©Operative Dentistry, 2022, **47-3**, E162-E173

# Use of Computerized Microtomography, Energy Dispersive Spectroscopy, Scanning Electron Microscopy, and Atomic Force Microscopy to Monitor Effects of Adding Calcium to Bleaching Gels

LC Mendonça • MLA Rodrigues • AA Bicalho • GR daSilva • PS Quagliatto • CJ Soares

**Clinical Relevance**

Bleaching teeth with hydrogen peroxide gels containing calcium does not prevent mineral loss at the enamel surface. However, the demineralized regions do not exhibit an increase in surface roughness.

## SUMMARY

**Objectives: The aim of this study was to evaluate the mineral content, expressed by calcium (Ca) and phosphate (P), in dental enamel exposed to bleaching agents using micro-computed tomography (micro-CT), scanning electron**

Ludmila Cavalcanti de Mendonça, DDS, MS, PhD student, Biomechanics Group, Department of Operative Dentistry and Dental Materials, professor at Technical School of Health, Federal University of Uberlândia, Uberlândia, MG, Brazil

Maria de Lara Araújo Rodrigues, DDS student, Biomechanics Group, Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

Aline Arêdes Bicalho, DDS, MS, PhD, Biomechanics Group, Department of Operative Dentistry and Dental Materials, professor at Technical School of Health, Federal University of Uberlândia, Uberlândia, MG, Brazil

Gisele Rodrigues da Silva, DDS, MS, PhD, professor, Biomechanics Research Group, Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

Paulo Sérgio Quagliatto, DDS, MS, PhD, professor, Biomechanics Research Group, Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

*Carlos José Soares, DDS, MS, PhD, professor and chairman, Biomechanics Research Group, Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

*Corresponding author: Avenida Pará, 1720, Bloco 4L, Anexo A, Sala 42, Campus Umuarama, Uberlândia - Minas Gerais - Brazil, 38405-320; e-mail: carlosjsoares@ufu.br

http://doi.org/10.2341/20-217-L

**microscopy (SEM), energy dispersive spectroscopy (EDS), and atomic force microscopy (AFM).**

**Methods: Sixty bovine dental enamel specimens were randomly divided into three groups (n=20): HP35ca (bleached using 35% hydrogen peroxide with Ca); HP35wca (bleached using 35% hydrogen peroxide without Ca); and control (without bleaching). Five specimens from each group were used for SEM and EDS analyses, 10 specimens were used for AFM analysis, and the remaining five specimens were used for micro-CT analysis. The pH of the gels was measured using a pH meter. The EDS and micro-CT data were analyzed using one-way ANOVA and Pearson's correlation test. The AFM data were analyzed using one-way ANOVA ($\alpha$=0.05).**

**Results: The weight percentages of Ca and P obtained using EDS were similar between the bleached and control groups. Small, superficial changes were observed by SEM in the HP35wca group. The HP35ca group showed similar patterns to the control group. AFM results showed no significant changes in the enamel roughness in any of the tested groups. No significant difference in the volume or depth of structural enamel loss was found between gels with and without Ca. No mineral loss was observed in the dentin substrate. The EDS and micro-CT analysis data exhibited a high correlation ($p$<0.001).**

**Conclusion: The addition of Ca to the bleaching gel had no beneficial effect on the bleached tooth enamel in terms of composition, mineral loss, and surface roughness. Micro-CT results exhibited a high correlation with the EDS results.**

## INTRODUCTION

Chromatic alterations in teeth compromise the esthetics of the smile, adversely affecting the social and emotional behavior of patients.[1] Bleaching procedures are the preferred method for treating tooth discoloration because they involve a simple and minimally invasive protocol.[1] Although there have been many studies on bleaching treatments, the performance of bleaching agents has not been demonstrated fully.[1]

Hydrogen peroxide is the most commonly used bleaching agent.[1] Different theories have been proposed to explain its bleaching mechanism, which involves the penetration of hydrogen peroxide and its decomposition into oxygen molecules capable of breaking down pigment macromolecules. However, studies have claimed that free radicals released by hydrogen peroxide are unstable and unspecific and react with the inorganic enamel matrix in addition to the pigmented organic molecules.[2-5] Hydrogen peroxide can diffuse through the tooth enamel and dentin, releasing free radicals that oxidize the chromophores of molecules.[6,7] These chromophores, rich in electrons that absorb specific wavelengths of visible light, break down when attacked by free radicals.[8,9] Free radicals attack the double bonds responsible for the color of chromophores, thus making the teeth appear lighter in color.[8,9] Another theory suggested that peroxide causes minor morphological changes in the enamel that increase its opacity due to the dispersion of light and hide the underlying dentin layer.[10-13] Whitening agents can also function by oxidizing the fluorescent components in dentin, such as dentin phosphoproteins. Hydrogen peroxide can whiten the dentin by oxidizing the aromatic amino acids in the dentin phosphoprotein.[14]

Several studies claim that bleaching is a completely safe procedure.[15-17] However, enamel demineralization upon bleaching can cause alterations in the tooth such as an increase in roughness, reduction in microhardness, and changes in the superficial micromorphology.[18-22] To prevent demineralization (especially the loss of calcium and phosphate ions) and the reduction of enamel hardness during tooth bleaching,[23] calcium and fluoride are added to the gel composition.[24] A significant increase in enamel permeability and roughness and a decrease in microhardness compared to the untreated control group have been reported after bleaching with 35% hydrogen peroxide gel with Ca or fluoride.[21] Bleaching with 10% carbamide peroxide showed that the enamel was susceptible to mineral loss during the whitening treatment, but this loss was minimized by the addition of F and Ca to the whitening agents.[24]

Different methods have been used to evaluate the changes that occur after tooth enamel bleaching, including quantitative tests to assess the changes in physical properties and mineral composition via biochemical measurements and qualitative evaluations using different imaging techniques.[19,22,25-28] However, micro-computed tomography is of particular interest to researchers because it can quantify the enamel loss at the surface as well as the subsurface.[29]

The clinical relevance of the undesirable effects of dental bleaching on tooth structures has yet to be addressed.[15-22] The methods used for analyzing enamel mineral loss after bleaching are important to confirm the effect of bleaching, obtain new information, and determine the association between different and complementary methods. Therefore, the aim of this study was to evaluate the mineral loss in dental enamel exposed to bleaching agents by micro-computed

tomography (micro-CT), scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), and atomic force microscopy (AFM). The following hypotheses were tested: (1) micro-CT can be used as an alternative method to determine the loss of enamel and dentin structures; (2) the presence of calcium in the bleaching gel can reduce enamel mineral loss and retain the surface roughness; and (3) the enamel mineral loss occurs primarily at the surface of the enamel structure.

