1} HCl was added. After agitation for 1 h (Shaker, SK-300, Lab. Companion, Kimpo City, Korea), the samples were diluted (1:10) and partly neutralized to avoid the HA powder precipitation. Aliquots of 5 µL were taken from the samples and added to 50 µL of deionized water and Arsenazo. For calibration, standards containing 40 to 200 µg Ca/mL were used. The Ca analysis was performed using a spectrophotometer (Microplate Spectrophotometer EONC, Biotek, USA) with a wavelength of 650 nm by adopting the Arsenazo III colorimetric method [13]. Phosphorus was measured by the molybdate method [14] with a wavelength of 660 nm and using aliquots of 20 µL from the samples, which were subsequently added to a mixture of 50 µL molybdate solution and 20 µL of reactive reducer. Standards containing 1.5 to 24 µg P/mL were used. The Ca and P analyses were realized in duplicate (Anexo C).

Fluoride analysis (alkali-soluble and acid-soluble F)

For fluoride analysis, 5 mg of HA powder was weighed into pre-weighed micro-centrifuge tubes, and 2.0 mL of 1 mol L^{-1} KOH was added for alkali-soluble F extraction, according to Caslavska et al.'s method [15]. After 24 h of continuous

agitation (Shaker, SK-300, Lab. Companion, Kimpo City, Korea), the samples were centrifuged (Combi – 514R) for 20 min at 2900 × *g*. A 0.5 mL aliquot of the supernatant was neutralized with 0.5 mL of TISAB II (total ionic strength adjustment buffer) modified with 1 mol L^{-1} HCl (0.82 mL HCl/L). Alkali-soluble F was determined by using a specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and reference electrode (Analyser, Sao Paulo, Brazil) connected to an ion analyzer (Orion 720A+ Thermo Scientific, Beverly, MA, USA). For determination of acid-soluble F, the precipitate was washed three times with deionized water and once with methanol. After methanol evaporation (overnight at 60°C), 1 mL of 1 mol L^{-1} HCl was added, and the samples were homogenized for 30 s by vortexing, and subsequently agitated for 1 h at room temperature. 0.5 mL aliquot of these samples was then added to 0.5 mL of TISAB II modified with 20 g NaOH/L. Samples were analyzed for acid-soluble F as described for alkali-soluble F. The F analyses were performed in duplicate (Anexo D).

Phosphorous and fluoride analysis in suspension during the HA treatment

F and P concentrations were determined in supernatants removed from the suspensions during the HA treatment. P was determined using an aliquot of 100 µL of sample and standards, plus 50 µL molybdate and 20 µL of reactive reducer through the colorimetric method described by Fiske & Subbarow [14]. The F was determined by a specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and inverted reference electrode (Orion 900100) coupled to an ion analyzer (Orion 720 A+, Thermo Scientific, Beverly, MA, USA). The electrode was calibrated with standards containing 0.25 to 4.00 µg F / mL and 4.0 to 64.0 µg F / mL under the same conditions of samples. Aliquots of 40 µL of samples and the same volume of TISAB II were dispensed on the active tip of the reference electrode. Analyses were performed in duplicate. Afterwards, the adsorption of F and TMP to hydroxyapatite was calculed from the initial concentrations of these compounds in the solutions and the concentration during the HA treatment (Anexo E).

Energy-dispersive X-ray spectroscopy (EDX)

Samples (n=1) of each group of HA were prepared and placed in sample holder to the EDX analysis in order to quantify the atomic percentage Ca, P, F and oxygen (O). It was performed in order to complement and support the discussion of

the results obtained by structural as well as biochemical analysis. HA powders were dropped onto a specific holder and the characterization was carried out using a scanning electron microscope (Carl Zeiss, model EVO LS-15, NTS, LTD, Germany) at a voltage of 20kV (× 500-1000 magnification) associated with energy-dispersive X-ray spectrophotometer (Oxford Instrument, Inca X-act) with 133eV resolution.

X-Ray Diffraction (XRD)

Samples (n=1) of each F group of HA associated with TMP concentrations at 0, 0.4, 1.0, 6.0 and 10%, were prepared and placed in sample holder to the XRD analysis. Powder XRD was performed at room temperature, using Cu-Kα radiation (Ultima IV X-ray diffractometer, Rigaku Corp., Osaka, Japan) generated at a voltage of 40 kV and a current of 20 mA. The scanning range (2θ) was from 10 to 60° with a step size of 0.02°. The CRYSTMET database (Toth Information Systems Inc., Ottawa, Canada) was used for phase identification. The crystallite sizes were estimated using the Scherrer equation ($d= K \lambda/ \beta \cos \theta_\beta$), where d is the diameter dimension of the crystalline particle, λ is the wavelength of the incident X-ray (1.542 Å), β is the line broadening at half the maximum intensity (FWHM) diffraction line width of the diffraction peak, θ_β is the Bragg angle obtained from the XRD pattern, and K (0.9) is the slope factor.

FTIR Spectroscopy (FTIR)

Samples (n=1) of each F group of HA associated with TMP concentrations at 0, 0.4, 1.0, 6.0 and 10%, were analyzed by FTIR Spectroscopy. First of all, the samples were mixture with powder potassium bromide (KBr), in the proportion of 1 mg of sample to 600 mg of KBr. Soon after, a pellet was made with 170 mg of this mixture (sample plus KBr). The infrared absorbance spectra were recorded by the absorbance method in an FTIR spectrophotometer (Nexus 670, Nicolet Instrument Corporation Madison, USA) using 128 scans at 4 cm^{-1} resolution in the spectral range between 400 and 4,000 cm^{-1}. The intensity of the absorption band was divided by the pellet thickness, and the coefficient of absorption (in cm^{-1}) was measured regarding the baseline joining the points of lowest absorbance on the peak using the subtraction of a straight line. Thus, the absorption coefficient values obtained were compared among the groups evaluated. The error of absorption coefficient measurements was of the 0.005 order.

Data Analysis

For statistical analysis, SigmaPlot 12.0 was used, and the significance limit was set at 5%. Ca, P, Ca/P ratio, alkali and acid soluble F, F and P data showed normal (Shapiro Wilk test) and homogeneous (Cochran's test) distribution and were subjected to two-way ANOVA followed by Student-Newman-Keuls' test. Correlation between Ca and P in HA (Pearson's test) was calculated to quantify the relationships between these ions under all conditions studied. FTIR and XRD were described as a function of the presence of specific bands obtained from different treatment submitted to the pH-cycle. FTIR data were analyzed as absorption coefficient and data obtained from XRD were evaluated from the diameter values of crystallite sizes. EDX data were described as atomic percentage of the elements.

Results

The XRD pattern obtained for the synthetic HA is shown in Figure 1a. This pattern was compared with that available at the CRYSTMET database, confirming that the material obtained by the method described above consists only of HA. The diffractograms obtained for all samples were similar to the pattern seen at CRYSTMET, but differences in the size of crystals were observed (Figure 2). A reduction of the crystallites was seen with increased TMP concentrations in the most of groups. The treatment of HA with the 500 ppm F solution associated to 1% TMP promoted an increase of its crystallinity when compared to the synthetic HA (Figure 2d). Solutions containing F without TMP led to an increase of the HA crystals in comparison to synthetic HA, moreover, an increase of the F concentration in the groups without TMP promoted an increase in the size of the crystallites.

