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Enamel Mineral Content Changes After Bleaching With High and Low Hydrogen Peroxide Concentrations: Colorimetric Spectrophotometry and Total Reflection X-ray Fluorescence Analyses

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Clinical Relevance

Hydrogen peroxide concentrations and the composition and application protocol of each bleaching agent can influence calcium and phosphorous content in enamel, with the results varying according to the methodology used.

SUMMARY

The purpose of this study was to evaluate the calcium (Ca) and phosphorous (P) content in enamel bleached with high and low concentrations of hydrogen peroxide (HP) using Total Reflection X-Ray Fluorescence (TXRF) and colorimetric spectrophotometry (SPEC). Forty-eight sound human third molars were used.

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Their roots were embedded in polystyrene resin and immersed for seven days in an artificial saliva solution. Then they were distributed into six groups to receive the bleaching treatments. The agents of high HP concentration (for in-office use) evaluated were Whiteness HP Maxx/FGM (35% HP), Whiteness HP Blue/FGM (35% HP, 2% calcium gluconate), Pola Office+/SDI (37.5% HP, 5% potassium nitrate), and Opalescence Boost/Ultradent (38% HP, 1.1% ion fluoride, 3% potassium nitrate); these agents were applied to enamel in three sessions. The agents of low HP concentration (for home use) evaluated were Pola Day/SDI (9.5% HP) and White Class 10%/FGM (10% HP, potassium nitrate, calcium, fluoride), and these agents were applied for 14 days. Enamel microbiopsies were evaluated by TXRF and SPEC analysis before the bleaching treatment (baseline), during the treatment, and 14 days after the end of the treatment. For TXRF, the Kruskal-Wallis test showed that Ca and P were not influenced by agent ($p>0.05$). For SPEC, Pola Office+, Opalescence Boost, Pola Day, and White Class 10% caused a decrease of Ca over time; there was a significant decrease of P over time to Pola Office+ and White Class 10%. The Spearman test showed no correlation between the Ca ($p=0.987$; $r^2=-0.020$) and P ($p=0.728$, $r^2=0.038$) obtained by SPEC and TXRF. For TXRF and SPEC, changes in Ca and P during bleaching occurred independently of the HP concentration used.

INTRODUCTION

Hydrogen peroxide (HP) is the most widely used agent for tooth bleaching, and it can be used either at high concentrations (35% to 40%) using in-office techniques or at low concentrations (5% to 10%) for at-home bleaching.[1,2] Despite the application of faster bleaching protocols, which reduce the contact time between bleaching agent and tooth structure, changes in enamel and dentin micromorphology may occur, such as the presence of erosions and/or porosity[3,4] and changes in mineral content[5-8] as well as in surface microhardness,[3,6,9-12] which may be related to the composition of bleaching agents, concentration, pH values, and different application techniques.

Considering the increased risk of enamel demineralization or loss of mineral content due to teeth bleaching, the addition of calcium (Ca) and/or fluoride to the composition of bleaching gels could be beneficial.[4,9,11,13-19] Borges and others[17] observed that adding 2% calcium gluconate to a hydrogen peroxide gel resulted in reduced enamel erosion, with a significant increase in enamel microhardness after bleaching with hydrogen peroxide at 35% enriched with calcium and fluoride.[18]

However, Oliveira and others[20] found no significant increase in enamel microhardness when using a bleaching agent at low concentration (10% carbamide peroxide) enriched with calcium fluoride for home use. Soares da Costa and others[16] showed that high-concentration chairside bleaching treatments with or without calcium decreased enamel microhardness. Basting and others[7] measured calcium and phosphate content via enamel microbiopsy and spectrophotometry,[5,21] verifying that the mineral content within the human dental enamel structure following high-concentration bleaching was generally similar whether or not calcium was featured in its composition. Consequently, questions remain regarding the advantages of incorporating calcium and fluoride in the composition of high- or low-concentration bleaching agents as a result of the contradictory results observed in different studies.

Various methods of analysis can be applied to evaluate changes in dental substrate in terms of mineral content, such as Fourier Transform infrared spectroscopy,[8] energy-dispersive X-ray spectroscopy,[6,11] Fourier Transform Raman spectroscopy,[22-24] atomic absorption spectroscopy,[13,25] surface microhardness,[9,12,22,26,27] induced plasma mass spectrometry,[14] total reflection X-Ray fluorescence,[25] and spectrophotometric or colorimetric analysis.[5,7] Total reflection X-ray fluorescence analysis (TXRF), which causes exposure of ions through the phenomenon of fluorescence,[25] allows the study of the atomic and molecular structure of matter, identifying and quantifying chemical elements, especially in small volumes.[28] This could be advantageous when using the enamel microbiopsy technique,[5,7] especially in clinical studies that aim to preserve the dental structure when evaluating changes in mineral content.[5] By using the technique of enamel microbiopsy, the method of colorimetric spectrophotometry (SPEC) may also be useful to quantify the minerals extracted from the dental substrate;[5,7] however, there are no studies comparing these methods.

Therefore, the aim of this study was to evaluate the Ca and phosphate (P) contents in human enamel before, during, and after treatment with bleaching

agents containing either high- or low-concentration hydrogen peroxide with or without calcium and/or fluoride. Mineral content was evaluated by both SPEC and TXRF, and the two methods were also verified for correlation.