## METHODS AND MATERIALS

### Preparation of Specimens — Bleaching Procedures

Incisor teeth of bovine animals of equal ages were extracted immediately after sacrificing the animals. The teeth were stored in distilled water at -10°C for a maximum of 30 days. Specimens of dimensions 5 mm × 5 mm × 4 mm (approximately 1.5 mm: enamel and 2.5 mm: dentin) were obtained from the central region of the buccal surface of each tooth using a water-cooled, low-speed diamond saw (Buehler Ltd, Lake Bluff, IL, USA). The specimens were randomly divided into three groups (N=20): HP35ca [bleached using 35% hydrogen peroxide with Ca ions (Whiteness HP Blue Calcium - FGM, Joinvile, Brazil)], HP35wca [bleached using 35% hydrogen peroxide without Ca ions (Whiteness HP Maxx - FGM, Joinvile, Brazil)], and control (without the application of a bleaching gel). Five specimens from each group were used for SEM and EDS analyses, 10 specimens for AFM analysis, and the remaining five specimens for micro-CT analysis. For the micro-CT and AFM analyses, the specimens from the bleached groups were analyzed before and after the bleaching procedures, while the specimens from the control group were analyzed before and after immersion in artificial saliva. The tooth enamel surfaces of the specimens were regularized using 600-, 1000-, 1200-, and 1500-grit abrasive papers (Arotec, Cotia, SP, Brazil) and polished with a polishing cloth and 6-, 3-, 1-, and 0.5-μm diamond pastes (Arotec) in a polishing machine (Arotec) to standardize the surface. The lateral and bottom surfaces were covered with nail polish to isolate the contact of the products only to the specimen buccal surface during the treatments (Rísque, SP Brazil). The HP35wca group was treated for two 40-min sessions, with a 7-day interval. The HP35ca group was treated for two 45-min sessions, with three applications per session, every 15 min; there was also a 7-day interval between sessions. All bleaching procedures were performed according to the manufacturer's instructions (Table 1). The specimens were rinsed with a distilled water spray after each session and then immersed in artificial saliva at 37°C until the next application of the whitening gel. After the last session for each group, the specimens were rinsed and stored in distilled water. The specimens were ultrasonicated in distilled water for 10 min before all tests. The debris layer produced during specimen preparation was not intentionally removed before analyses.

### Micro-CT Analysis

Herein, micro-CT (a 3D imaging technique that utilizes X-rays to see inside a sample, slice by slice)

Table 1: *Product Composition, pH Values, and Manufacturer's Recommendations for Use*

| Material | Group | Treatment | Composition | Batch Number | pH | Manufacturer |
|---|---|---|---|---|---|---|
| Whiteness HP Blue Calcium - HP 35% | HP35ca | 2 sessions of 40 min, with an interval of 7 days | HP 35% (after mixing the phases), thickeners, inherent pigment, neutralizing agents, calcium gluconate, glycol and purified water | 010319 | 8.3 (0.3) | FGM (Joinville, SC, Brazil) |
| Whiteness HP Maxx HP 35% | HP35wca | 2 sessions of 45 min each (3 applications of 15 min), with an interval of 7 days | HP 35% (after mixing the phases), thickeners, mixture of dyes, glycol, inorganic filler and deionized water | 060619 | 6.8 (0.1) | FGM (Joinville, SC, Brazil) |
| Specimen without contact with bleaching gel | Control | Storage in artificial saliva | 1.5 mm Ca and 0.9 mm P in 0.1 mm Tris buffer solution | — | 7.0 (0.1) | — |

was conducted (Nrecom software version 1.6.10.1; DataViewer software version 1.5.1.2; CTAn, version 1.13; CTVol, version 2.0; SkyScan Bruker Belgium). In the reconstructed image, the internal structure of the sample was analyzed. The reconstructed images were then overlaid using the DataViewer software (version 1.5.1.2, SkyScan, Bruker, Belgium). Comprehensive 3D image analysis capable techniques (such as morphometry, densitometry, and segmentation) and advanced image processing methods allowed the quantification of mineral loss at the surface and subsurface.[30]

The bleached tooth analysis methodology was based on a previous study that conducted micro-CT to evaluate the cusp deformation produced by resin composite restorations. The images of the prepared tooth (reference) and the image of the restored tooth (target) were overlaid, generating a difference in the volume of the image (Diff). This Diff image represents the volume of cusp deformation caused by the polymerization contraction of the composite resin restoration with high resolution.[30] A similar protocol was used in the present study to determine the structural alterations produced by the bleaching process.

In this study, specimens (n = 5) were scanned using a high-resolution micro-CT instrument with a resolution of 0.35 mm (Bruker, Kontich, Belgium). The device was set to the following configuration: 100 kV and 100 mA, a 0.11 mm Cu filter, an image pixel size of 13 μm, a resolution of 1632 × 1092, and a 0.6° rotation pitch. Three image slices were generated over 1850 ms with 20 random movements, resulting in 1692 image slices. NRecon software (Bruker) was used to reconstruct the images by adjusting to the appropriate parameters for smoothing and beam-hardening artifact correction. The images of each specimen before and after treatment were overlaid using DataViewer software (Bruker) and the difference between them (Diff) was used to characterize the mineral loss in the entire sample (enamel and dentin) on a cubic millimeter scale. These differences were measured by 3D morphometric analysis using CTAn software (Bruker) that also allowed the measurement of mineral loss in millimeters via 2D morphometric analysis. In a 2D longitudinal cross-section view of the specimen, a micrometer was used to measure the enamel and dentin mineral loss, which was represented by the difference in depth in the overlapping images. To guide the overlay process, cavities were made at the base of the specimen with spherical drills and subsequently filled with composite resin.

Prior to the study, pilot tests were performed to confirm the accuracy of the technique by superimposing two different scans of the same specimen, one without treatment and one when stored in distilled water, which showed no significant difference in the calculated tissue volume (Figure 1A). However, significant mineral loss was detected in the treated specimens stored in distilled water, as marked by the dark gray line at the specimen surface (Figure 1B).

## SEM and EDS Analysis

The specimens were vacuum-plated with gold (Balzers, Berlin, Germany) and analyzed at a magnification of 20,000× (Zeiss, Jena, Germany) by SEM. The EDS software, model INCA X-act (Oxford, Abingdon-on-Thames, United Kingdom), was calibrated based on the information that the sample was covered with a 56-nm thick gold layer; therefore, the software was able to perform the adjusted calculation. The content of calcium (Ca) and phosphate (P) ions (wt%) on the enamel surface was measured using EDS (Oxford, Abingdon-on-Thames, United Kingdom). The Ca/P ratio was calculated for each specimen and compared to the stoichiometric ratio of hydroxyapatite (1.67). Five measurements were made per specimen in the area corresponding to the 20,000× magnification image. This image was consistently used for all measurements and was defined during the pilot experiment. EDS analyses were normalized to a 100% windowless detector that determines semi-quantitative evaluation