The infrared absorption spectra of the HA synthetized are presented in the Figure 1b. The characteristic bands of the HA corresponding to the functional groups of the phosphates (PO_4^{3-}) and hydroxyl (OH^-) were observed at all spectra. The phosphate presented absorption bands between 960 and 1,000 cm^{-1} (symmetric stretching - ν_1), 1,000 and 1,200 cm^{-1} (asymmetric stretching - ν_3), 540 and 580 (angular vibration - ν_4) and 600 and 620 cm^{-1} (angular vibration - ν_4). The OH^- band was observed in the region of 630-650 cm^{-1}. Alterations in the intensity of most of these bands were observed when the HA, treated with F and TMP, was submitted to a pH-cycle as described in the Table 1. The intensities of the

phosphate bands to all groups were lower when compared to the synthetic HA except for the band at 964 cm^{-1}, where the most of the samples presented an increase in the intensity of this band. Furthermore, it was observed that an increase at the TMP concentration promoted a reduction in the intensity of the phosphate bands.

The bands at 1418 and 1451 cm^{-1} are related to the vibrational mode ν_3 (stretching) of the carbonate group that presented lower intensity at the groups with F and TMP compared with the synthetic HA (Table 1). With the increase of TMP concentration, there was a reduction in the intensity of these bands. For all groups, a reduction in the intensity of the OH^- band at 634 cm^{-1} can be observed in relation to the HA synthetized. Monohydrogen phosphate (HPO_4^{2-}) ions can be detected from the peaks at 875 and 868 cm^{-1} at the carbonated HA. In the groups with F and TMP, it was observed a reduction of this band in relation to the synthetic HA. On the other hand, the groups without F presented an increase in the intensity of this band (874 cm^{-1}) when compared to the HA synthetized.

Figure 3 shows Ca and P concentrations in HA, and Ca/P ratio among the groups tested. Increases in the percentage of TMP in the solutions led to lower Ca content in HA for all groups ($p<0.05$) (Anexo F). A similar pattern was seen for P concentrations in HA. Further values for the variation of Ca and P according to the TMP concentration are shown in Figures 3a and 3b. Ca/P ratios were greatly influenced by both F and TMP in the solutions (Figure 3c). For the F-free solution, TMP did not affect Ca/P ratio at any concentration tested ($p>0.05$). On the other hand, a dose-dependent trend between TMP and Ca/P ratio was observed for the F solutions without TMP. Samples with TMP concentration between 0.4% and 1% presented significantly higher values of Ca/P ratio for the 250 ppm F, as well as between 0.4% and 2% for the 500 ppm F solution. A strong positive correlation ($r=0.820$, $p<0.001$) was observed between Ca and P in the HA structure (Figure 3d). EDX analysis showed the % atomic of Ca, P, F and O. (Figures 4) (Anexo G).

Regarding alkali-soluble and acid-soluble F, the overall pattern showed that fluoride levels in both post-treated and post-cycled HA were related to F concentrations in the solutions, regardless the TMP concentrations (Figure 5). In the groups with no F, alkali-soluble and acid-soluble F were not affected by TMP in the solutions for both substrates. For post-treated HA, alkali-soluble F was significantly reduced for TMP concentrations between 0% and 0.8% when compared with higher

TMP concentrations for the 250 and 500 ppm F solutions, while only minor changes were observed for the 100 ppm F solution (Figure 5a). Acid-soluble F concentrations were inversely related to TMP concentrations in the solutions (Figure 5c) for all F concentrations tested. For post-cycled HA, alkali-soluble F increased according to TMP concentrations for the 250 and 500 ppm F solutions, while only the 100 ppm F solution with TMP at 6% presented a slight increase. Samples of HA treated with 500 ppm F and TMP between 2% and 10% showed the highest alkali-soluble F among all samples. Alkali-soluble F values in post-cycle samples were lower than those for the post-treatment samples.

Estimated F and TMP adsorption to HA is shown in Figure 6. Increase of TMP concentration led to an increase of P adsorption in the HA structure for the 0 and 100 ppm F, while lower adsorption was seen for the solutions with the highest concentrations of fluoride (250 and 500 ppm F) (Figure 6a). Fluoride adsorbed was proportional to the F concentration presented in the solutions and related to the alkali-soluble F on HA (Figures 6b and 5b).

Discussion

This study evaluated the structural and biochemical alterations of HA treated with F and TMP using a pH-cycle model, in order to provide additional data for a better understanding on the mechanisms of action of this phosphate. The present results showed that chemical and physical properties of HA can be significantly modified by the presence of F and TMP in combination, in comparison with F or TMP alone, thus leading to the rejection of the study's null hypothesis.

The pH-cycle method used showed a reduction in the Ca/P ratio of the control group (no TMP or F), which was mainly caused by loss of Ca from HA. Although the pH-cycle model was able to lead a reduction in the Ca concentration and Ca/P ratio in the experimental groups (Figure 3), there was no change in the basic crystalline arrangement (Figure 2). In the present study, the increase in the percentage of TMP in the treatment solutions promoted a reduction in the P concentration for all groups with F when compared to that of the HA. According to Rodríguez-Lorenzo et al. [16], this reduction could be related to the occurrence of P in the form of HPO_4^{2-} (866-879 cm^{-1}) in the sample, which occupies PO_4^{3-} sites. The HPO_4^{2-} band (866-879 cm^{-1}) is related to the formation of a calcium-deficient HA (Ca/P 1.5-1.6) [17] as HA synthesized in this experiment. The peak intensity in this

region is higher when the Ca/P ratio or Ca concentration [18,19] is lower. These data are in line with the results obtained by FTIR and chemical analysis. The peak intensity in this region was higher in the groups without F when compared to that with F and TMP and was associated with a lower Ca/P ratio and the Ca and P concentration (Table 1). On the other hand, the decrease in the intensity of the carbonate (ν_3) bands observed in the groups with F and TMP are related to well-mineralized apatites phases [20].

According to Freund et al. [21], the absorption band at 631 cm^{-1} reacts to the introduction of F into the OH^- chains. In particular, this band shifts markedly to higher wavenumber and decreases in intensity. In addition, new bands appear nearby. The treatment of the HA with F and TMP promoted no displacement of this absorption band in the present study. Furthermore, new bands were not observed in the spectra of the groups (Table 1). In addition, there was no change in the basic crystalline arrangement as shown in Figure 2. It indicates that, the F and TMP did not modified the structure of the HA after the pH-cycle. However, it can be adsorbed on the HA as it was observed in this study (Figure 6). Thus, it would be interesting evaluate more accurately these chemical interactions in order to understand how TMP could be adsorbed.

The incorporation of F ions into the apatite structure was shown to increase its crystallinity and the Ca/P ratio, what is in line with previous data showing that HA samples treated with 1100 ppm F presented a higher crystallinity and Ca/P ratio than the HA treated with deionized water [11]. In the present study, Ca/P ratios increased according to the F concentration in the solutions, being dose-dependent. The solution containing 250 ppm F associated with TMP between 0.4 and 0.8% showed the highest Ca/P ratio for this F concentration which are in agreement with an *in vitro* study conducted by Missel et al. [22], that observed an improved reduction of bovine enamel demineralization when 250 ppm F was associated with TMP at 0.25 and 0.5% in dentifrices. Furthermore, the solution containing 500 ppm F associated with TMP among 0.4 and 2% showed also the highest Ca/P ratio for this F concentration and, mainly, promoted an increase in the crystallinity of the HA (Figure 2d), what is also in line with *in vitro* data showing that HA treated with 500 ppm F associated to 1 and 3% TMP increased twice the alkali-soluble F content and precipitated an HA with a Ca/P ratio more similar to synthetic HA [23].