METHODS AND MATERIALS

Experimental Design

The samples were comprised of aqueous solutions of mineral obtained from the enamel surface via microbiopsy from 48 human third molars distributed into the following study factors (n=8):

- Bleaching agents containing hydrogen peroxide with or without calcium and/or fluoride on six levels. Four levels of high-concentration bleaching agents: Whiteness HP Maxx/FGM (35% hydrogen peroxide); Whiteness Blue HP/FGM (35% hydrogen peroxide + 2% calcium gluconate); Pola Office+/SDI (37.5% hydrogen peroxide + 5% potassium nitrate); Opalescence Boost/Ultradent (38% hydrogen peroxide + 1.1% fluoride and 3% potassium nitrate)—and two levels of low-concentration bleaching agents: Pola Day/SDI (9.5% hydrogen peroxide, + 5% fluoride); White Class 10%/FGM (10% hydrogen peroxide + potassium nitrate + calcium + fluoride);
- Treatment times on six levels: T0 = control: before applying the bleaching agent (baseline); T1 = immediately after the first application of the bleaching agent; T2 = seven days after starting treatment; T3 = after the second application of high-concentration hydrogen peroxide or after the eighth application of low-concentration hydrogen peroxide; T4 = after 14 days of starting treatment; T5 = after the third application of high-concentration hydrogen peroxide or after the 15th application of low-concentration hydrogen; and T6 = 15 days after the completion of treatment.

Ca and P values were obtained from the enamel microbiopsy samples in micrograms per milliliter (μg/mL) using SPEC and TXRF. For TXRF, only T0, T1, T3, T5, and T6 were evaluated, with n = 3.

Tooth Preparation and Dental Bleaching

Forty-eight unerupted freshly extracted human third molars were cleaned using a rotary brush, pumice, periodontal curettes, and scalpel blades and were stored in 0.1% thymol solution for up to six months. The teeth showed no evidence of cracks, wear, or fractures, and the coronal enamel was free from spots.

Polyvinyl chloride rings measuring 20 mm in diameter and 20 mm in height were used to embed the teeth in acrylic resin (Maxi Rubber, Diadema, SP, Brazil). The roots of the teeth were immersed into the resin 2 mm apically to the cementoenamel junction, maintaining the long axis of the tooth perpendicular to the horizontal plane.

The teeth were randomly divided into six groups (n=8) according to high or low hydrogen peroxide concentration (Table 1). Prior to bleaching, the teeth were stored at 37°C ± 1°C in a bacteriological incubator (Odontobrás Ind E Com Equip Med Odont Ltda, Ribeirão Preto, SP, Brazil) immersed in 20 mL of artificial saliva solution for seven days before the application of bleaching agents. The artificial saliva solution used was the same reported in previous studies.[7,26,29]

The teeth were removed from the artificial saliva and dried with jets of air for five seconds to perform the initial microbiopsy T0 (baseline). The bleaching agents were then applied following the instructions and application times recommended by the manufacturers (Table 1). The pH value of the bleaching agents was measured using a benchtop pH meter (MS Tecnopon Special Equipment Ltd, Piracicaba, SP, Brazil) at different times (baseline and after seven, 15, 30, and 40 minutes), coinciding with half of the time and total application time for each bleaching agent. The bleaching agent was dispensed directly from the syringe or after mixing the freshly prepared gel.

Immediately upon completion of the first bleaching application an enamel microbiopsy was taken (T1). Teeth receiving the high-concentration hydrogen peroxide treatment were stored separately in bottles containing 20 mL of artificial saliva for seven days, changing the solution every two days. The bleaching treatment was repeated twice for a total of 15 days (three clinical sessions, each session intercalated by a seven-day immersion in artificial saliva). Enamel microbiopsies from these groups were taken at the times established in the experimental design. The teeth receiving low-concentration hydrogen peroxide were also stored separately in bottles containing 20 mL of artificial saliva, changing the solution every two days. The bleaching agents were applied daily for a total of 15 days. The teeth were removed from the storage solution and dried prior to daily application of the bleaching treatment and enamel biopsies at certain times, as established in the experimental design.