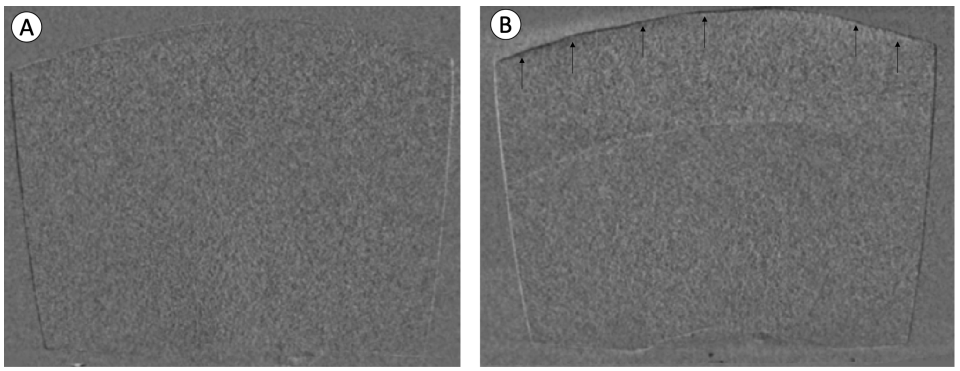


Figure 1. *(A) Representative image of the superposition of two scans of the same sample without any treatment and (B) representative image of the overlay of the previous scan image with the post-bleach image of the same sample.*

of lighter elements. The acquisition time was 30 s per measurement.

### AFM Analysis

To determine the alteration of the surface roughness of the specimens, scans (in the positive X-axis direction) were performed on the specimens using an AFM machine in the dynamic force mode (Shimadzu, Chiyoda-ku, Tokyo). The probes (Tap190Al-G - Budget Sensors), which are designed to work at resonance frequencies between 160 and 220 kHz, performed the scans at the frequencies between 160 and 170 kHz without specimen distortion, damage, or removal from the surface. The scan speed (rate) was set to 0.5 Hz, collecting data from an area of 30 × 30 mm (resolution of 512 × 512 pixels) in approximately 18 min with a scan speed of 2 s per line. The equipment settings were as follows: operating point between 0.100 and 0.160, integral gain fixed at 1800, and proportional gain at 0. Using the Gwydion analysis software (version 2.57, open-source software for scanning probe microscopy data processing (http://gwyddin.net), 10 measurements (five vertical and five horizontal line scans) were extracted and the roughness of the specimen was determined.

### Measurement of pH

The pH of the bleaching gels was measured with a pH meter (Adwa, Szeged, Hungary), which monitored the degree of acidity or alkalinity via an electrode coupled to a potentiometer (potential difference meter). The pH measurements were calibrated using a standard buffered potassium chloride solution. Three measurements were taken for each gel and an average was obtained. The pH electrode was calibrated with standard solutions before each measurement to ensure the sensitivity of the pH meter.

### Statistical Analysis

After checking the data from micro-CT and EDS for normality (Shapiro-Wilks) and homogeneity (Levene), the volume and depth loss data for enamel and dentin obtained from micro-CT analysis and the volume losses of Ca and P measured using EDS were analyzed by one-way ANOVA. Surface roughness data measured by AFM were analyzed by one-way repeated measures ANOVA. All tests were performed using Sigma Plot (Systat Software Inc, Chicago, IL, USA) at a level of significance ($\alpha$) of 0.05.

## RESULTS

The means and standard deviations of the pH values of the HP35ca and HP35wca groups are shown in Table 1. The means and standard deviations of the loss of enamel (μm) in terms of depth and total volume loss ($mm^3$) are shown in Table 2. One-way ANOVA demonstrated no significant difference in the volume ($p$=0.001) or depth of enamel loss ($p$=0.001) of HP35wca and HP35ca groups. However, the bleached groups showed significant differences compared to the control group. No mineral loss was detected in the dentin substrate in any of the groups. The losses of the enamel structure in the HP35wca and HP35ca groups were located close to the surface. Micro-CT images showed similar volume losses for HP35wca and HP35ca (Figure 2).

The means and standard deviations of the Ca and P (wt%) compositions are listed in Table 3. The Ca ($p$=0.955) and P ($p$=0.393) contents analyzed by EDS were similar between the bleached and control groups. SEM images revealed superficial alterations in HP35wca, such as pores and depressions, and images obtained from the HP35ca group showed slight alterations (Figure 3).

The means and standard deviations of the surface roughness (Ra) are listed in Table 4. The Ra values were similar for all groups before ($p$=0.690) and after treatment ($p$=0.630). No significant variation was found before and after bleaching HP35ca ($p$=0.340), HP35wca ($p$=0.213), and control ($p$=0.412) groups. The AFM images showed no significant changes in any of the tested groups (Figure 4). The correlation coefficient for EDS data (Ca and P) and micro-CT data (depth and volume) is shown in Figure 5. Pearson's correlation exhibited high values between the Ca and P percentages measured by EDS and the depth and volume of mineral losses measured by micro-CT ($p$<0.001) for all combinations.

Table 2: *Means and Standard Deviations of Depth of Mineral Loss and Total Volume Between the Experimental Groups Obtained by Micro-CT, Calculated by One-Way ANOVA*[a]

| Group | Depth of Loss (mm) ($p$<0.001) | Total Loss ($mm^3$) ($p$<0.001) |
|---|---|---|
| HP35ca | 0.0326 (0.0008) B | 0.4691 (0.2090) B |
| HP35wca | 0.0338 (0.0008) B | 0.4488 (0.2150) B |
| Artificial Saliva (control) | 0.0064 (0.0008) A | 0.0005 (0.0002) A |

[a] *Uppercase letters establish relationships among columns. Different uppercase letters indicate statistically significant differences (p>0.05). The standard deviations are presented in parentheses.*

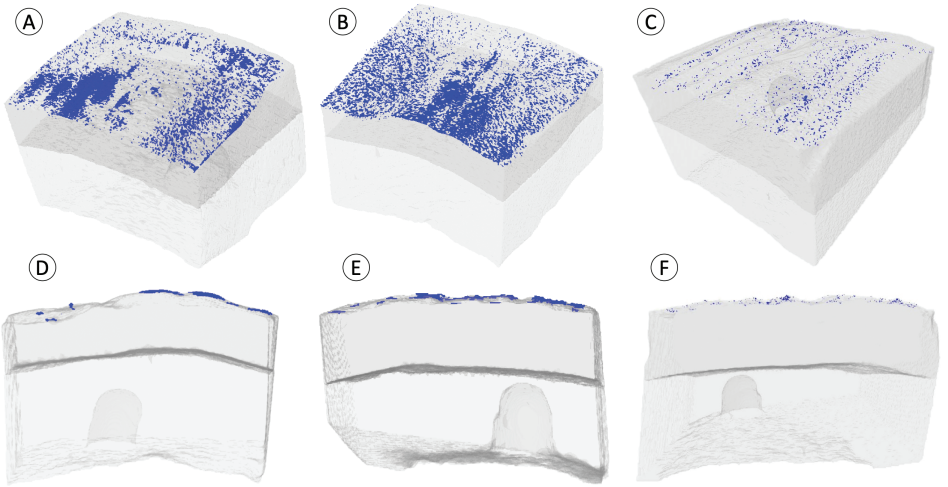


Figure 2. *Micro-CT images demonstrate similar volumes and depths of the loss of enamel structure in bleached groups. In the control group, only the difference related to the deviation of precision from the real measurement of the object is measured by the software. Representative image of depth loss for (A) HP35wca, (B) HP35ca, and (C) control. Representative image of enamel surface demonstrating volume loss for (D) HP35wca, (E) HP35ca, and (F) control.*