After pH-cycle, formation of alkali-soluble F on HA increased for the 500 ppm F group associated to TMP at higher concentrations (2 to 10%). This observation helps to explain why the addition of 3% TMP to low-fluoride dentifrices (500 µg F/g) led to an anticaries action similar to that of standard dentifrice using a pH-cycling model and bovine enamel specimens [24]. The authors also showed that this association also increased fluoride and calcium present in enamel, showing results similar to those of a standard dentifrice (containing 1100 ppm F). However, in the present study, a reduction of acid-soluble F incorporation on the HA for all F concentrations associated with TMP was observed, what is in agreement with results obtained using a similar protocol [25]. The effect of TMP and F has been related with the TMP adsorption on enamel surface that seems to involve the same binding sites as those for F and can, thus, interfere with its action depending on the TMP concentration. TMP form a “barrier” on the enamel surface that could limit acid diffusion and allow the deposition of F as CaF_2, which is helpful in the remineralization process and would be released during acid challenges [26].

HA is usually produced from wet chemical synthesis, due to its simplicity, low cost, and easy application in industrial production [27]. It is important mention that in this study an *in vitro* model was used to simulate dissolution and precipitation for the evaluation of the effect of TMP and/or F on HA. However, this is a chemical model and therefore it presents limitations, especially related to the inability to reproduce the complex intraoral conditions. Such as, the saliva and the acquired pellicle are extremely important in the de- and remineralization process as well for adsorption of ions and molecules to the HA structure.

Conclusion

To conclude, the combination of F and TMP promoted changes in the biochemical and physical properties of HA. At appropriate TMP:F molar ratios, this association can precipitate a more crystalline HA and with lower amount of impurities. It was also observed that a lower TMP adsorption on the HA structure occurred in the presence of F, thus reinforce the hypothesis that TMP and F compete for the same binding sites in the HA. Thus, the action mechanism seems to be related with the TMP adsorption on enamel surface by binding to HA.

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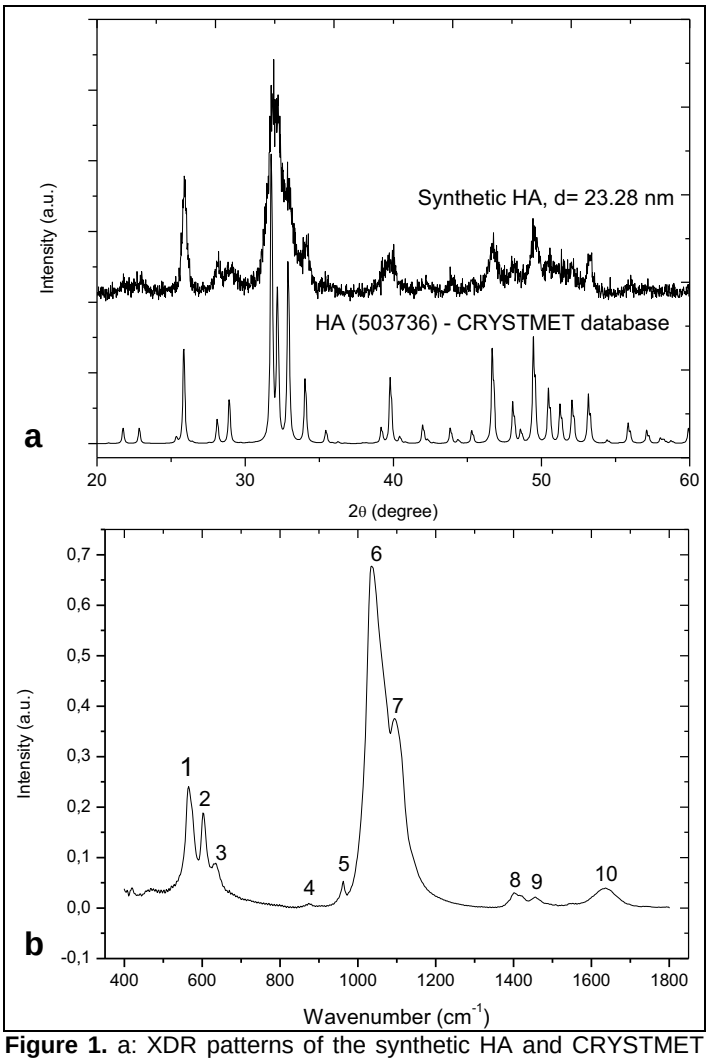


Figure 1. a: XDR patterns of the synthetic HA and CRYSTMET database. b: HA FTIR spectra obtained for synthetic HA (Peaks 1,2: PO_4^{3-}; 3: OH^- 4: HPO_4^{2-}, 5, 6 and 7: PO_4^{3}; 8 and 9: CO_3^-; 10: H_20).

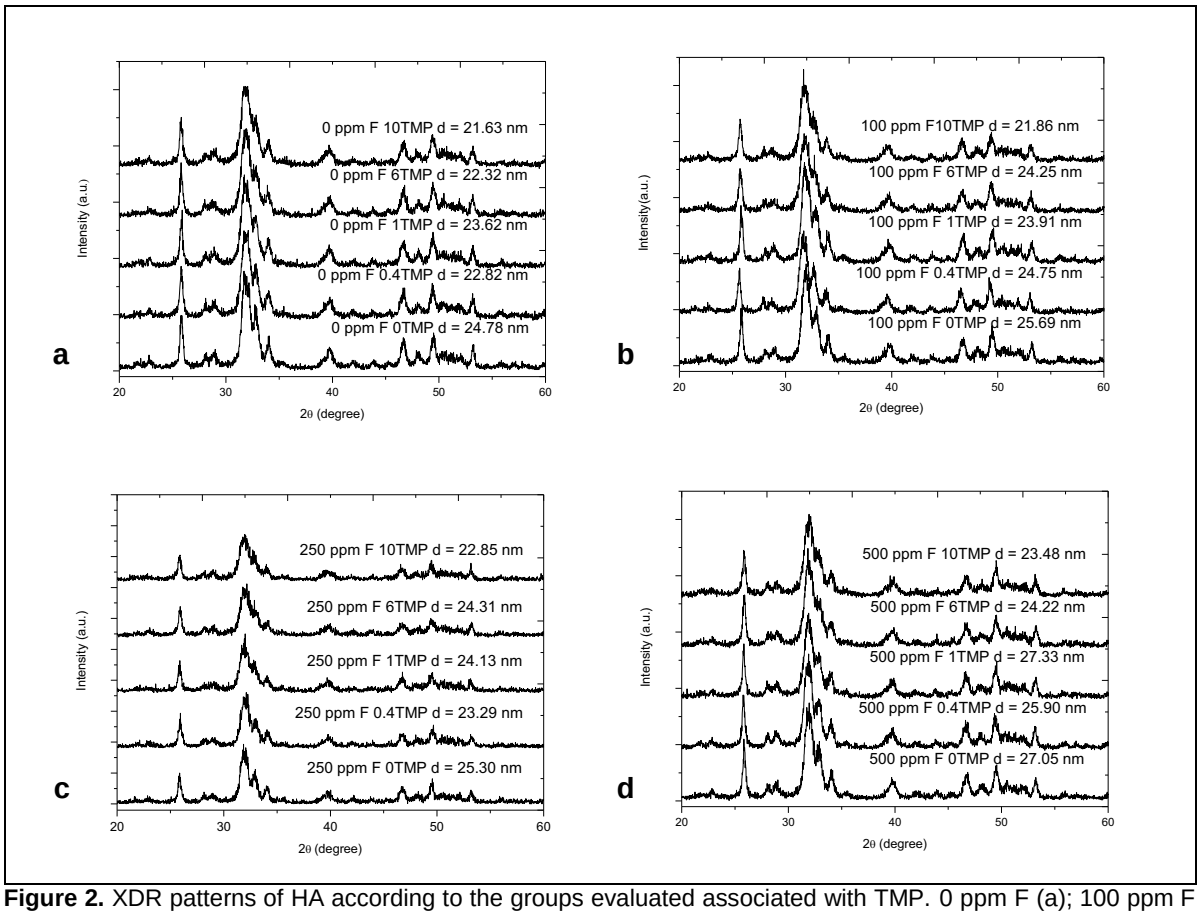


Figure 2. XDR patterns of HA according to the groups evaluated associated with TMP. 0 ppm F (a); 100 ppm F (b); 250 ppm F (c); 500 ppm F (d). The diameter values (nm) of the crystal after pH-cycle according to the fluoride concentration (ppm F) and percentage of TMP are represented by the letter d.