Table 1: *Treatment Agents, Composition, Protocol of Use, and pH Values*

Hydrogen Peroxide	Commercial Brand/ Manufacturer (City, State, Country) Lot No.	Composition (According to the Manufacturer)	Time Application in Each Session/ Total No. of Sessions or Total Days of Treatment	pH Values				
				Baseline	7 min	15 min	30 min	40 min
High concentrations	Whiteness HP Maxx/FGM (Blumenau, PR, Brazil) P72199	35% Hydrogen peroxide, thickener, dyes, glycol, inorganic filler, deionized water	15 min × 3 = 45 min/3 sessions	5.70	5.54	5.40	—	—
	Whiteness HP Blue Calcium/FGM (Blumenau, PR, Brazil) 150514	35% Hydrogen peroxide, thickener, violet dye, neutralizing agents, 2% calcium gluconate, glycol, deionized water	40 min/3 sessions	8.0	—	8.09	—	7.89
	Pola Office+/SDI (Melbourne, Victoria, Australia) 132020	37.5% Hydrogen peroxide, 5% potassium nitrate	8 min × 4 = 32 min/3 sessions	7.41	8.21	7.27	—	—
	Opalescence Boost/ Ultradent Products (South Jordan, Utah, USA) DOO6A	38% Hydrogen peroxide, 1.1% fluoride, 3% potassium nitrate	15 min × 3 = 45 min/3 sessions	6.98	7.01	6.74	—	—
Low concentrations	Pola Day 9.5%/SDI (Melbourne, Victoria, Australia) P130308Z	9.5% Hydrogen peroxide, <47% additives, 30% glycerol, 20% water, 0.1% flavoring, potassium nitrate	30 min/15 d	6.07	—	6.07	6.13	—
	White Class 10%/ FGM (Blumenau, PR, Brazil) 140114	10% Hydrogen peroxide, neutralized carbopol, 5% potassium nitrate, sodium fluoride, aloe vera, calcium gluconate (concentration not available), stabilizing, moisturer, deionized water	30 min/15 d	5.49	—	5.47	6.00	—

Enamel Microbiopsy Technique

Enamel microbiopsy was performed according to the method described by Amaral and others[5] and Basting and others.[7] The tooth surfaces used for biopsy were buccal, lingual, and proximal for each tooth, which were rinsed with water for five seconds and air-dried for five seconds. Each biopsy was taken from a different surface every time. The solutions containing the microbiopsies were frozen at −18°C (CRM 32ABANA, Consul Biplex Frost Free 320, Multibrás SA Appliances, Joinville, SC, Brazil). When carrying out chemical analyses, the samples were thawed at room temperature.

Chemical Analyses Via Spectrophotometry

The concentrations of Ca and P were established following the method previously published by Amaral and others[5] and Basting and others.[7] Ca concentration was determined via an endpoint colorimetric Arsenazo III method, in which calcium reacts with Arsenazo III in acidic medium to form a blue color complex, in which intensity is proportional to calcium concentration in the sample, using the calcium-Arsenazo III kit (K051, Bioclin, Belo Horizonte, MG, Brazil). P concentrations were determined by the principle that phosphorus from mineral phosphates is transformed into phosphomolybdate, which is then

reduced by alpha-amino-naphthol-sulfonic acid to a blue reaction product, the color intensity of which is proportional to the inorganic phosphorus content in the sample. The quantification of Ca and P concentrations was performed in duplicate. Wavelength absorbance was evaluated in a Universal Microplate Reader (ELX800UV, Bio-Tek Instruments Inc, Winooski, Vt, USA) at 630 nm and concentrations were obtained in μg/mL.

Chemical Analyses Via TXRF

The mineral solutions obtained from the microbiopsy were taken to the Brazilian Synchrotron Light Laboratory for analysis. As a result of the large number of samples obtained as well as the time needed for each analysis, a decision was made to include n = 3 for T0, T1, T3, T5, and T6 in the benefit of optimization and rational use of the equipment. The three samples from each group were randomly selected for evaluation.

A standard Scandium solution (20 ppm) was added to each sample to serve as a known reference for comparison for each sample. Five microliters of each solution was applied to the acrylic reflector of the equipment in triplicate. The reflector was then repositioned into its specific compartment in the Total Reflection X-Ray Fluorescence equipment so that the analysis using Synchrotron Radiation could be performed. The concentrations of Ca and P were quantified in μg/mL.

Statistical Analysis

The concentrations of Ca and P obtained by SPEC were analyzed using the mixed model method (PROC MIXED) via repeated-measures analysis of variance (ANOVA) and Tukey-Kramer test (α=0.05) (SAS Institute Inc, Cary, NC, USA; Release 9.3, 2011). For Ca and P concentrations obtained by TXRF, neither data homoscedasticity nor normality were obtained, even after data transformation. Nonparametric methods were therefore used: Kruskal-Wallis, Friedman, and Wilcoxon tests with Bonferroni correction. The Spearman correlation test was used to verify the correlation between the concentrations of Ca and P obtained via the SPEC and TXRF methods (α=0.05) at the matched evaluation times (T0, T1, T3, T5, and T6) (SPSS 20, SPSS Inc, Chicago, Ill, USA).

RESULTS

The data obtained by SPEC and analyzed by two-way ANOVA applied to Ca concentrations showed an interaction between the factors 'hydrogen peroxide with or without calcium and/or fluoride' and 'treatment times' (p=0.0001). Ca concentrations were not significantly influenced by the type of bleaching agent used (p=0.2822) but were influenced by time (p<0.001). The Tukey-Kramer test showed that at any time point Ca concentrations between the different bleaching agents did not differ (Table 2). For the bleaching agents Pola Office+ Opalescence Boost, Pola Day, and White Class, a significant reduction in calcium concentration was observed over time, with values at T6 significantly lower than those at T0. For Whiteness HP Maxx and Whiteness HP Blue and Calcium no significant difference in calcium concentration was observed at different time points. Regarding the concentration of P, two-way ANOVA showed no interaction between the factors 'hydrogen peroxide with or without calcium and/or fluoride' and 'treatment times' (p=0.0691). P concentrations were not significantly influenced by the type of bleaching agent used (p=0.0833), but they were affected by time (p<0.0001). The Tukey-Kramer test showed that at any time point, the concentration of P between different bleaching agents did not differ (Table 2). For the Pola Office+ agent, a significant reduction in the concentration of P was observed over time, with values at T2, T3, T5, and T6 being lower than values at T0. Regarding the White Class agent, a significant reduction in the concentration of P was observed at T4 when compared to T0. As for the remaining bleaching agents, no significant difference was observed over time.