## DISCUSSION

Micro-CT is a nondestructive method that allows the evaluation of the internal structure of the whitened enamel and can be used to evaluate the surface and subsurface enamel layers in 3D. The results of the study demonstrated that micro-CT can be recommended to assess the structural volume and depth loss of enamel and dentin tissues; therefore, the first hypothesis of our study was confirmed. Pilot tests performed prior to the study by overlaying two different scans of each specimen showed no significant differences when the images of specimens with no treatment were overlaid. In contrast, differences were observed between the treated specimens. In another study, the authors suggested that micro-CT was an adequate method to assess the mineral content of tooth enamel after the application of whitening gels.[31] However, the study tested only one group by applying 10% carbamide peroxide for two weeks, which caused enamel demineralization up to a depth of 50 micrometers below the enamel surface, and did not have a control group.[31] Although the effectiveness of the method in the analysis of the integrity of treated tissues has been proven, studies evaluating structural alterations after bleaching treatment using micro-CT are scarce.[32-35]

This study used overlapping images of the initial and final scans to show the surface alterations of enamel and the loss of enamel structure in terms of depth after using 35% hydrogen peroxide (HP) in-office bleaching gels, regardless of the calcium content in the gels. No mineral loss was observed in the dentin tissue using the resolution of the micro-CT analysis used in this study. New studies using nano-CT may detect mineral loss in the dentin; however, it is also important to consider the clinical relevance of minor mineral losses in the dentin caused by bleaching gels. Previous studies have shown similar morphological changes in enamel, and no changes in dentin have been reported after treatment with 37.5% HP and 35% HP. Ca and P decreased in the enamel and dentin with no significant differences between them or in relation to the untreated control specimens,[36] while another *in vitro* study with a 35% HP gel showed no evidence of deleterious effects of bleaching on enamel or dentin and suggested that studies reporting adverse effects on enamel and/or dentin actually reflected the pH of the formulation used.[37]

Table 3: *The Means and Standard Deviation of the Ca and P Values (wt%) and Ca/P Ratio in Enamel after Application of Bleaching Gels and in the Control Group Obtained by EDS*[a]

| Group | Calcium (Ca) (wt%) (*p*=0.955) | Phosphate (P) (wt%) (*p*=0.393) | Ca/P Ratio (*p*=0.021) |
|---|---|---|---|
| HP35ca | 36.5 (6.1) A | 17.5 (2.4) A | 2.1 (0.1) A |
| HP35wca | 37.3 (4.8) A | 19.3 (0.4) A | 2.1 (0.2) A |
| Control | 36.6 (2.3) A | 17.5 (2.9) A | 1.9 (0.1) A |

[a]*The same uppercase letters indicate that there was no significant difference among the groups analyzed by one-way ANOVA (p>0.05). No significant difference among groups within columns is observed. The standard deviations are presented in parentheses.*

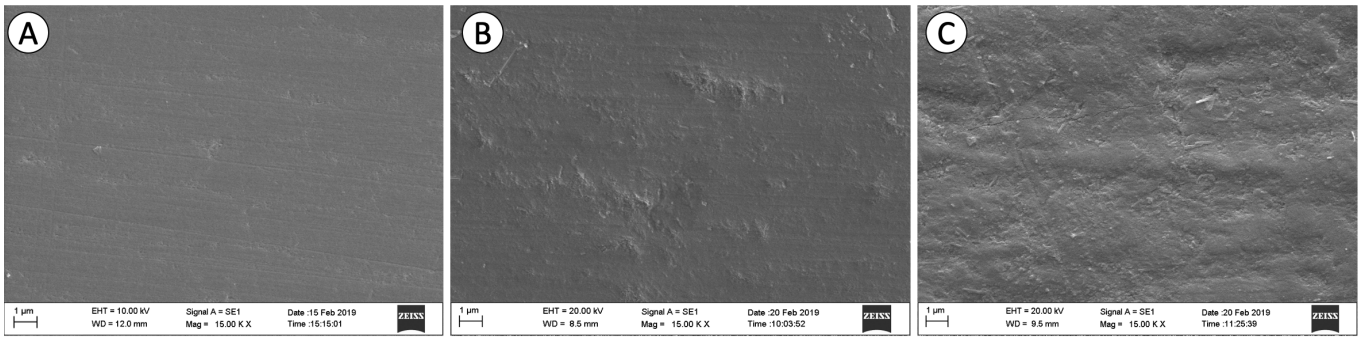


Figure 3. *Representative scanning electron microscopy images of the following tested enamels: (A) HP35wca application showing porosities and depressions; (B) HP35ca application showing slight surface alterations and areas with calcium deposition; and (C) no treatment (control group) showing no changes in the smooth polished surface.*

Ca is used in bleaching gels to achieve Ca ion supersaturation, preventing the dissolution of hydroxyapatite.[38] The optimal Ca concentration required during bleaching protocols is not well defined.[38] The addition of 0.5% calcium gluconate to a 35% HP gel was unable to prevent demineralization of the enamel[38;] therefore, this concentration was insufficient to supersaturate Ca ions relative to the enamel hydroxyapatite crystals.[38] A previous study found that bleaching enamel using a 35% HP gel containing sodium fluoride or calcium gluconate resulted in higher microhardness values than using gels without these compounds and that high concentrations of calcium gluconate in bleaching gels had a positive effect on enamel, but not at low concentrations in gels.[39]

Although studies have reported that the addition of Ca or fluoride to the 35% HP bleaching gel can reduce demineralization of the enamel surface,[40] it cannot fully prevent it or remineralize the subsurface enamel.[25] The increase in the permeability and roughness of the enamel surface and the decrease in the microhardness of the enamel are not prevented when Ca and F ions are added to 35% HP bleaching gels.[21] The results of the present study demonstrate the loss of the enamel structure in terms of volume by micro-CT in the studied groups; however, there was no significant difference in the results obtained by bleaching using HP gels with or without Ca. Therefore, the second hypothesis of this study was rejected because the presence of Ca of a certain concentration in the tested bleaching gel did not inhibit the mineral loss of the enamel structure. It is important to emphasize that the percentages of Ca and P on the enamel surface, which are the main constituents of hydroxyapatite crystals, were similar in both the bleached and control groups. Therefore, the 35% HP gel used for the 80-90 min in-office bleaching technique caused no significant change in the mineral composition of the tooth. Demineralization in some regions promoted the redistribution of minerals, as reported in previous studies,[34] which may explain the EDS results in our study. This result corroborates the results of those studies that used the same bleaching gels and determined from the EDS results that there was no statistically significant loss of Ca and P during treatment.[41]

