

Effects of Er:YAG laser irradiation and manipulation treatments on dentin components, part 2: energy-dispersive X-ray fluorescence spectrometry study

Luís Eduardo Silva Soares

Vale do Paraíba University, UNIVAP
Dental Materials and Operative Dentistry Department
School of Dentistry Research and Development Institute,
IP&D
Laboratory of Biomedical Vibrational Spectroscopy, LEVB
Av. Shishima Hifumi, 2911
12244-000 São José dos Campos, São Paulo
Brazil

Ana Maria do Espírito Santo

Vale do Paraíba University, UNIVAP
Research and Development Institute, IP&D
Laboratory of Biomedical Vibrational Spectroscopy, LEVB
Av. Shishima Hifumi, 2911
12244-000 São José dos Campos, São Paulo
Brazil

Aldo Brugnera Junior

Fátima Antônia Aparecida Zanin
Vale do Paraíba University, UNIVAP
Research and Development Institute, IP&D
Dental Laser Center
Av. Shishima Hifumi, 2911
12244-000, São José dos Campos, São Paulo
Brazil

Airton Abrahão Martin

Vale do Paraíba University, UNIVAP
Research and Development Institute, IP&D
Laboratory of Biomedical Vibrational Spectroscopy, LEVB
Av. Shishima Hifumi, 2911
12244-000 São José dos Campos, São Paulo
Brazil

1 Introduction

Etching is one of the most fundamental steps in the restoration of teeth by adhesion of composite resin. Acid etching on dentin with phosphoric acid is effective for removing the smear layer and also for enlarging the dentin tubules or decalcifying the superficial intertubular dentin.¹ The smear layer is the accumulated layer of mechanically polished debris. Etching is utilized as a pretreatment before adhesion of composite resin for removal of the smear layer. The technique exposes fresh tooth surfaces and facilitates direct contact of the adhesive and hydrogen bonding. Etching also enhances mechanical bonding through an interlocking effect in the roughened interface between the resin and the tooth.²

Address all correspondence to: Prof. Dr. Luís Eduardo Silva Soares, Universidade do Vale do Paraíba, UNIVAP, Instituto de Pesquisa e Desenvolvimento, IP&D, Laboratório de Espectroscopia Vibracional Biomédica, LEVB. Av. Shishima Hifumi, 2911, Urbanova, CEP 12244-000, São José dos Campos, SP, Brazil. Tel: +55 12 39471165; Fax: +55 12 39471165; E-mail: lsoares@univap.br

Abstract. The effects of laser etching, decontamination, and storage treatments on dentin components were studied by energy-dispersive X-ray fluorescence spectrometry (EDXRF). Thirty bovine incisors were prepared to expose the dentin surface and then divided into two main groups based upon the decontamination process and storage procedure: autoclaved (group A, $n=15$) or stored in aqueous thymol solution (group B, $n=15$). The surfaces of the dentin slices were schematically divided into four areas, with each one corresponding to a treatment subgroup. The specimens were either etched with phosphoric acid (control subgroup) or irradiated with erbium-doped yttrium-aluminum-garnet (Er:YAG) laser (subgroups: I-80 mJ, II-120 mJ, and III-180 mJ). Samples were analyzed by micro-EDXRF, yielding three spectra for each area (before and after treatment). Surface mappings covering an area of 80×60 points with steps of $20 \mu\text{m}$ were also performed on selected specimens. The amount of Ca and P in group A specimens decreased significantly ($P < 0.05$) after the acid etching and the Ca/P ratio increased ($P < 0.001$). Er:YAG laser-etching using lower laser energies did not produce significant changes in dentin components. The mapping data support the hypothesis that acid etching on dentin produced a more chemically homogeneous surface and thus a more favorable surface for the diffusion of adhesive monomers. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3103287]

Keywords: dentin; Er:YAG laser; manipulation treatment; energy-dispersive X-ray fluorescence spectrometry; dentin mapping.