Table 1. Absorption coefficient obtained in the FTIR analysis according to the groups evaluated regarding the F and TMP concentration

Groups		Wavenumber (cm^{-1})								
µg F/g	TMP%	**565**	**603**	**634**	**874**	**964**	**1,039**	**1,094**	**1,418**	**1,451**
	HA	4.879	3.740	1.650	0.151	0.695	17.459	8.920	0.590	0.457
	0	4.132	3.056	1.253	0.220	1.175	13.264	7.366	0.427	0.351
	0.4	4.100	3.180	1.300	0.100	0.881	13.230	7.312	0.302	0.205
0	1	3.488	2.800	1.272	0.120	0.828	10.939	6.283	0.263	0.218
	6	3.812	2.936	1.248	0.102	0.815	12.179	6.827	0.280	0.243
	10	3.997	3.183	1.529	0.197	0.887	12.165	6.966	0.234	0.196
	0	4.911	4.159	1.614	0.152	0.965	16.464	9.426	0.541	0.473
	0.4	4.389	3.440	1.210	0.121	0.945	14.459	7.967	0.368	0.349
100	1	3.682	3.166	1.234	0.103	0.758	11.922	6.624	0.289	0.243
	6	3.951	3.393	1.318	0.064	0.767	12.730	7.054	0.301	0.262
	10	4.259	3.445	1.405	0.089	0.732	13.350	7.227	0.319	0.261
	0	4.732	3.972	1.637	0.191	0.929	14.803	7.871	0.433	0.365
	0.4	3.810	3.177	1.454	0.078	0.671	11.610	6.422	0.405	0.370
250	1	4.412	3.647	1.604	0.143	0.742	13.302	7.223	0.384	0.318
	6	3.353	2.725	1.250	0.058	0.586	9.861	5.735	0.261	0.261
	10	3.860	3.201	1.540	0.084	0.638	10.595	6.297	0.327	0.327
	0	3.185	2.712	1.193	0.058	0.522	9.260	5.412	0.305	0.286
	0.4	3.804	3.093	1.312	0.103	0.717	12.131	6.525	0.378	0.339
500	1	3.379	2.746	1.173	0.056	0.561	10.459	5.889	0.351	0.302
	6	3.559	2.803	1.314	0.103	0.619	11.128	5.379	0.316	0.248
	10	3.742	3.144	1.515	0.066	0.519	11.016	6.266	0.349	0.299

* 565, 603, 964, 1039 and 1094 cm^{-1} correspond to phosphate bands; 874 cm^{-1} correspond to monohydrogen phosphate (HPO_4^{2-}); the carbonate vibrational mode is located at regions of 1418 and 1451 cm^{-1} and the OH band was observed at 634 cm^{-1}.

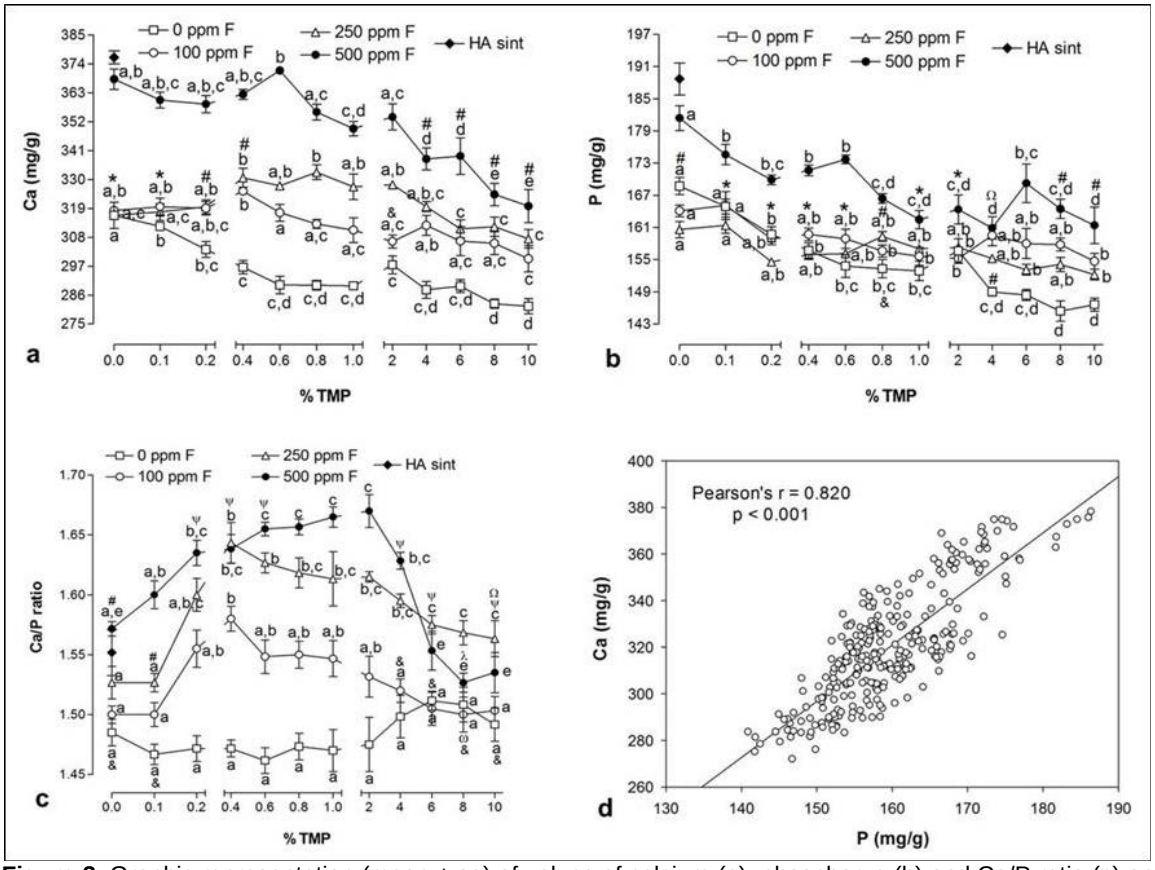


Figure 3. Graphic representation (mean ± se) of values of calcium (a), phosphorus (b) and Ca/P ratio (c) on hydroxyapatite synthetized and after pH-cycle according to the fluoride and TMP concentration. Correlation: concentration of calcium and phosphorus on hydroxyapatite (d). Distinct letters show significant differences between the % TMP for each fluoride concentration (Student-Newman-Keuls, p <0.05). (*) 0 ppm F = 100 ppm F = 250 ppm F; (&) 0 ppm F = 100 ppm F; (Φ) 0 ppm F = 250 ppm F; (#) 100 ppm F = 250 ppm F; (λ) 0 ppm F = 500 ppm F; (Ω) 100 ppm F = 500 ppm F; (ψ) 250 ppm F = 500 ppm F; (ω) 100 ppm F = 250 ppm F = 500 ppm F.