Table 4: *Means and Standard Deviations of Ra (nm) between the Experimental Groups Obtained by AFM — One-Way Repeated Measures ANOVA*[a]

| Group | Initial Ra (nm) ($p$=0.690) | Final Ra (nm) ($p$=0.630) |
|---|---|---|
| HP35ca | 3.7 (1.3) Aa | 2.7 (0.8) Aa |
| HP35wca | 3.4 (1.2) Aa | 2.8 (1.1) Aa |
| Artificial saliva (control) | 3.1 (0.4) Aa | 2.7 (0.8) Aa |

[a]*The same uppercase letters indicate that the Ra values were similar for all groups before and after treatment. The same lowercase letters indicate that no significant variation was found before and after bleaching the HP35ca (*p*=0.340), HP35wca (*p*=0.213), and control (*p*=0.412) groups. The standard deviations are presented in parentheses.*

Hydroxyapatite is a hydrated calcium phosphate from the mineral group of apatite, whose stoichiometric chemical formula is $Ca_{10}(PO_4)_6(OH)_2$ with a molar Ca/P ratio of 1.67.[42] The Ca/P ratios of the groups were calculated using the compositions of Ca and P obtained by microanalysis in EDS.[43] The calculated Ca/P ratio varied between 1.89 to 2.13 and showed no significant differences between the groups. Therefore, the presence or absence of calcium in the bleaching gel composition caused no significant alteration in the Ca/P molar ratio. In a previous study, where in a whitening treatment with 10% carbamide peroxide (CP) containing calcium (Ca) or amorphous calcium phosphate (ACP) was conducted, concluded that the

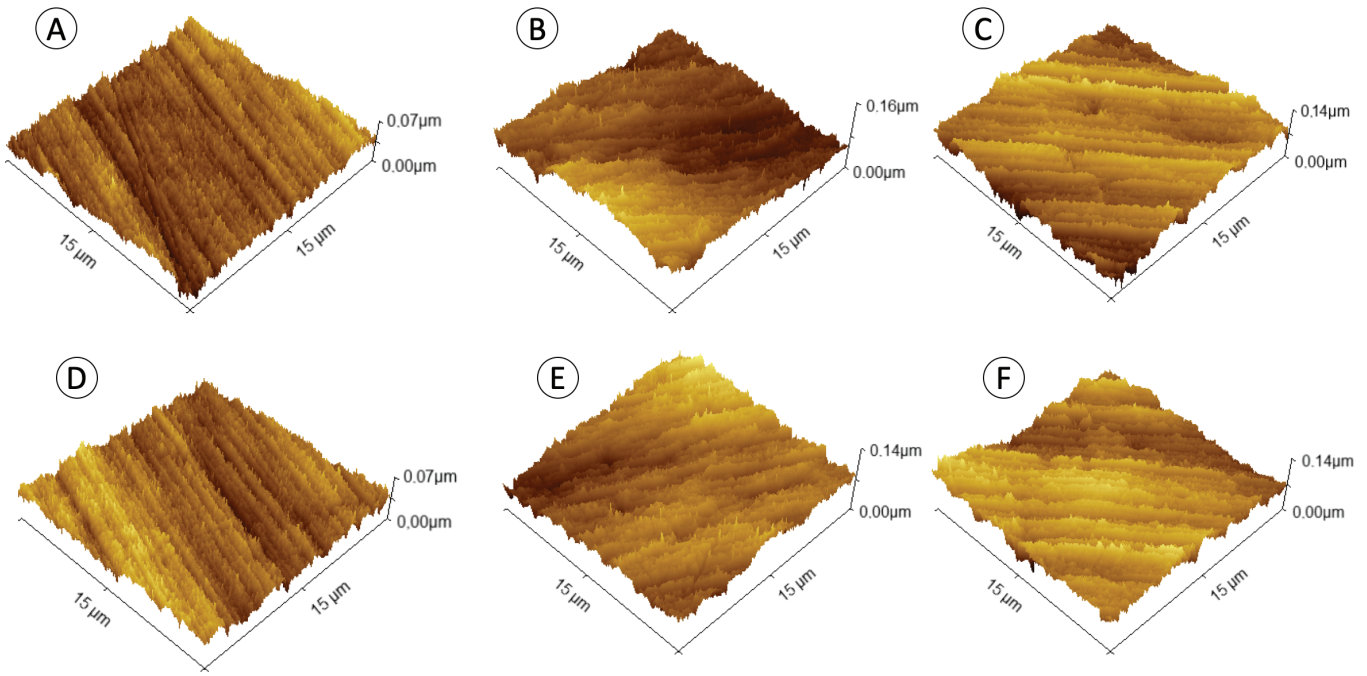


Figure 4. *Representative images of AFM of the following tested enamels: (A) and (D) representative images of the HP35wca group showing no significant changes on the enamel surface; (B) and (E) representative images of the HP35ca; and (C) and (F) without treatment (control group), showing no significant changes on the enamel surface.*

enamel microhardness decreased after the whitening process, regardless of the presence of Ca or ACP. However, no significant change in the enamel Ca/P ratio was detected, indicating that the bleaching gels have an erosive potential, causing enamel softening without promoting surface loss, irrespective of the presence of calcium or ACP ions.[44]

The concentration and duration of bleaching as well as the pH of bleaching agents can influence the mineral loss of bleached enamel.[22,45] An acidic pH causes changes in the mineral composition of the enamel structure,[4,46] contributing to enamel surface erosion.[33] No morphological or chemical alteration was found in the enamel surface in neutral or alkaline bleaching

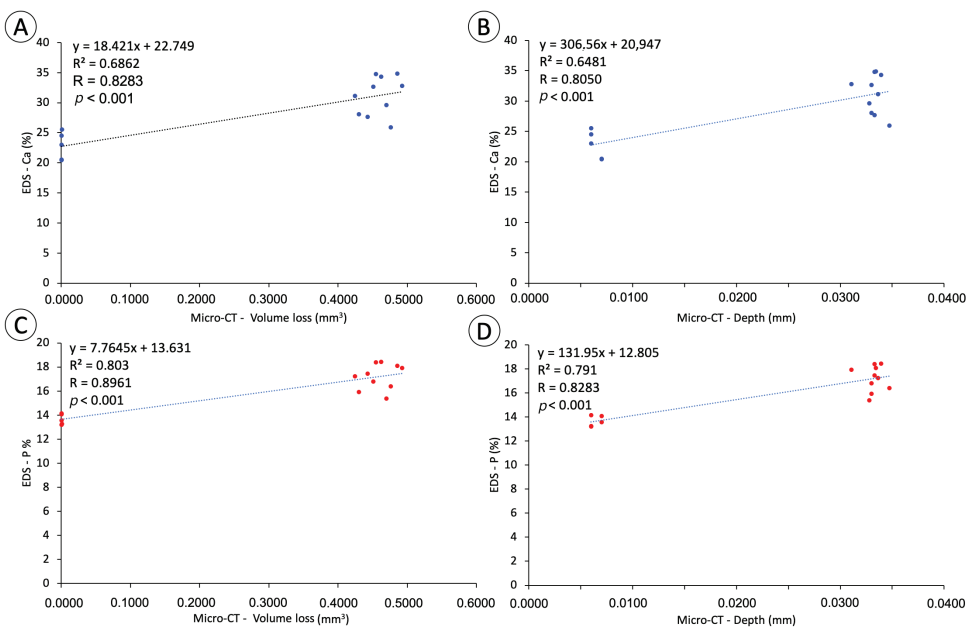


Figure 5. *Pearson's correlation between EDS and micro-CT data. Correlation between: (A) Ca percentage and volume of enamel tissue loss; (B) Ca percentage and depth of enamel tissue loss; (C) P percentage and volume of enamel tissue loss; and (D) P percentage and depth of enamel tissue loss; p < 0.001 for all tested correlations.*