Regarding the results obtained by TXRF, the Kruskal-Wallis test showed that at each time point Ca concentration was not significantly affected by the bleaching agent used (p>0.05) (Table 3). Only for the bleaching agent Pola Office+ did the Friedman test show that time significantly influenced the concentration of Ca (p=0.027), with no difference between T5 and T6, whereas these concentrations were lower than the concentrations at T1 and T3. At T0, the concentration of Ca was intermediate and did not differ over time (Wilcoxon multiple comparisons test with Bonferroni correction). For the remaining bleaching agents, time did not significantly influence the concentration of P (Friedman test, p>0.05). The Kruskal-Wallis test showed that P concentration was not significantly affected by the bleaching agent used at any time point, (p>0.05). Only for the bleaching agent Pola Day did the Friedman test show that time significantly influenced the concentration of P (p=0.043), with greater concentrations

Table 2: *Calcium and Phosphorous Concentration Means (±Standard Deviation Values) by Colorimetric Spectrophotometry (SPEC) According to Groups and Time (μg/mL)*[a]

Time	High Hydrogen Peroxide Concentration				Low Hydrogen Peroxide Concentration	
	Whiteness HP Maxx	Whiteness HP Blue Calcium	Pola Office+	Opalescence Boost	Pola Day	White Class
Calcium						
T0	10.34 ± 0.86 Aa	10.67 ± 0.89 Aa	11.10 ± 0.80 Aa	10.85 ± 1.03 Aa	10.91 ± 0.99 Aa	11.33 ± 0.99 Aa
T1	8.60 ± 0.64 Aa	8.98 ± 1.46 Aa	9.08 ± 0.80 Aab	8.46 ± 0.51 Ab	9.34 ± 0.69 Aabc	10.29 ± 1.92 Aab
T2	9.84 ± 0.73 Aa	9.81 ± 0.78 Aa	9.32 ± 0.87 Aab	9.15 ± 0.80 Aab	9.33 ± 1.06 Aabc	8.45 ± 0.98 Abcd
T3	9.76 ± 1.58 Aa	9.70 ± 1.45 Aa	9.51 ± 1.56 Aab	9.26 ± 1.66 Aab	7.83 ± 1.58 Ac	7.58 ± 0.60 Acd
T4	9.23 ± 1.41 Aa	10.16 ± 1.52 Aa	9.08 ± 1.06 Aab	10.12 ± 0.99 Aab	8.53 ± 0.73 Abc	8.61 ± 1.12 Abcd
T5	9.67 ± 0.48 Aa	9.04 ± 0.71 Aa	8.82 ± 0.48 Ab	9.29 ± 0.64 Aab	10.04 ± 1.43 Aab	9.36 ± 1.04 Abc
T6	9.37 ± 1.36 Aa	9.60 ± 1.16 Aa	8.99 ± 1.24 Ab	8.89 ± 1.45 Ab	8.00 ± 1.91 Ac	7.38 ± 0.40 Ad
Phosphorous						
T0	3.90 ± 0.94 Aa	3.55 ± 1.21 Aa	5.50 ± 1.98 Aa	3.52 ± 1.33 Aa	4.12 ± 1.30 Aa	4.52 ± 1.67 Aa
T1	3.13 ± 0.78 Aa	4.18 ± 0.91 Aa	4.68 ± 1.91 Aab	3.91 ± 0.85 Aa	4.32 ± 1.92 Aa	4.27 ± 1.75 Aab
T2	4.05 ± 1.54 Aa	2.86 ± 0.67 Aa	3.14 ± 0.93 Ab	2.71 ± 0.71 Aa	2.42 ± 0.71 Aa	2.94 ± 0.70 Aab
T3	2.66 ± 0.42 Aa	2.90 ± 0.66 Aa	2.85 ± 0.77 Ab	2.14 ± 0.41 Aa	2.80 ± 1.00 Aa	2.68 ± 0.99 Aab
T4	2.47 ± 0.76 Aa	2.84 ± 0.85 Aa	3.26 ± 0.57 Aab	2.42 ± 0.76 Aa	3.09 ± 1.42 Aa	2.38 ± 0.53 Ab
T5	3.15 ± 0.70 Aa	3.26 ± 0.70 Aa	2.82 ± 0.52 Ab	3.49 ± 1.11 Aa	2.68 ± 0.71 Aa	3.19 ± 1.20 Aab
T6	2.61 ± 0.87 Aa	2.59 ± 0.45 Aa	2.92 ± 0.74 Ab	3.10 ± 0.76 Aa	2.92 ± 0.99 Aa	2.66 ± 0.50 Aab

Abbreviations: T0, control: before applying the bleaching agent (baseline); T1, immediately after the first application of the bleaching agent; T2, seven days after starting treatment; T3, after the second application of high-concentration hydrogen peroxide or after the eighth application of low-concentration hydrogen peroxide; T4, after 14 days of starting treatment; T5, after the third application of high-concentration hydrogen peroxide or after the 15th application of low-concentration hydrogen; T6, 15 days after the completion of treatment.