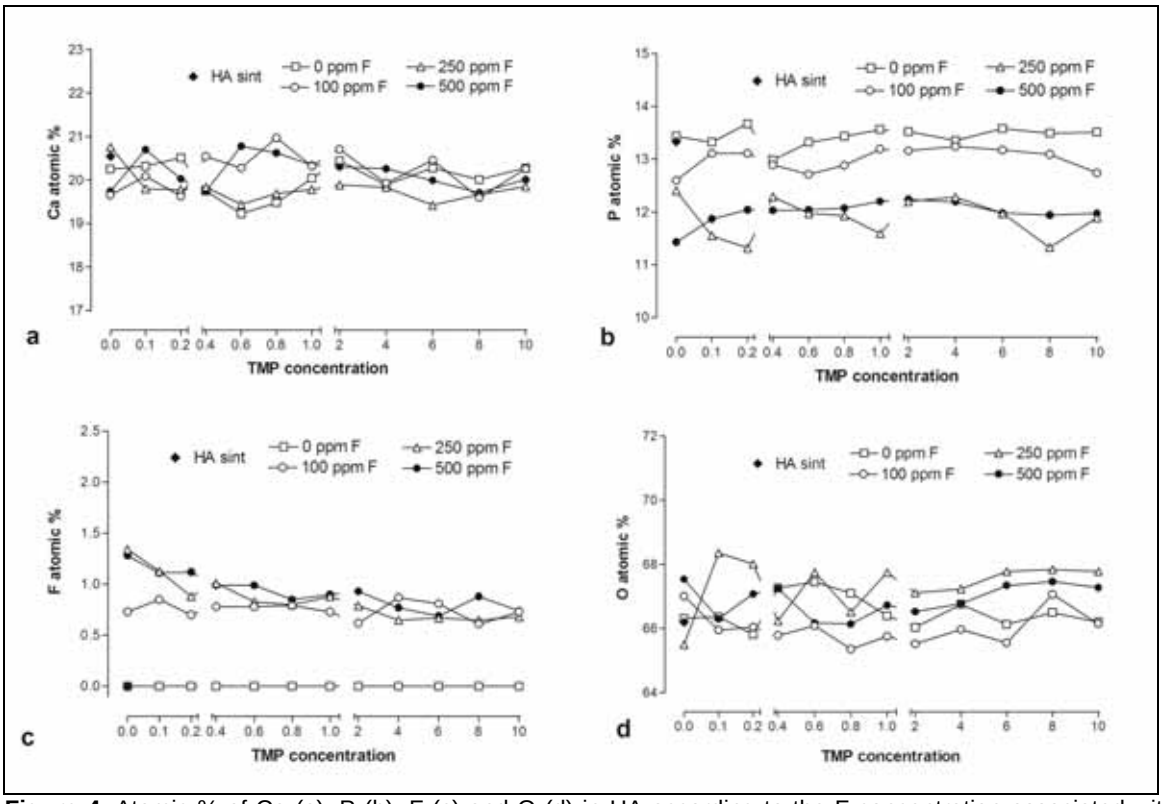


Figure 4. Atomic % of Ca (a), P (b), F (c) and O (d) in HA according to the F concentration associated with TMP.

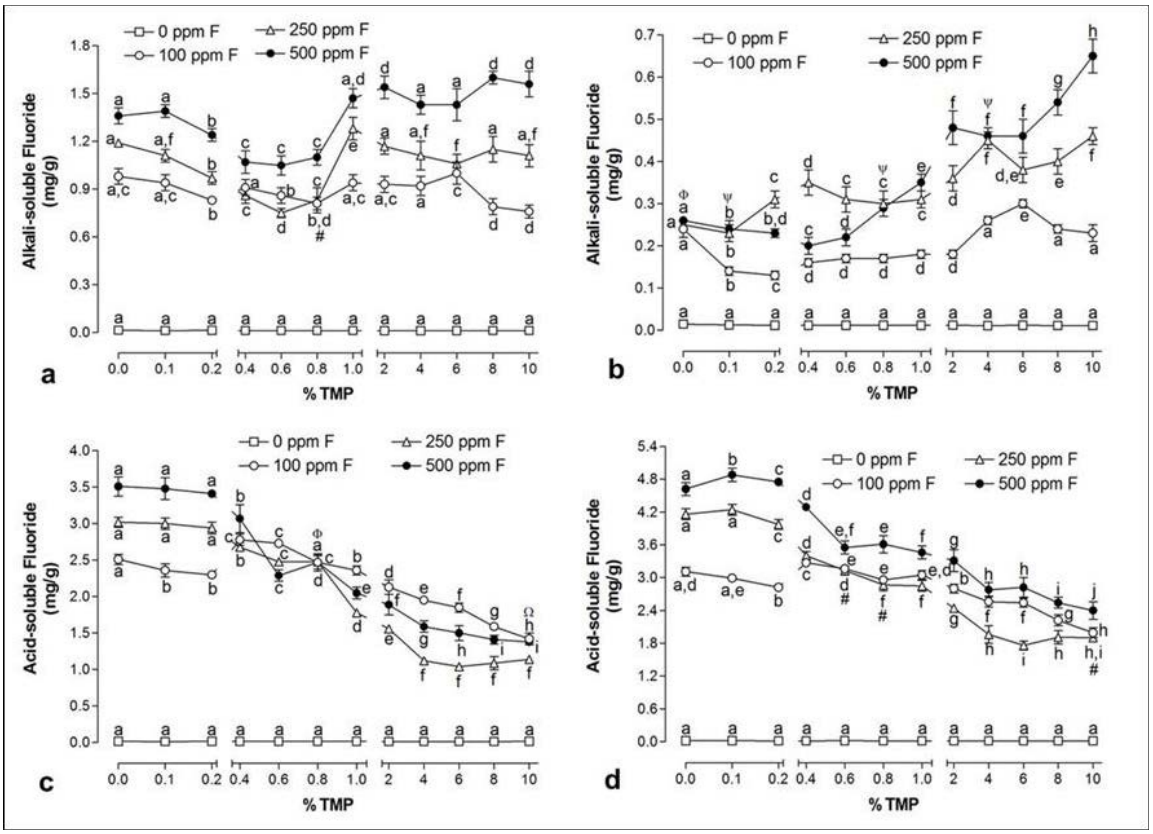


Figure 5. Graphical representation (mean ± se) of alkali-soluble F on HA after treatment (a) and after pH-cycle (b); acid-soluble F on HA after treatment (c) and after pH-cycle (d). Distinct letters show significant differences between the % TMP for each fluoride concentration (Student-Newman-Keuls, $p < 0.05$). (#) 100 ppm F = 250 ppm F; (ψ) 250 ppm F = 500 ppm F; (λ) 0 ppm F = 500 ppm F; (Φ) 100 ppm F = 250 ppm F = 500 ppm F; (Ω) 100 ppm F = 500 ppm F. Concentrations (mg/g) (mean ± se) of alkali and acid soluble F in the synthetic HA were: 0.02 (0.0) and 0.01 (0.0), respectively.