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With the development of new adhesive materials, alternatives for dental structure conditioning have appeared as well. One of these innovations for substrate surface treatment is the use of erbium-doped yttrium-aluminum-garnet (Er:YAG) laser irradiation.³ Laser irradiation on dental hard tissue causes morphological and chemical changes. The extent of these changes is affected by the absorption characteristics of the tissues, so that the changes vary according to the type of laser and dental tissue.⁴

Dentists have yet to reach consensus regarding the correct parameters for Er:YAG laser use. The benefits of dentin laser etching prior to adhesive procedures are still controversial and the results of several studies are conflicting.⁵⁻⁹ Some studies report that the adhesion strength of laser-irradiated dentin is lower than that of nonirradiated dentin¹⁰⁻¹² due to the weaker mechanical properties of dentin after laser irradiation.¹² Some authors have also discussed the possible denaturation of col-

lagen fibrils.^{3,12} We modified the laser-irradiation parameters used in a previous investigation³ to determine whether it was possible to etch dentin without causing significant damage on inorganic and organic dentin components.

For the *in vitro* study, the chemical characteristics of teeth following specimen preparation must be carefully considered. An important factor that could affect the chemical content of teeth is the disinfection/sterilization method used to prepare the extracted teeth specimens.^{13,14} Several studies have evaluated the effects of sterilization methods on tooth tissues. As steam autoclaving is available in dental clinics; it is the easiest sterilization method.¹⁵ Despite its damage to collagen,¹³ autoclaving sterilization is still used in some studies.¹⁶ In other studies the methods used for tooth decontamination and storage treatment are not mentioned.⁶

The influence of tooth sterilization methods has been evaluated by analytical tools such as Fourier transform infrared spectroscopy (FTIR),¹⁷ bond strength study,¹⁸ and Raman spectroscopy.¹⁴ However, there are no previous reports of X-ray fluorescence microanalysis used to analyze sterilization effects on dentin components.

X-ray fluorescence is a nondestructive analytical technique based on the atomic emission of a given material's components when irradiated. The most common irradiation method utilizes an electron beam coupled with a scanning electron microscope (SEM).^{19,20} The interaction of the electron beam with the sample surface causes X-rays to be emitted by the atoms and ions in the top few micrometers of the sample surface. An electron is ejected from an inner shell of the atom; when an outer shell electron takes the place of the missing electron, energy is emitted in the form of X-ray radiation.²¹

Compositional changes due to laser irradiation were evaluated by atomic analytical studies in previous reports. Rohanizadeh et al. showed through X-ray fluorescence analysis of dentin that Nd:YAG laser irradiation reduced the calcium/phosphorus (Ca/P) ratio in comparison to nonirradiated specimens.⁴ Lin et al.¹⁹ used SEM-energy-dispersive X-ray spectroscopy (EDX) to evaluate Nd:YAG laser irradiation of dentin at a range from 150 mJ/pulse, 10 pps, 4 s, to 150 mJ/pulse, 30 pps, 4 s. The authors verified that the Ca/P ratio of the irradiated area increased proportionally with the elevation in irradiation energy.

This analytical tool has also been used to study the chemical composition of dentin after Er:YAG laser irradiation.^{20,22} Hossain et al.²⁰ showed through atomic analysis by SEM-EDX that the quantities of Ca and P (weight percent) were increased in cavity floors prepared by the erbium, chromium doped yttrium, scandium, gallium, garnet (Er,Cr:YSGG) laser. However, in these studies, the laser parameters employed to prepare the cavities used higher energies than those used to etch the dentin. Analysis was conducted using a combination of SEM and X-ray fluorescence. Therefore, to the best of our knowledge, X-ray fluorescence microanalysis and chemical mapping of the dentin surface after etching have not yet been completed.

The aim of this *in vitro* study was to investigate, using energy-dispersive X-ray fluorescence spectrometry (EDXRF), the effects of Er:YAG laser irradiation on dentin components. The effects of decontamination and storage processes on dentin components were also evaluated.