[a] *Means followed by the same letter (capital letters in rows and lowercase letters in column) do not differ from each other ($p \leq 0.05$) for calcium (Ca) and phosphate (P) independently.*

at T1 when compared to other times, during which no significant differences in concentrations were observed (Wilcoxon multiple comparisons test with Bonferroni correction). For the remaining bleaching agents, time did not significantly influence the concentration of P (Friedman test, $p>0.05$) (Table 3). Figure 1 illustrates the comparisons of the mean Ca and P concentrations (μg/mL) obtained by SPEC and TXRF over time and study group.

The Spearman test revealed no correlation between the concentrations of Ca ($p=0.987$; $r^2=-0.020$) and P ($p=0728$; $r^2=0.038$) obtained by the SPEC and TXRF methods (Figure 2).

DISCUSSION

The results for the concentration of Ca and P by TRFX showed no differences among bleaching agents at any time point. Although no other studies have quantitated the mineral content of the enamel throughout the bleaching procedure using the same agents as those in the present study, thus preventing some comparisons, it is important to note that the results obtained using SPEC did not correlate with those obtained from TXRF via the Spearman test.

The TXRF method was used by Wang and others[25] when analyzing the amount of calcium leached from the enamel after treatment with a sodium chloride–based bleaching agent (Rapid White/Rapid White Products) and a hydrogen peroxide–based high-concentration agent at 38% (Opalescence Xtra Boost/Ultradent Products Inc). These authors reported that for the hydrogen peroxide–based agent, the presence of potassium in the solutions collected (by applying distilled water) decreased the detection limit of the method, in which the presence of calcium could only be evaluated via atomic absorption spectroscopy. Consequently, TXRF, though accurate, could yield results biased by contaminants in the solutions,[25] since there is a risk of bleaching gel residue on the enamel surface, which may have affected the detection of other elements. Despite the fact that calcium belongs to the group of alkaline earth metals in the periodic table, it can react with other elements when in contact with water, forming hydroxides,[30] thereby affecting the detection accuracy of this chemical element in the solution used. On the other hand, phosphorus is not a metal and

Table 3: *Calcium and Phosphorous Concentration Means (±Standard Deviation Values) by X-ray Fluorescence by Total Reflection (TRXF) According to Groups and Time (μg/mL)[a] (Ext.)*

Time	High Hydrogen Peroxide Concentration			
	Whiteness HP Maxx	Whiteness HP Blue Calcium	Pola Office+	Opalescence Boost
Calcium				
T0	2.26E-04 ± 2.87E-04 Aa	6.79E-05 ± 2.68E-05 Aa	1.90E-04 ± 2.21E-04 Aab	5.57E-05 ± 4.33E-05 Aa
T1	5.48E-05 ± 2.22E-05 Aa	1.30E-04 ± 1.47E-04 Aa	1.68E-03 ± 1.87E-03 Aa	7.02E-05 ± 5.90E-05 Aa
T3	5.47E-05 ± 1.16E-05 Aa	3.16E-03 ± 3.28E-03 Aa	5.46E-03 ± 9.28E-03 Aa	6.86E-05 ± 3.40E-05 Aa
T5	2.08E-04 ± 2.50E-04 Aa	5.57E-05 ± 5.34E-05 Aa	5.14E-05 ± 7.76E-06 Ab	6.23E-05 ± 6.34E-06 Aa
T6	7.87E-05 ± 4.84E-05 Aa	4.84E-05 ± 1.06E-05 Aa	4.44E-05 ± 2.15E-05 Ab	3.22E-03 ± 5.41E-03 Aa
Phosphorous				
T0	8.03E-05 ± 1.24E-04 Aa	4.47E-05 ± 5.92E-05 Aa	1.16E-04 ± 1.80E-04 Aa	1.70E-05 ± 1.45E-05 Aa
T1	1.48E-05 ± 8.66E-06 Aa	8.02E-04 ± 1.36E-03 Aa	1.24E-03 ± 1.37E-03 Aa	3.33E-04 ± 5.61E-04 Aa
T3	2.11E-05 ± 9.20E-06 Aa	6.54E-04 ± 4.07E-04 Aa	1.50E-03 ± 2.57E-03 Aa	1.08E-05 ± 5.52E-06 Aa
T5	8.92E-05 ± 1.34E-04 Aa	5.43E-05 ± 6.08E-05 Aa	8.20E-05 ± 1.23E-04 Aa	1.03E-05 ± 2.61E-06 Aa
T6	1.31E-05 ± 3.78E-06 Aa	1.29E-05 ± 6.38E-06 Aa	3.95E-05 ± 4.64E-05 Aa	1.11E-03 ± 1.88E-03 Aa

Abbreviations: T0, control: before applying the bleaching agent (baseline); T1, immediately after the first application of the bleaching agent; T2, seven days after starting treatment; T3, after the second application of high-concentration hydrogen peroxide or after the eighth application of low-concentration hydrogen peroxide; T4, after 14 days of starting treatment; T5, after the third application of high-concentration hydrogen peroxide or after the 15th application of low-concentration hydrogen; T6, 15 days after the completion of treatment.