solutions.[47] The tested HP35ca group had a basic pH (8.3), while the HP35wca group had an acidic pH (6.8), which may justify some of the changes in the enamel observed in the SEM images before and after bleaching. A representative HP35wca SEM image suggests the presence of pores and depressions on the enamel surface. The presence of Ca ions in the HP35ca group may have promoted crystal formation on the enamel surface.[48,49] However, the loss of enamel minerals was concentrated near the surface for groups treated with or without Ca, confirming the third hypothesis of our study. The application of silver nitrate to dental structures after bleaching with 35% HP gels in a previous study demonstrated moderate penetration of the enamel gel through the surface and subsurface prisms to a greater depth through the cracks and microcracks present in the enamel structure.[18] Transverse morphological observations with SEM in another study revealed morphological changes limited to a depth of less than 5 μm (0.005 mm) below the enamel surface with 30% HP gels for 30 or 180 min of immersion.[32] In this study, micro-CT showed significantly higher mineral loss for both bleached groups HP35wca group (0.033 mm) and HP35ca group (0.032 mm) than the control group (0.0064 mm).

The overlapping technique of micro-CT scans has proven to be a promising method to assess the loss of tooth structure caused by tooth whitening. A strong correlation was found between the EDS and micro-CT findings. The percentage of Ca and volume of enamel tissue loss (R=0.8283), the percentage of Ca and depth of enamel tissue loss (R=0.8050), the percentage of P and volume of enamel tissue loss (R=0.896), and the percentage of P and depth of enamel tissue loss (R=0.8283) are indicators of the efficiency of this method. Pilot tests also confirmed the accuracy of the technique by superimposing two different sweeps of the same specimen stored in distilled water, without treatment. There was no significant difference in the calculated tissue volume (Figure 1A), while significant mineral loss was detected in the treated samples and those stored in distilled water (Figure 1B).

In our study, AFM analysis showed no significant alteration in the surface roughness for all tested groups. Another study evaluated the effects of using in-office 35-40% HP bleaching gels with or without Ca or F on teeth and found that the 35% HP gel without Ca exhibited a slight increase in Ra, which was statistically different from the control.[50] On the other hand, another study that carried out bleaching using 20-45% CP gels and 9.5-38% HP gels reported no effect on the surface roughness.[51] A study carried out using 35% HP gels with and without calcium in the composition showed that the addition of calcium gluconate and the high and stable pH of the calcium-containing gel reduced tooth sensitivity in the study participants.[52] In our study, the presence of Ca in the bleaching gel showed no benefit; however, the study was designed using simulated artificial saliva containing Ca and P. For patients having a different saliva composition, the presence of Ca in the bleaching gel may prevent enamel demineralization.

One of the limitations of this study is the use of bovine teeth instead of human teeth. The majority of human teeth available for laboratory studies are extracted third molars. Because it is difficult to obtain human anterior incisors, the alternative bovine teeth were chosen. In this study, we opted to use bovine enamel due to its histological and structural similarity to human enamel.[53] Bovine enamel exhibits a reproducible surface, especially when its buccal surface is polished; hence, it can be safely used in a study that requires serial measurements.[54] Several related studies have used specimens of bovine teeth due to the difficulty in controlling the testing parameters with human teeth and the morphological variability of human teeth.[55] Other limitations are related to the use of gels with similar concentrations and classifications as well as different treatment protocols. The non-inclusion of other control groups indicated that saliva effects were not accounted for; therefore, the resolution limits of micro-CT could not be verified. This oversight did not consider the possible accumulation of debris resulting from the regularization and polishing of samples. In the present study, the control group, stored in saliva, did not have significantly different micro-CT, SEM, and AFM results. Therefore, the ions present in the formulation of the saliva did not interfere with the results. Artificial saliva was used to simulate the clinical environment and was replaced daily.[27] Studies *in situ* and *in vivo* have shown that the presence of saliva promotes remineralization on the enamel surface and does not make it porous.[56] Future studies are needed in order to test different resolutions of micro-CT; different devices with higher resolutions such as nano-CT can investigate different products with greater variability. A previous study evaluated whether there were significant long-term clinical benefits or side effects caused by the addition of ACP to CP16% whitening gel. The effects on tooth color, gingival health, and dentin hypersensitivity were evaluated after 90 and 180 days. After 180 days, the ACP group retained nearly 10% more of the original whitening treatment compared to that of the control. No other significant differences were found between groups. Tooth sensitivity, soft tissue health, and gingival health remained similar to baseline levels, proving the long-term safety of whitening treatment.[57]

Although we performed an *in vitro* evaluation using bovine teeth with artificial saliva, which does not accurately reproduce the clinical environment, these results have important clinical significance because they indicate that bleaching with a 35% HP gel can cause enamel demineralization regardless of the presence or absence of calcium in the gel. However, this demineralization did not change the surface roughness and the Ca and P levels on the surface of the treated enamel. Treatment with whitening gels is generally safe,[34] as long as it is performed by respecting the particularities of each patient and by a dentist. It is necessary to relativize the amount of mineral loss observed in this study with the clinical performance of bleaching procedures. Any adverse effects associated with use of the bleaching gel are temporary, easily controlled, and often disappear within minutes or hours of treatment.

## CONCLUSIONS

Within the limitations of this study design, the following conclusions were drawn.

1. The micro-CT method was able to assess the loss of enamel structure in terms of volume and depth with a high correlation with EDS results.
2. The addition of Ca to the bleaching gel composition was not able to prevent enamel surface demineralization; that was minimal and superficial.
3. The enamel underwent mineral loss primarily near the surface regardless of whether bleaching gel, with or without Ca, was used; however, no alteration in surface roughness was observed.

### Acknowledgments

This study was supported by CNPq grants 434598/2018-6 and FAPEMIG grants APQ-02105-18.