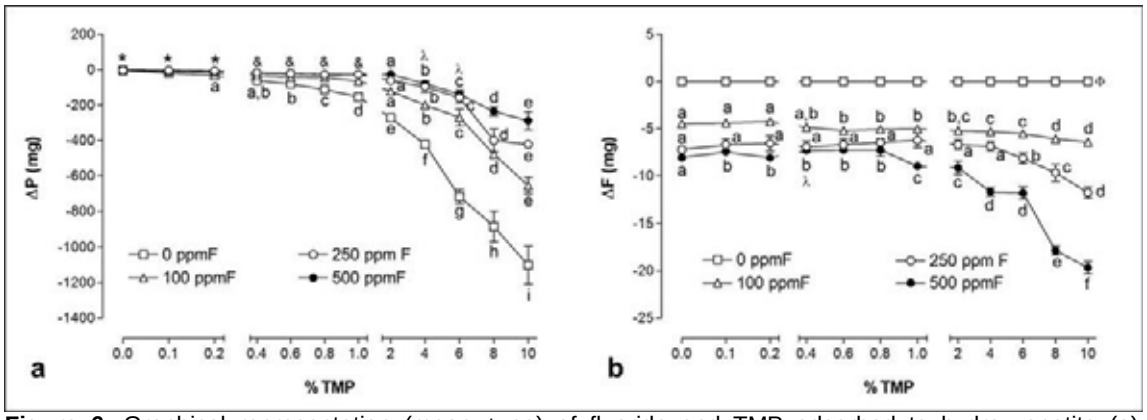


Figure 6. Graphical representation (mean ± se) of fluoride and TMP adsorbed to hydroxyapatite (a) adsorption of TMP (expressed through the amount of phosphorus), (b) adsorption of fluoride. Distinct letters show significant differences between the %TMP for each fluoride concentration (Student-Newman-Keuls, $p < 0.05$). (*) All comparisons show similarity; (&) 100 ppm F = 250 ppm F = 500 ppm F; (λ) 250 ppm F = 500 ppm F; (Φ) no difference among the %TMP in 0 ppm F group.

Anexos

ANEXO A

SÍNTESE DA HA



1. Adição de Fosfato de Amônio dibásico 0,3 mol L^{-1} à solução de Nitrato de Cálcio 1 mol L^{-1} sob agitação constante à 37°C para a precipitação da HA.

2. O precipitado foi filtrado utilizando-se um funil de Buchner acoplado a um sistema de bomba a vácuo, seco em estufa à 70°C.







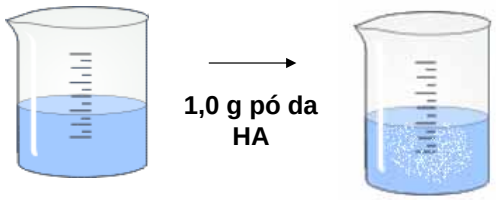
3. O precipitado foi triturado utilizando-se um almofariz de ágata e um moinho de bolas (Pulverisette 7).

4. O pó de HA foi triturado em um pó fino com aproximadamente 108 µm de diâmetro com auxílio de peneiras granulométricas.



ANEXO B

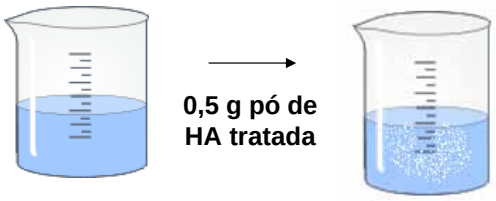
TRATAMENTO DA HA E CICLO DE pH



1. 1,0 g do pó de HA sintética foi suspenso em 100 mL de cada solução experimental.

2. A suspensão foi mantida em estufa por 2 min sob agitação constante à 37°C. Imediatamente, o precipitado foi filtrado, seco, triturado e armazenado para o processo de ciclagem de pH.





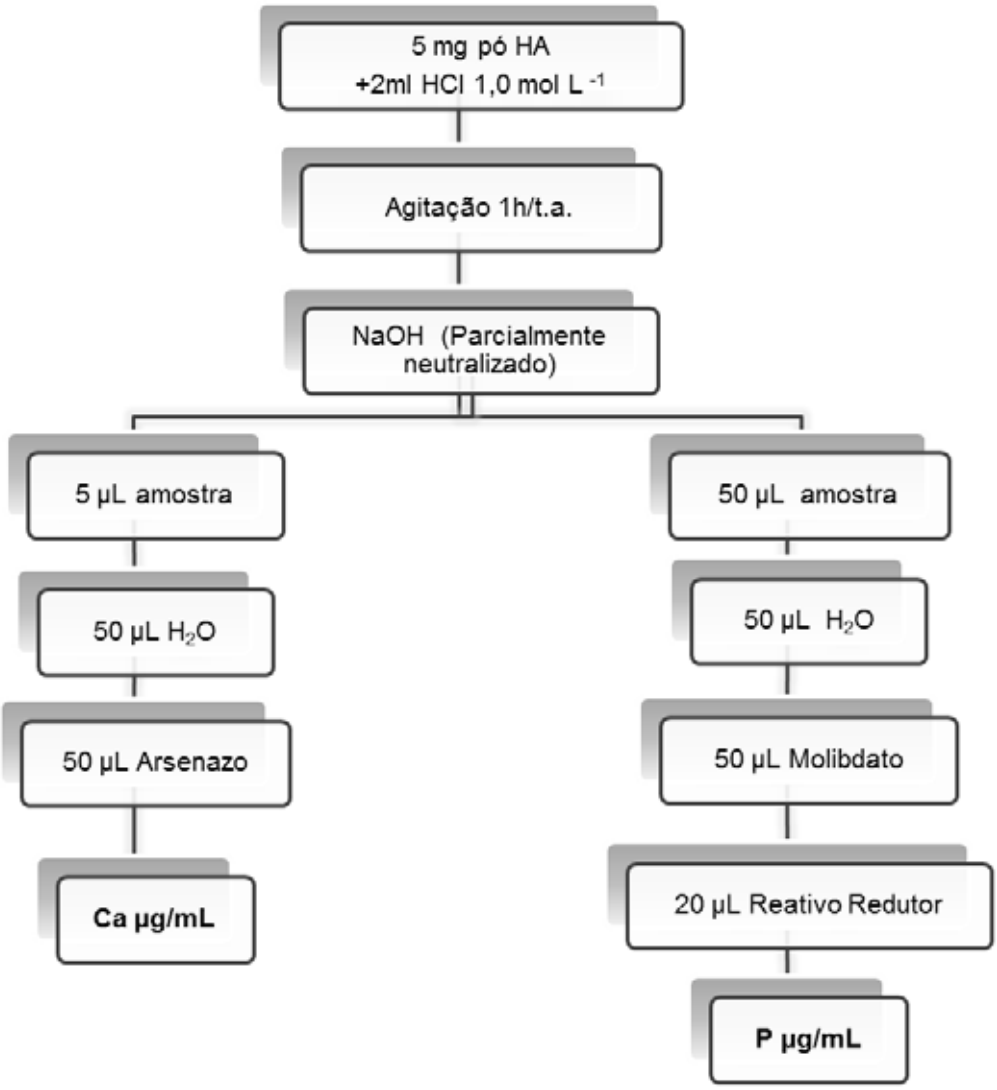
3. 0,5 g do pó de HA tratado com a respectiva solução de TMP e F foi suspensa em 100 mL de água deionizada.