2 Materials and Methods

2.1 Specimen Preparation

This study was approved by the Ethics Committee of the University of Vale do Paraíba (A073/CEP/2007). Thirty bovine incisor teeth were obtained from bovine jaws. All specimens were stored in saline solution (Aster Produtos Médicos LTDA, Sorocaba, SP, Brazil) at 9 °C prior to use. After extraction, the remaining soft tissue was removed from the tooth surface with a dental scaler (7/8; Duflex, Rio de Janeiro, RJ, Brazil). The teeth were polished with a paste of pumice (S. S. White, Rio de Janeiro, RJ, Brazil) and water using a Robinson brush (Viking-KG Sorensen, Barueri, SP, Brazil) in a low-speed hand-piece (KaVo do Brasil SA, Joinville, SC, Brazil).^{3,14}

After the cleaning procedure, teeth were divided into two major groups according to the manipulation treatment (sterilization or storage). Group A consisted of fifteen teeth which were autoclaved at 121 °C for 15 min (Biodont-Alpax, Brazil) in a flask filled with sterile saline (Farmavale & Cia-LBS Laborasa Ind. Farm., Ltd., Brazil), closed tightly and stored at 9 °C.¹⁴ Group B was comprised of fifteen teeth stored in 0.1% thymol aqueous solution at 9 °C for one week. To prepare the dentin specimens, the teeth were washed for 24 h with filtered water to eliminate thymol residues.^{3,14}

The buccal enamel surface was removed by means of a water-cooled low-speed diamond disk at 250 rpm with a 100-g load. The surface was ground for 1 min with wet 600-grit silicon carbide paper at 150 rpm to expose the dentin layer and to produce a smooth surface.^{3,14} Ultrasonic cleaning (Maxiclean 1450, Merse, Campinas, SP, Brazil) with distilled water was performed for 5 min in order to remove excess debris and the smear layer. The specimens were then stored in saline solution in a refrigerator at 9 °C for one week.

2.2 Surface Treatment

A reference point was created with a diamond disk in the proximal enamel of the samples with a low-speed hand-piece (KaVo do Brasil SA, Joinville, SC, Brazil). The specimens' surfaces were schematically divided into four areas and each area of the sample received a different treatment, generating four subgroups as described in Table 1.

Specimens were removed from the saline solution. Laser irradiation was performed in noncontact mode by an Er:YAG laser (KaVo Key Laser II, Germany, $\lambda=2.94 \mu\text{m}$, beam diameter=1 mm) with the no. 2051 hand-piece at a focal distance of 12 mm, with cooling water spray (20 mL/min) and a total energy value of 12 J. Irradiation of the control group quadrant was avoided and a visual distance was maintained between the sides of each group. After the irradiation procedure, acid etching was performed in the control group area using 37% phosphoric acid gel (FGM, Brazil) for 15 s. The etched surface was then rinsed with an air-water spray for 15 s.

2.3 EDXRF Measurements

Semiquantitative elemental analyses of calcium (Ca) and phosphorus (P) were carried out by an energy-dispersive micro X-ray fluorescence spectrometer, model μ -EDX 1300, Shimadzu (Kyoto, Japan), equipped with a rhodium X-ray

Table 1 Description of group treatments.

Groups	Manipulation process
Group A	Autoclaving 121 °C (15 min, with pressure of 1.1 kgf/cm ²)
Group B	0.1% aqueous thymol solution (one week)
Subgroups	Surface treatments
Control group (CG)	37% phosphoric acid (15 s)
Group I (GI)	Er:YAG laser (80 mJ, 3 Hz, 12 J, 153 pulses)
Group II (GII)	Er:YAG laser (120 mJ, 3 Hz, 12 J, 103 pulses)
Group III (GIII)	Er:YAG laser (180 mJ, 3 Hz, 12 J, 70 pulses)

tube and a Si(Li) detector cooled by liquid nitrogen (N₂). The spectrometer was coupled to a computer system for data acquisition and processing. The voltage in the tube was set at 50 kV, with automatic adjustment of the current and incident beam diameter of 50 μm.

Three spectra from each area subgroup were collected before and after the treatments. Three points within the same line were selected so that the first point was located in the center of the irradiated area and two other points were selected 1 mm from the center. The measurements were performed with a count rate of 100 s per point (live time) and a dead time of 25%. The energy range of scans was 0.0–40.0 eV. The equipment was calibrated and adjusted using certified commercial stoichiometric hydroxyapatite (Aldrich, synthetic Ca₁₀(PO₄)₆(OH)₂, grade 99.999%, lot 10818HA) as a reference. The measurements were collected using classic parameters for Ca and P X-ray emission. The elements oxygen (O) and hydrogen (H) were used as a chemical balance. Energy calibration was performed using equipment-specific internal standards.