### Conflict of Interest

The authors have no financial interest in any of the companies or products mentioned in this article

*(Accepted 6 August 2021)*

## REFERENCES

1. McEvoy SA (1989) Chemical agents for removing intrinsic stains from vital teeth. II. Current techniques and their clinical application *Quintessence International* **20(6)** 379-384.
2. Sun G (2000) The role of lasers in cosmetic dentistry *Dental Clinics of North America* **44(4)** 831-850.
3. Zalking M, Arwas JR, Goldman A, & Roststein I (1996) Surface morphology changes in human enamel, dentin and cementum, following bleaching: A scanning electron microscopy study *Endodontics and Dental Traumatology* **12(2)** 82-88.
4. Hegedüs C, Bistey T, Flóra-Nagy E, Keszthelyi G, & Jenei A (1999) An atomic force microscopy study on the effect on the bleaching agents on enamel surface *Journal of Dentistry* **27(7)** 509-515.
5. Park HJ, Kwon TY, Nam SH, Kim HJ, Kim KH, & Kim YJ (2004) Changes in bovine enamel after treatment with 30% hydrogen peroxide bleaching agents *Dental Materials Journal* **23(4)** 517-521.
6. Borges AB, Torres CR, de Souza PA, Caneppele TM, Santos LF, & Magalhaes AC (2012) Bleaching gels containing calcium and fluoride: Effect on enamel erosion susceptibility *International Journal of Dentistry* 347848.
7. Torres CR, Crastechini E, Feitosa FA, Pucci CR, & Borges AB (2014) Influence of pH on the effectiveness of hydrogen peroxide whitening *Operative Dentistry* **39(6)** E261-E268.
8. Borges AB, Zanatta RF, Barros AC, Silva LC, Pucci CR, & Torres CR (2015) Effect of hydrogen peroxide concentration on enamel color and microhardness *Operative Dentistry* **40(1)** 96-101.
9. Dannacher JJ (2006) Catalytic bleach: Most valuable applications for smart oxidation chemistry *Journal of Molecular Catalysis A: Chemical* **251(1/2)** 159-176.
10. Markovic L, Jordan RA, Lakota N, & Gaengler P (2007) Micromorphology of enamel surface after vital tooth bleaching *Journal of Endodontics* **33(5)** 607-610.
11. Pinto CF, Oliveira R, Cavalli V, & Giannini M (2004) Peroxide bleaching agent effects on enamel surface microhardness, roughness and morphology *Brazilian Oral Research* **18(4)** 306-311.
12. Cavalli V, Arrais CA, Giannini M, & Ambrosano GM (2004) High-concentrated carbamide peroxide bleaching agent effects on enamel surface *Journal of Oral Rehabilitation* **31(2)** 155-159.
13. Fu B, Hoth-Hannig W, & Hannig M (2007) Effects of dental bleaching on micro- and nano-morphological alterations of the enamel surface *American Journal of Dentistry* **20(1)** 35-40.
14. Jiang T, Guo YR, Feng XW, Sa Y, Yang X, Wang M, Li P, & Wang YN (2018) Hydrogen peroxide might bleach natural dentin by oxidizing phosphoprotein *Journal of Dental Research* **97(12)** 1339-1345.
15. Cadenaro M, Breschi L, Nucci C, Antoniolli F, Visintini E, Prati C, Matis BA, & Di Lenarda R (2008) Effect of two in-office whitening agents on the enamel surface *in vivo*: A morphological and non-contact profilometric study *Operative Dentistry* **33(2)** 127-134.
16. Spalding M, Taveira LA, & De Assis GF (2003) Scanning electron microscopy study of dental enamel surface exposed to 35% hydrogen peroxide: Alone, with saliva, and with 10% carbamide peroxide *Journal of Esthetic and Restorative Dentistry* **15(3)** 154-164.
17. Unlü N, Cobankara FK, Altinöz C, & Ozer F (2004) Effect of home bleaching agents on the microhardness of human enamel and dentin *Journal of Oral Rehabilitation* **31(1)** 57-61.
18. Mendonça LC, Naves LZ, Garcia LFR, Correr-Sobrinho L, & Soares CJ, Quagliatto PS (2011) Permeability, roughness and topography of enamel after bleaching: tracking channels of penetration with silver nitrate *Brazilian Journal of Oral Sciences* **10** 1-6.

19. Soares DG, Ribeiro AP, Sacono NT, Loguércio AD, Hebling J, & Costa CA (2013) Mineral loss and morphological changes in dental enamel induced by a 16% carbamide peroxide bleaching gel *Brazilian Dental Journal* **24(5)** 517-521.

20. Attia ML, Cavalli V, do Espírito Santo AM, Martin AA, D'arce MB, Aguiar FH, Lovadino JR, do Rego MA, Cavalcanti AN, & Liporoni PC (2015) Effects of bleaching agents combined with regular and whitening toothpastes on surface roughness and mineral content of enamel *Photomedicine and Laser Surgery* **33(7)** 378-383.

21. Rauen CA, Filho JCC, Bittecourt BF, Gomes GM, Gomes JC, & Gomes OMM (2015) Effect of bleaching agents containing fluoride or calcium on enamel microhardness, roughness and permeability *Journal of Oral Science* **14(4)** 262-266.

22. Ferreira AF, Perez FM, Limeira Júnior F de A, de Moura M de F, & de Sousa FB (2016) Graded changes in enamel component volumes resulted from a short tooth bleaching procedure *Archives of Oral Biology* **65** 52-58.

23. Lee KH, Kim HI, Kim KH, & Kwon YH (2006) Mineral loss from bovine enamel by a 30% hydrogen peroxide solution *Journal of Oral Rehabilitation* **33(3)** 229-233.

24. Cavalli V, Rodrigues LK, Paes-Leme AF, Brancalion ML, Arruda MA, Berger SB, & Giannini M (2010) Effects of bleaching agents containing fluoride and calcium on human enamel *Quintessence International* **41(8)** e157-165.

25. Cavalli V, Rosa DAD, Silva DPD, Kury M, Liporoni PCS, Soares LES, & Martins AA (2018) Effects of experimental bleaching agents on the mineral content of sound and demineralized enamels *Journal of Applied Oral Science* **4(26)** e20170589.

26. Sasaki RT, Catelan A, Bertoldo E, Venâncio PC, Groppo FC, Ambrosano GM, Marchi GM, Lima DA, & Aguiar FH (2015) Effect of 7.5% hydrogen peroxide containing remineralizing agents on hardness, color change, roughness and micromorphology of human enamel *American Journal of Dentistry* **28(5)** 261-267.

27. Silveira J, Coutinho S, Marques D, Castro J, Mata A, Carvalho ML, & Pessanha S (2018) Raman spectroscopy analysis of dental enamel treated with whitening product - Influence of saliva in the remineralization *Spectrochimica Acta Part A, Molecular and Biomolecular Spectroscopy* **198** 145-149.

28. Coceska E, Gjorgievska E, Coleman NJ, Gabric D, Slipper IJ, Stevanovic M, & Nicholson JW (2016) Enamel alteration following tooth bleaching and remineralization *Journal of Microscopy* **262(3)** 232-244.