4. Para o ciclo de pH, adicionou-se HNO_3 ajustando o pH para 4,0. Após 30 min, adicionou-se NaOH ajustando o pH para 7,0. Após 30 min, o precipitado foi filtrado, seco, triturado e armazenado para as análises bioquímicas.



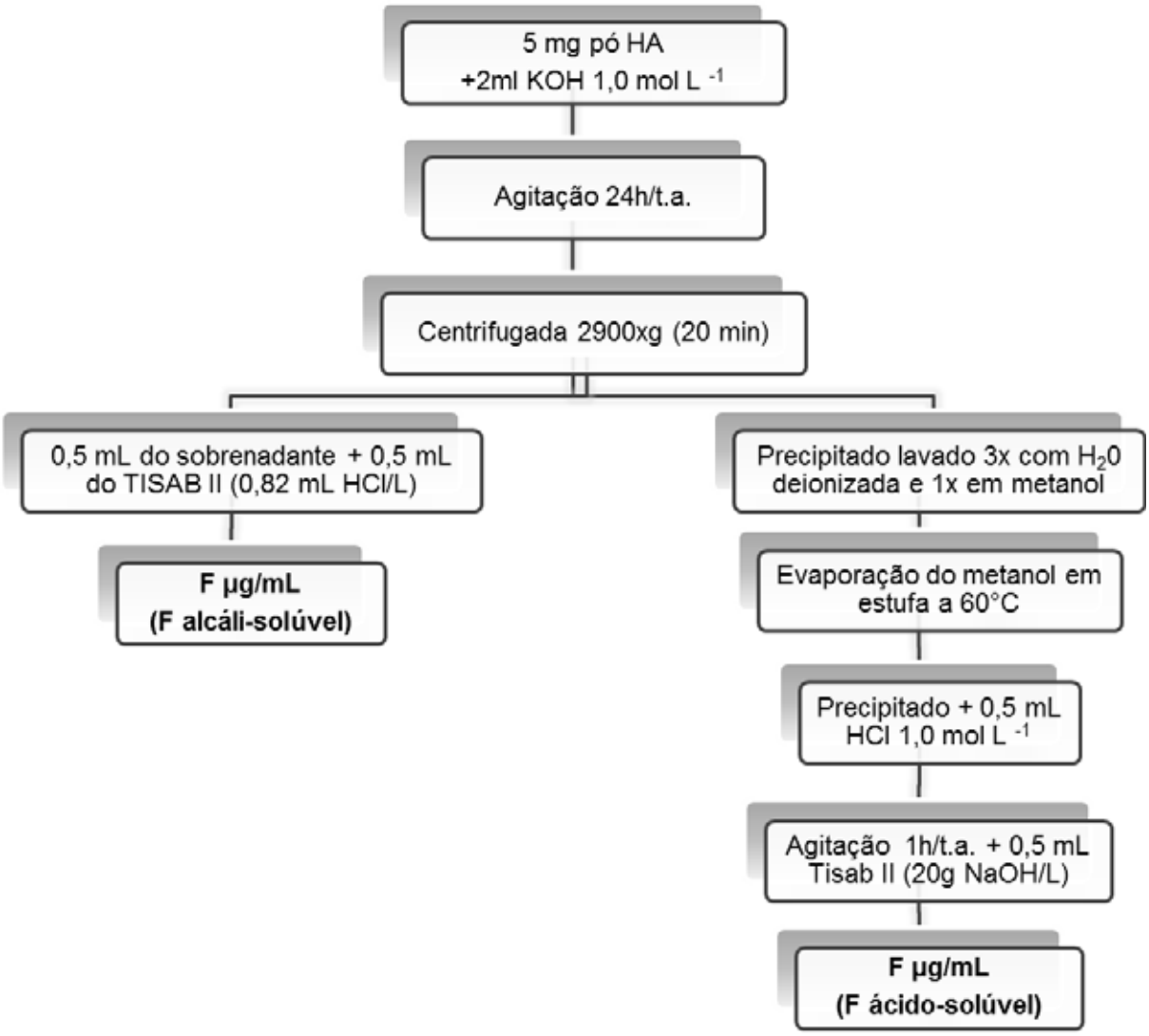
ANEXO C

ANÁLISE DE CÁLCIO E FÓSFORO NA HA



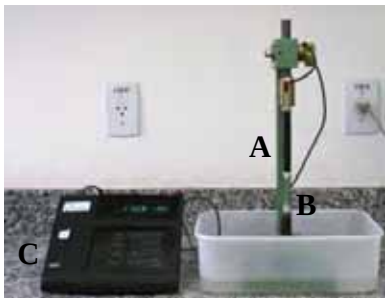
ANEXO D

ANÁLISE DE F ÁLCALI- E ÁCIDO-SOLÚVEL NA HA



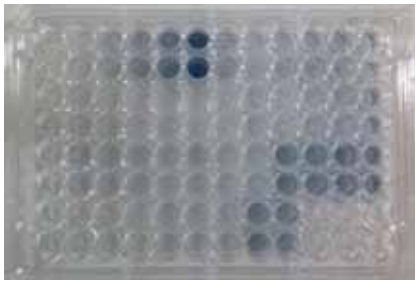
ANEXO E

ANÁLISE DE FLUORETO E TMP NO SOBRENADANTE COLETADO APÓS O TRATAMENTO DA HA



1. A – Eletrodo específico para fluoreto 9409 BN (Orion); B – Microeletrodo de referência invertido; C – Analisador de íons Orion 720 A.

2. O TMP foi determinado pela análise de P no sobrenadante coletado após o tratamento da HA adotando o método de Fiske & Subbarow, 1925.



ANEXO F

RESULTADOS DAS ANÁLISES DE CÁLCIO, FÓSFORO E FLUORETO NA HA

Tabela 2. Concentração média (DP) em mg/g de Ca, P e µg/g de F (DP) álcali- e ácido-solúvel ligado a HA de acordo com as concentrações de TMP no grupo contendo 0 ppm F

%TMP	Variáveis				Ca/P
	Ca	P	Álcali-solúvel	Ácido-solúvel	
0	329,4 (3,9)	165,4 (5,3)	14,5 (2,3)	18,3 (1,1)	1,54 (0,05)
0,1	318,3 (11,3)	163,0 (5,8)	12,3 (1,0)	16,4 (1,1)	1,51 (0,03)
0,2	311,9 (8,3)	159,1 (4,8)	11,4 (0,3)	14,9 (0,5)	1,52 (0,05)
0,4	329,6 (6,2)	175,8 (5,1)	11,6 (0,4)	15,1 (1,2)	1,46 (0,02)
0,6	297,1 (13,5)	154,5 (8,8)	11,4 (0,3)	15,6 (0,4)	1,49 (0,08)
0,8	328,7 (13,5)	174,6 (3,8)	11,4 (0,3)	15,6 (0,4)	1,46 (0,07)
1,0	279,7 (13,5)	152,9 (4,4)	11,4 (0,1)	13,8 (0,7)	1,42 (0,13)
2,0	297,5 (8,4)	156,6 (5,6)	12,0 (1,2)	14,3 (0,8)	1,47 (0,06)
4,0	285,6 (7,9)	149,0 (7,0)	12,0 (1,2)	14,3 (0,8)	1,49 (0,09)
6,0	295,6 (14,9)	147,6 (7,4)	11,3 (0,4)	15,1 (1,2)	1,55 (0,08)
8,0	280,2 (9,3)	145,6 (4,4)	11,2 (0,3)	13,8 (0,5)	1,49 (0,05)
10	279,1 (10,4)	145,5 (8,0)	11,1 (0,6)	14,1 (0,5)	1,49 (0,11)