[a] Means followed by the same letter (capital letters in rows and lowercase letters in column) do not differ from each other ($p \leq 0.05$) for calcium (Ca) and phosphorous (P) independently. Scientific notation: 1.0E-03" = 1.0×10^{-3}.

is part of the nitrogen group, which is highly reactive and highly oxidative when in contact with oxygen from the air. Additionally, P is an element with low electrical conductivity,[31] and these characteristics may have also influenced the results. In this respect, the standard deviations were high for all groups, illustrating that the coefficient of variation reflected high data dispersion and heterogeneity. Although the high variation of TXRF results would justify the unnecessary performance of the correlation analysis, the lack of a correlation emphasizes that one of the methods is preferable to the other.

When the SPEC method was used, the negative control group was established as the baseline data for each group, when no bleaching agent was applied. An interesting and important result can be observed for the Ca and P baseline values that show no differences among the groups (represented by the rows of Table 2). This result (no differences at the baseline time point) allowed comparisons among groups (represented by the columns of the Table 2) according to each treatment time. No difference was found in Ca and P concentrations at any time point for the different bleaching agents. Basting and others[7] assessed the Whiteness HP Maxx and the Whiteness HP Blue agents and also found similar results for the times evaluated in the present study using the same methodology.

From the high-concentration hydrogen peroxide–based bleaching agents for use in office, the Pola Office+ was shown to reduce the concentration of Ca and P over time. The Pola Office+ agent has slightly alkaline pH values, which would not be responsible for the change in mineral content. For this reason, one would expect the Whiteness HP Maxx agent, due to its acidic pH values (5.4 after 15 minutes of mixing, which is a value close to the critical pH for enamel demineralization), to have caused greater changes in mineral content,[9] which was not the case.

Hydrogen peroxide–based bleaching agents feature an acidic pH and are therefore recognizably superior in terms of product stability, which is important in terms of the degradation reactions of the bleaching agent. However, despite the risk of enamel erosion due to the low pH of the bleaching agents, Xu and others[24] and Sa and others[27] reported that such a characteristic has not been implicated in significant changes in enamel composition. Araujo and others[32] also observed that the acidic pH of hydrogen peroxide bleaching agents was not the cause of changes to enamel microhardness. This finding could be attributed to the composition of bleaching agents, which includes no calcium and/or fluoride, unlike other bleaching products. According to the manufacturer, the Pola Office+ agent only has potassium nitrate added to its composition, which is used to reduce the dentinal sensitivity caused by tooth bleaching.[33] Potassium nitrate would not,

Table 3: Extended.

Time	Low Hydrogen Peroxide Concentration	
	Pola Day	White Class
Calcium		
T0	4.79E-05 ± 8.58E-06 Aa	6.67E-05 ± 4.81E-05 Aa
T1	5.25E-05 ± 4.16E-05 Aa	6.27E-05 ± 1.45E-05 Aa
T3	5.21E-05 ± 3.44E-05 Aa	7.00E-05 ± 5.21E-05 Aa
T5	4.29E-05 ± 2.35E-05 Aa	8.27E-05 ± 6.27E-05 Aa
T6	1.06E-04 ± 1.25E-04 Aa	1.30E-03 ± 6.27E-05 Aa
Phosphorous		
T0	1.55E-05 ± 4.17E-06 Aa	9.64E-06 ± 6.44E-06 Aa
T1	7.36E-06 ± 7.96E-06 Ab	2.11E-04 ± 2.65E-04 Aa
T3	9.64E-05 ± 1.32E-04 Aa	5.80E-05 ± 4.46E-05 Aa
T5	1.60E-05 ± 3.96E-06 Aa	3.43E-05 ± 5.17E-06 Aa
T6	7.21E-05 ± 5.34E-05 Aa	3.71E-03 ± 2.11E-04 Aa

however, be expected to affect the mineral content of the enamel, unlike sodium fluoride, which is found in Opalescence Boost.

As a result of their mechanism of deposition onto the dental substrate,[33] sodium fluoride present in high-concentration hydrogen peroxide bleaching agents could contribute to the maintenance of the mineral content or even enamel remineralization.[9,18] Such an effect was not, however, observed for Opalescence Boost, which revealed a decrease in the concentration of Ca over the treatment period, even at pH values that were close to neutral. Phosphate concentrations were, however, similar for Opalescence Boost over time. A reduction in the concentration of Ca may be related to the mineral content of the bleaching agent, which is subsaturated in relation to the dental enamel, even when fluoride is added to its composition.