2.3.1 EDXRF mapping

Elemental distribution maps were performed for one specimen from each group (A and B) in order to determine the surface distribution of the elements Ca and P after treatment. For each subgroup, the maps were scanned using 50 kV in real-time scanning acquisition (1 s per point), covering an area of 80 × 60 points with steps of 20 μm and scan time of 320 min per area per group. The elemental mapping parameters were set similarly to those used for scanning measurements by points.

2.4 Statistical Analysis

The measurements of Ca, P, and the Ca/P ratio obtained by X-ray fluorescence were analyzed using InStat software (GraphPad Software, Inc., San Diego, CA, USA). Statistical analyses were performed using the difference between normal and treated values yielded by the X-ray fluorescence results.

Comparisons between decontamination and storage treatment groups subjected to the same type of surface etching were also performed. Those comparisons were performed using the Mann–Whitney U test. Comparisons among surface treatments in the same decontamination/storage treatment groups were performed using the one-way ANOVA test at a 95% confidence level and the Tukey–Kramer multiple comparisons test.

3 Results and Discussion

All experimental samples showed lower Ca/P weight ratios than those of stoichiometric hydroxyapatite, which is calculated as 2.15. This observation indicates that the dental hydroxyapatite (as biological hard tissue) is nonstoichiometric, as reported in the literature.²³ Statistical comparisons of Ca and P content as well as Ca/P weight ratio were performed for normal and treated specimens in all experimental groups (Table 2, horizontal comparisons). Statistical analysis showed that the amounts of Ca (Ca weight %) and P (P weight %) decreased significantly in the autoclaved specimens and in those treated with phosphoric acid (A_CG), as compared with the autoclaved untreated specimens (Table 3, rows 1 and 2). Therefore, for this group the weight ratio (Ca/P) increased significantly (Table 3, row 4). However, no significant differences were found between the quantities of Ca and P, in gram atom percentage, before and after treatments in the specimens stored in thymol and treated with phosphoric acid (B_CG) (P > 0.05).

Lower quantities of phosphorus were found in the acid-etched specimens that had been autoclaved than in those treated with thymol (Table 3, row 3). The Ca/P weight ratio increased significantly for the acid-etched specimens that had previously been autoclaved as compared to those treated with thymol (Table 3, row 5).

Differences among etching treatments were found only in the autoclaved specimens, as shown by the Tukey–Kramer test. Calcium levels were lower in the acid-etched specimens than in those subjected to laser irradiation of 120 mJ (P < 0.01) (Table 2, column 3). Phosphorus levels were lower in the acid-etched specimens than in those subjected to laser irradiation of 80 mJ (P < 0.05), 120 mJ (P < 0.001), and 180 mJ (P < 0.01) (Table 2, column 5). The Ca/P weight ratio was higher in the acid-etched specimens than in those that were laser-irradiated (P < 0.01) (Table 2, column 7).

The results of the elemental analysis in the present study showed that the amounts of calcium (Ca weight %) and phosphorus (P weight %) were reduced in the autoclaved specimens treated with 37% phosphoric acid (15 s). The calcium-to-phosphorus weight ratio also increased significantly. Acid etching dissolved peritubular and intertubular dentin, significantly removing chemical compounds containing Ca and P. Heat and pressure effects (121 °C and pressure of 1.1 kgf/cm²) likely explain the intense effect observed in autoclaved specimens as opposed to those treated with thymol. For this group (A_CG), the results are in agreement with previous studies obtained by dispersive Raman spectroscopy.¹⁴ White et al.¹⁷ used FTIR spectroscopy to investigate the sterilization effects of steam autoclaving on whole tooth roots. They found that this treatment induced a loss of mineral and collagen components (as shown by reduction in amine and

Table 2 Mean and standard deviations ($n=15$) of calcium and phosphorus percentages and Ca/P weight ratios obtained by X-ray fluorescence, before (normal) and after (treated) dentin surface treatment. *