Tabela 3. Concentração média (DP) em mg/g de Ca, P e µg/g de F (DP) álcali- e ácido-solúvel ligado a HA de acordo com as concentrações de TMP no grupo contendo 100 ppm F

%TMP	Variáveis				Ca/P
	Ca	P	Álcali-solúvel	Ácido-solúvel	
0	306,9 (13,7)	165,7 (9,1)	250,2 (22,9)	3182,7 (98,7)	1,44 (0,04)
0,1	322,5 (10,6)	167,0 (9,7)	142,8 (13,3)	2995,0 (75,6)	1,50 (0,05)
0,2	317,9 (13,5)	159,5 (3,3)	127,6 (9,1)	2818,7 (43,2)	1,54 (0,07)
0,4	326,2 (7,5)	158,2 (4,5)	159,3 (7,1)	3274,7 (18,4)	1,60 (0,06)
0,6	314,2 (10,0)	159,6 (5,3)	165,5 (8,9)	3152,0 (80,9)	1,53 (0,06)
0,8	312,1 (3,1)	157,3 (4,0)	174,9 (7,9)	2957,3 (67,6)	1,54 (0,04)
1,0	312,5 (12,4)	150,4 (5,8)	183,3 (14,2)	3050,4 (79,7)	1,61 (0,07)
2,0	344,1 (17,5)	164,5 (12,3)	180,2 (9,7)	2801,3 (78,6)	1,62 (0,06)
4,0	373,9 (18,8)	179,8 (6,4)	258,4 (14,7)	2555,8 (99,3)	1,61 (0,05)
6,0	369,0 (21,3)	175,0 (11,4)	305,4 (26,2)	2540,6 (79,0)	1,64 (0,05)
8,0	362,0 (14,1)	170,9 (8,3)	243,6 (8,9)	2216,7 (96,3)	1,64 (0,07)
10	347,5 (28,2)	168,2 (7,6)	231,8 (15,3)	1994,3 (87,5)	1,60 (0,07)

Tabela 4. Concentração média (DP) em mg/g de Ca, P e µg/g de F (DP) álcali- e ácido-solúvel ligado a HA de acordo com as concentrações de TMP no grupo contendo 250 ppm F

%TMP	Variáveis				Ca/P
	Ca	P	Álcali-soluvél	Ácido-solúvel	
0	319,8 (23,7)	157,9 (8,1)	292,7 (27,2)	4162,9 (94,5)	1,53 (0,08)
0,1	307,1 (22,5)	160,7 (5,4)	227,6 (22,5)	4317,0 (98,2)	1,51 (0,03)
0,2	310,8 (14,0)	152,0 (7,0)	304,1 (31,8)	3966,5 (85,8)	1,59 (0,06)
0,4	329,8 (21,7)	151,3 (6,2)	328,7 (50,4)	3399,0 (75,9)	1,72 (0,05)
0,6	325,7 (5,7)	152,3 (5,4)	294,5 (47,2)	3127,7 (77,2)	1,66 (0,07)
0,8	328,2 (11,1)	153,4 (6,8)	295,5 (44,6)	2874,0 (96,2)	1,68 (0,07)
1,0	327,2 (11,7)	153,5 (2,5)	310,4 (18,4)	2851,0 (94,5)	1,65 (0,04)
2,0	331,3 (12,6)	154,7 (4,4)	363,8 (31,7)	2443,9 (60,1)	1,69 (0,06)
4,0	284,7 (17,0)	152,4 (3,7)	439,6 (14,8)	1899,9 (98,0)	1,43 (0,08)
6,0	317,3 (13,4)	149,5 (3,2)	388,5 (42,0)	1758,7 (77,4)	1,64 (0,05)
8,0	312,0 (9,0)	151,2 (3,7)	399,5 (49,1)	1869,3 (92,8)	1,60 (0,02)
10	307,4 (8,6)	149,4 (1,7)	467,0 (35,0)	1898,6 (43,8)	1,59 (0,04)

Tabela 5. Concentração média (DP) em mg/g de Ca, P e µg/g de F (DP) álcali- e ácido-solúvel ligado a HA de acordo com as concentrações de TMP no grupo contendo 500 ppm F

%TMP	Variáveis				Ca/P
	Ca	P	Álcali-solúvel	Ácido-solúvel	
0	368,1 (9,6)	180,1 (7,3)	207,1 (10,0)	4616,9 (90,5)	1,58 (0,03)
0,1	363,1 (14,0)	174,5 (4,8)	234,0 (27,0)	4882,8 (93,9)	1,61 (0,03)
0,2	358,6 (8,1)	168,9 (1,5)	229,6 (6,8)	4747,3 (58,8)	1,65 (0,04)
0,4	365,8 (9,7)	171,6 (2,6)	191,3 (40,7)	4291,7 (71,8)	1,65 (0,04)
0,6	372,8 (4,0)	172,7 (3,1)	217,2 (17,5)	3545,6 (95,0)	1,67 (0,02)
0,8	355,6 (7,4)	166,3 (2,1)	291,1 (24,3)	3611,3 (92,0)	1,66 (0,02)
1,0	336,1(12,5)	163,8 (4,9)	335,1 (56,8)	3456,3 (92,9)	1,59 (0,06)
2,0	345,4 (22,6)	164,3 (6,7)	436,1 (33,2)	3118,7 (92,9)	1,63 (0,07)
4,0	338,1 (16,2)	163,0 (8,0)	454,7 (30,2)	2689,4 (90,0)	1,61 (0,06)
6,0	338,8 (16,9)	171,5 (9,8)	468,1 (60,2)	2756,1 (93,0)	1,53 (0,05)
8,0	329,3 (20,3)	164,9 (5,0)	557,1 (55,0)	2536,3 (95,5)	1,55 (0,07)
10	319,8 (15,5)	160,6 (8,4)	682,7 (66,8)	2403,8 (94,8)	1,54 (0,05)

ANEXO G

RESULTADOS DA ANÁLISE DE ENERGIA DISPERSIVA DE RAIOS-X (EDX)

Tabela 6. Valores da porcentagem (%) dos elementos atômicos Ca, P, O, e F na HA de acordo com as concentrações de F e de TMP

ppm F	%TMP	% dos elementos atômicos				Ca/P
		Ca	P	O	F	
0	0	20,2	13,4	66,3	-	1,51
	0,4	19,7	13,0	67,3	-	1,52
	1,0	20,0	13,5	66,4	-	1,48
	6,0	20,3	13,6	66,1	-	1,49
	10	20,2	13,5	66,2	-	1,50
100	0	19,6	12,6	67,0	0,73	1,56
	0,4	20,5	12,9	65,8	0,78	1,59
	1,0	20,3	13,1	65,8	0,73	1,54
	6,0	20,4	13,2	65,6	0,81	1,55
	10	20,2	12,7	66,1	0,73	1,59
250	0	20,7	12,4	65,5	1,34	1,67
	0,4	19,8	12,3	66,2	1,01	1,61
	1,0	19,8	11,6	67,8	0,88	1,71
	6,0	19,4	11,9	67,8	0,67	1,62
	10	19,8	11,9	67,7	0,68	1,67
500	0	19,7	11,4	67,5	1,28	1,73
	0,4	19,7	12,0	67,2	0,99	1,64
	1,0	20,3	12,2	66,7	0,90	1,67
	6,0	20,0	12,0	67,3	0,69	1,67
	10	20,0	12,0	67,3	0,74	1,67

*-: Este elemento na análise não foi detectado.