Only Whiteness HP Blue Calcium (containing calcium gluconate) and HP Maxx Whiteness (which displays neither calcium nor fluoride in its formulation) were among the high-concentration hydrogen peroxide agents that were able to maintain the concentrations of Ca and P in the enamel over time. In addition to containing neutralizing agents that could favor neutral pH values, Whiteness HP Blue Calcium features calcium in its composition in a saturated form, which would promote mineral deposition onto the enamel surface, or Ca incorporation into hydroxyapatite, preventing demineralization of the substrate, as shown by Borges and others[18] and Cavalli and others,[15] who evaluated agents containing calcium chloride, and Borges and others,[19] who

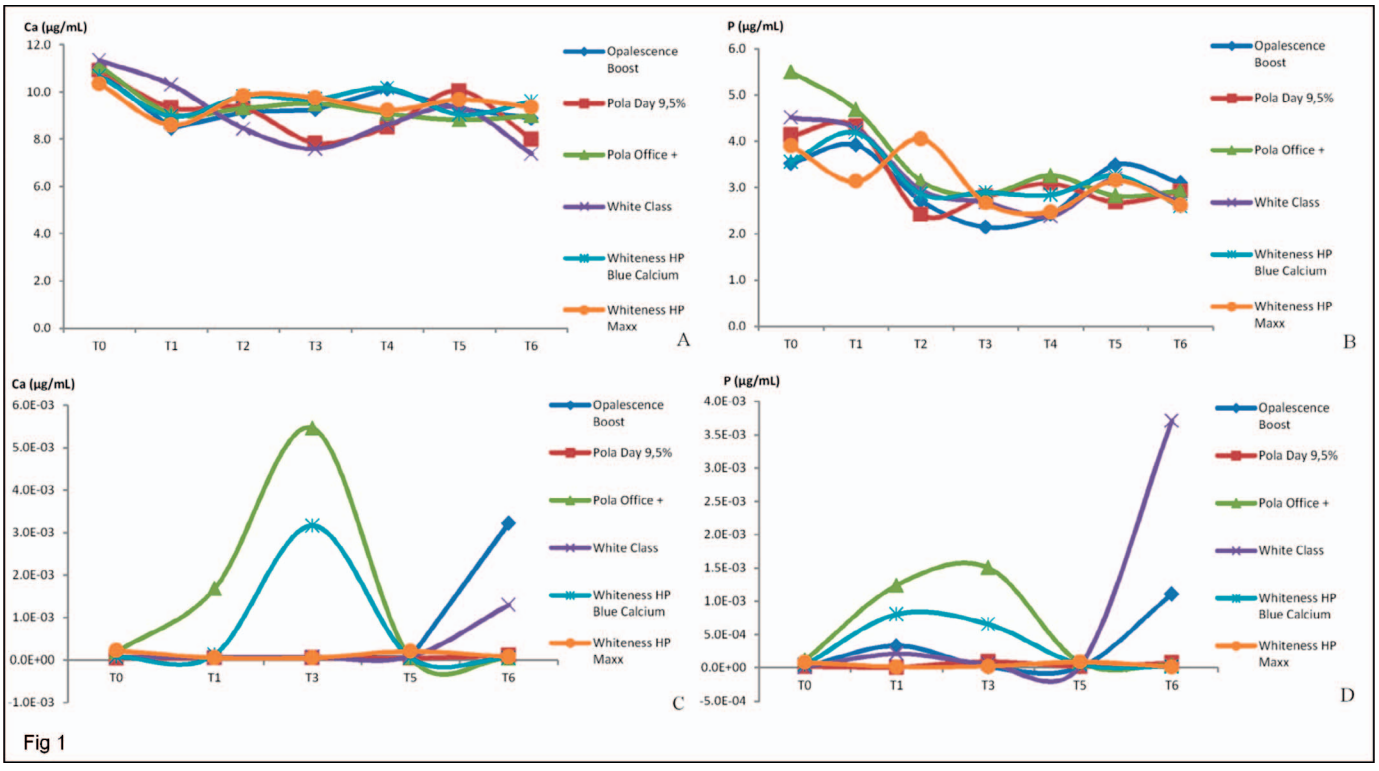

Figure 1. *Ca and P concentration means (in µg/mL) by colorimetric spectrophotometry (A and B) and total reflection X-ray fluorescence analyses (C and D) according to bleaching agents over time.*

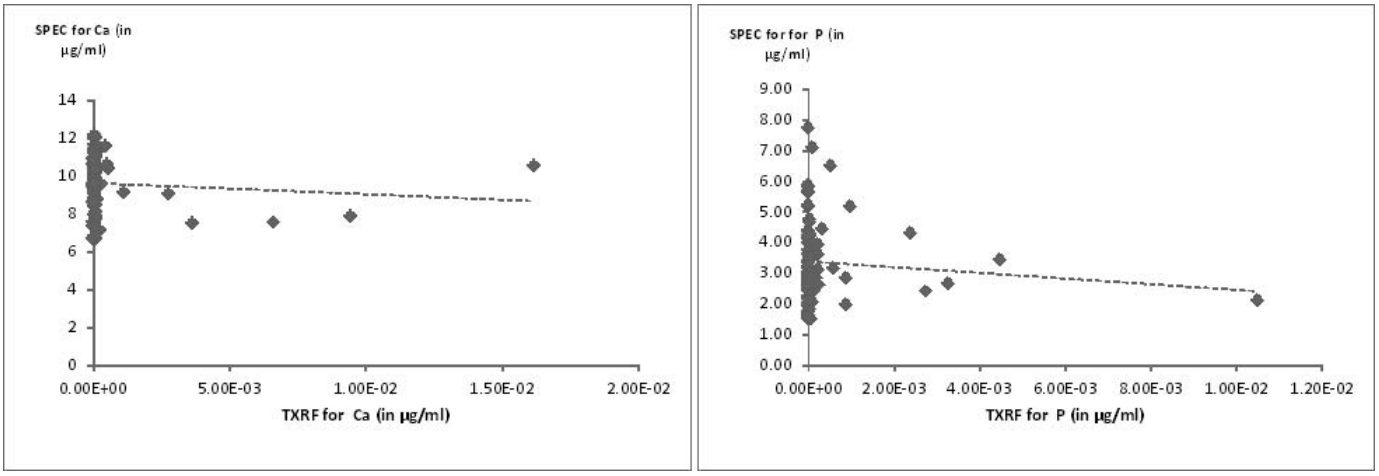

Figure 2. *Spearman correlation of Ca (A) and P (B) concentrations by colorimetric spectrophotometry and total reflection X-ray fluorescence analyses.*