29. Gomes MN, Rodrigues FP, Silikas N, & Francci CE (2018) Micro-CT and FE-SEM enamel analyses of calcium-based agent application after bleaching *Clinical Oral Investigations* **22(2)** 961-970.

30. Oliveira LRS, Braga SSL, Bicalho AA, Ribeiro MTH, Price RB, & Soares CJ. (2018) Molar cusp deformation evaluated by micro-CT and enamel crack formation to compare incremental and bulk-filling techniques *Journal of Dentistry* **(74)** 71-78.

31. Efeoglu N, Wood D, & Efeoglu C (2005) Microcomputerised tomography evaluation of 10% carbamide peroxide applied to enamel *Journal of Dentistry* **33(7)** 561-567.

32. Efeoglu N, Wood DJ, & Efeoglu C (2007) Thirty-five percent carbamide peroxide application causes *in vitro* demineralization of enamel *Dental Materials* **23(7)** 900-904.

33. Ushigome T, Takemoto S, Hattori M, Yoshinari M, Kawada E, & Oda Y (2009) Influence of peroxide treatment on bovine enamel surface--cross-sectional analysis *Dental Materials Journal* **28(3)** 315-323.

34. Tanaka R, Shibata Y, Manabe A, & Miyazaki T (2010) Micro-structural integrity of dental enamel subjected to two tooth whitening regimes *Archives of Oral Biology* **55(4)** 300-308.

35. Oldoini G, Bruno A, Genovesi AM, & Parisi L (2018) Effects of amorphous calcium phosphate administration on dental sensitivity during in-office and at-home interventions *Dentistry Journal (Basel)* **6(4)** 52.

36. Llena C, Esteve I, & Forner L (2018) Effects of in-office bleaching on human enamel and dentin. Morphological and mineral changes *Annals of Anatomy* **217** 97-102.

37. Sulieman M, Addy M, Macdonald E, & Rees JS (2004) A safety study *in vitro* for the effects of an in-office bleaching system on the integrity of enamel and dentine *Journal of Dentistry* **32(7)** 581-90.

38. Torres C, Zanatta RF, Silva TJ, & Borges AB (2019) Effect of calcium and fluoride addition to hydrogen peroxide bleaching gel on tooth diffusion, color, and microhardness *Operative Dentistry* **44(4)** 424-432.

39. Furlan I, Bridi E, Amaral F, França F, Turssi C, & Alinhavo (2017) Effect of high- or low-concentration bleaching agents containing calcium and/or fluoride on enamel microhardness *General Dentistry* **65(3)** 66-70.

40. Alexandrino L, Gomes Y, Alves E, Costi H, Rogez H, & Silva C (2014) Effects of a bleaching agent with calcium on bovine enamel *European Journal of Dentistry* **8(3)** 320-325.

41. Moreira RF, Santos FP, Santos EA, Dos Santos RS, Dos Anjos MJ, & de Miranda MS (2017) Analysis of the chemical modification of dental enamel submitted to 35% hydrogen peroxide "in-office" whitening, with or without calcium *International Journal of Dentistry* **2017** 4646789.

42. SRM 2910b; Hydroxyapatite; National Institute of Standards and Technology, U.S. Department of Commerce: Gaithersburg, MD, USA, 2018.

43. Miculescu F, Luță C, Constantinescu AE, Maidaniuc A, Mocanu AC, Miculescu M, Voicu ȘI, & Ciocan LT (2020) Considerations and influencing parameters in EDS microanalysis of biogenic hydroxyapatite *Journal of Functional Biomaterials* **11(4)** 82.

44. Moura CW, Catelan A, Zanatta RF, Cavalcanti AN, Soares LE, Martins KV, & Liporoni PC (2019) Effects of bleaching using 10% carbamide peroxide with calcium or amorphous calcium phosphate on enamel mineral content and hardness *Acta Odontológica Latinoamericana* **32(3)** 126-132.

45. Balladares L, Alegría-Acevedo LF, Montenegro-Arana A, Arana-Gordillo LA, Pulido C, Salazar-Gracez MT, Reis A, & Loguercio AD (2019) Effects of pH and application technique of in-office bleaching gels on hydrogen peroxide penetration into the pulp chamber *Operative Dentistry* **44(6)** 659-667.

46. Pinto CF, Oliveira R, Cavalli V, & Giannini M (2004) Peroxide bleaching agents effects surface microhardness, roughness and morphology *Brazilian Oral Research* **18(4)** 306-311.

47. Xu B, Li Q, & Wang Y (2011) Effects of pH values of hydrogen peroxide bleaching agents on enamel surface properties *Operative Dentistry* **36(5)** 554-562.

48. Chen HP, Chang CH, Liu JK, Chuang SF, & Yang JY (2008) Effect of fluoride containing bleaching agents on enamel surface properties *Journal of Dentistry* **36(9)** 718-725.

49. Borges AB, Dantas RL, Caneppele TM, Borges AL, & Rocha Gomes Torres C (2013) Effect of remineralizing agents on the bleaching efficacy of gels *General Dentistry* **61(7)** 67-71.

50. Vieira I, Vieira-Junior WF, Pauli MC, Theobaldo JD, Aguiar FH, Lima DA, & Leonardi GR (2020) Effect of in-office bleaching gels with calcium or fluoride on color, roughness, and enamel microhardness *Journal of Clinical and Experimental Dentistry* **12(2)** e116-e122.

51. De Carvalho AC, de Souza TF, Liporoni PC, Pizi EC, Matuda LA, & Catelan A (2020) Effect of bleaching agents on hardness, surface roughness and color parameters of dental enamel *Journal of Clinical and Experimental Dentistry* **12(7)** e670-e675.

52. Kossatz S, Martins G, Loguercio AD, & Reis A (2020) Tooth sensitivity and bleaching effectiveness associated with use of a calcium-containing in-office bleaching gel *Journal American Dental Association* **143(12)** e81-e87.

53. Kwon YH, Huo MS, Kim KH, Kim SK, & Kim YJ (2002) Effects of hydrogen peroxide on the light reflectance and morphology of bovine enamel *Journal of Oral Rehabilitation* **29(5)** 473-477.

54. Manning MS & Edgar WM (1992) Intra-oral models for studying de- and remineralization in man: methodology and measurement *Journal of Dental Ressearch* **71(Spec Nº)** 895-900.

55. Lenherr P, Allgayer N, Weiger R, Filippi A, Attin T, & Krastl G. (2012) Tooth discoloration induced by endodontic materials: A laboratory study *International Endodontic Journal* **45(10)** 942-949.

56. Attin T, Schmidlin PR, Wegehaupt F, & Wiegand A (2009) Influence of study design on the impact of bleaching agents on dental enamel microhardness: A review *Dental Materials* **25(2)** 143-157.

57. Giniger M, Spaid M, MacDonald J, & Felix H (2005) A 180-day clinical investigation of the tooth whitening efficacy of a bleaching gel with added amorphous calcium phosphate *Journal of Clinical Dentistry* **16(1)** 11-6.