used 2% calcium gluconate in 35% hydrogen peroxide. Calcium deposits on the enamel surface were observed via scanning electron microscopy when using Whiteness HP Blue Calcium,[16] which may reduce enamel susceptibility to erosion.[17] The beneficial effects of using bleaching agents containing calcium are still controversial, considering that no differences were observed regarding the enamel concentrations of Ca and P when using the Whiteness HP Maxx agent containing no calcium gluconate in its formulation. Since calcium gluconate is incompatible with strong oxidizing agents[34]—such as the free oxygen and perhydroxyl present in the byproducts released during the bleaching treatment[35]—the addition of this component to bleaching agents appears questionable, as observed by Basting and others.[7]

Regarding low-concentration hydrogen peroxide bleaching agents, higher concentrations of Ca[11,13,14] and P[11,14] have been observed in the enamel when compared to the high-concentration agents. The present study has shown, however, that both bleaching agents with low hydrogen peroxide concentration have caused a decrease in the concentrations of Ca. In terms of P, White Class has also caused a decrease in its concentration over time. Although both agents present acidic pH values, they feature remineralizing agents in their composition, which could avoid enamel demineralization. Regarding bleaching agents for home use, it is suggested[4,15] that the addition of calcium fluoride can minimize changes to the mineral content of the enamel. Fluoride is present in the composition of Pola Day, and calcium gluconate is part of the composition of White Class. One would therefore expect similar results with White Class to those observed with the high-concentration agent, which also contained calcium (Calcium Whiteness Blue HP) and did not cause changes in the concentrations of the minerals studied. Nonetheless, similar results on Ca and P concentrations for both agents containing calcium in the formulation were not observed.

Immersion in artificial saliva for 14 days after bleaching was not able to recover the initial concentration values of Ca and P in the enamel when using bleaching agents known to lead to changes in mineral content over time (Pola Office+, Opalescence Boost, Pola Day, and White Class). For home-use low–hydrogen peroxide agents, in particular, a significant reduction in Ca concentrations was observed. Other studies[6,11,12,16,26] using artificial saliva in the postbleaching period observed a recovery of the initial microhardness values due to the supersaturated minerals in the saliva, leading to enamel remineralization. Changes in mineral content may have been accompanied by erosive surface lesions, which may have prevented remineralization, or the post–treatment time was not sufficient to remineralize the tooth substrate.[16]

Considering the results of this study, one may suggest that changes in enamel calcium and phosphate contents during and after bleaching may occur regardless of the presence of fluoride or calcium in the composition of certain brands of bleaching agents, concentration of hydrogen peroxide and bleaching technique (office or home), or time of application of the bleaching agent and its pH, corroborating the study by De Abreu and others.[26] Changes in enamel mineral content may be related to the compositional characteristics of each bleaching gel brand, since not all manufacturers mention

all components and their percentages in the formulation. This study suggests that when choosing a bleaching approach, either in office with a high-concentration hydrogen peroxide agent or a home-bleaching method with a low-concentration hydrogen peroxide product, the agents should be selected according to their effects on the tooth structure, in which case Whiteness HP Maxx, Whiteness Calcium Blue HP, Opalescence Boost, and Pola Day were those that caused minimal or no change to the enamel concentrations of Ca and P.

Since no correlation was observed between the methods used to quantify the concentration of calcium and enamel phosphate, and in view of the limitations related to the accuracy of chemical element analysis by the TXRF method when using the enamel biopsy solution, as well as the laborious and restrictive evaluation protocols relating to the use of Synchrotron radiation beam, it can be suggested that the analysis by colorimetric spectrophotometry may yield more homogeneous results, with lower coefficients of variation as well as the possibility of using more repetitions per group.

CONCLUSIONS

Changes in enamel calcium and phosphate contents during and after bleaching may occur regardless of the presence of fluoride or calcium in the composition of certain brands of bleaching agents, concentration of hydrogen peroxide, and bleaching techniques used. Whiteness HP Maxx, Whiteness Calcium Blue HP, Opalescence Boost, and Pola Day caused minimal or no change to the enamel concentrations of calcium and phosphate. No correlation was observed between the methods (TXRF or colorimetric spectrophotometry) used to quantify the concentration of calcium and enamel phosphate.

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Regulatory Statement

This study was conducted in accordance with all of the provisions of the local human subjects' oversight committee guidelines and policies of São Leopoldo Mandic Research Ethics Committee. The approval code for this study is 32495614.0.0000.5374.

Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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