Group	Calcium (wt%)		Phosphorus (wt%)		Ca/P	
	Normal	Treated	Normal	Treated	Normal	Treated
A_CG	23.19 (4.06)	19.43 (3.78)A	11.71 (1.84)	8.88 (2.30) A	1.98 (0.08)	2.25 (0.28) A
A_GI	24.04 (3.54)	22.40 (4.22)	12.28 (1.42)	11.63 (1.87) B	1.95 (0.07)	1.90 (0.12) B
A_GII	24.00 (3.83)	25.61 (5.15) B	12.25 (1.63)	12.80 (2.16) B	1.95 (0.07)	1.98 (0.13) B
A_GIII	23.69 (3.87)	24.01 (5.87)	12.07 (1.59)	12.10 (2.82) B	1.96 (0.08)	1.96 (0.17) B
B_CG	23.05 (2.75)	21.49 (2.50)	11.71 (1.16)	10.80 (1.46)	1.97 (0.07)	2.00 (0.10)
B_GI	22.95 (2.55)	23.07 (5.61)	11.68 (1.11)	11.94 (2.74)	1.96 (0.07)	1.93 (0.14)
B_GII	23.88 (3.04)	24.77 (6.52)	12.24 (1.25)	12.41 (2.86)	1.95 (0.06)	1.97 (0.20)
B_GIII	24.69 (4.18)	23.73 (7.43)	12.05 (1.67)	12.15 (3.41)	1.96 (0.07)	1.95 (0.26)

* Tukey test showed a difference for values indicated with letters ($P<0.05$).

phosphate peaks) from the sample surface. Notably, dentin tissue releases its organic material at temperatures ranging from 100 to 400 °C and begins to lose carbonate at 100 °C.²⁴

When acid-etching treatment with phosphoric acid was compared to Er:YAG laser irradiation in specimens that had previously been autoclaved, it was observed that the acid removed more calcium than laser treatment with 120 mJ ($P<0.01$). Acid removed more phosphorus than laser irradiation with 80 mJ ($P<0.05$), 120 mJ ($P<0.001$), and 180 mJ ($P<0.01$). The Ca/P weight ratio also increased significantly in the acid-treated specimens, as compared to those that were laser-treated. This trend was not observed for the thymol-treated specimens, further demonstrating the impact of high-temperature autoclave treatment. Our study also showed that the amounts of Ca and P (weight %) increased in dentin irradiated with an Er:YAG laser. This result probably stems from the evaporation of organic components.^{4,20}

With regard to Ca and P content, the mapping of treated dentin showed an elemental uniformity in the control group (CG) specimens, when comparing groups A and B [Figs. 1(a) and 2(a) (Ca) and 3(a) and 4(a) (P)]. Isolated regions with blue spots in the GI and GII areas were observed for both decontamination treatments (groups A and B). This is shown in Figs. 1(b), 2(b), 1(c), and 2(c) (Ca) and 3(b), 4(b), 3(c), and 4(c), (P) and indicates lower calcium and phosphorus concentration than that found in the CG area. A significant demineralization (dark blue areas), as shown in Figs. 1(d) (Ca) and 3(d) (P), was found in the GIII_A area when compared to the GIII_B area, as shown in Figs. 2(d) (Ca) and 4(d) (P).

Elemental mapping by EDXRF showed that the autoclaving treatment caused greater demineralization after acid etching and laser irradiation than treatment with thymol solution. Acid etching produced demineralization that was chemically homogeneous compared to that induced by laser treatment, as shown by the yellow areas in the mapping. Surface distributions of calcium and phosphorus were more homogeneous in the acid-treated specimens of the group decontaminated with thymol than in the autoclaved samples. Laser treatment produced irregular patterns of Ca and P distribution throughout the dentin surface; 180 mJ of laser energy produced higher demineralization in autoclaved specimens compared to lower laser energies and compared to the specimens treated with thymol. Previous reports of Er:YAG laser etching on dentin, subjected to SEM analysis, showed a scaly, flaky surface of

Table 3 Results of Mann–Whitney unpaired-sample test.

Content	Group comparison	P-value
Calcium	A_CG normal versus A_CG treated	0.0145
	A_CG treated versus B_CG treated	0.0145
Phosphorus	A_CG normal versus A_CG treated	0.0012
	A_CG treated versus B_CG treated	0.0145
Ca/P ratio	A_CG normal versus A_CG treated	0.0005
	A_CG treated versus B_CG treated	0.0012

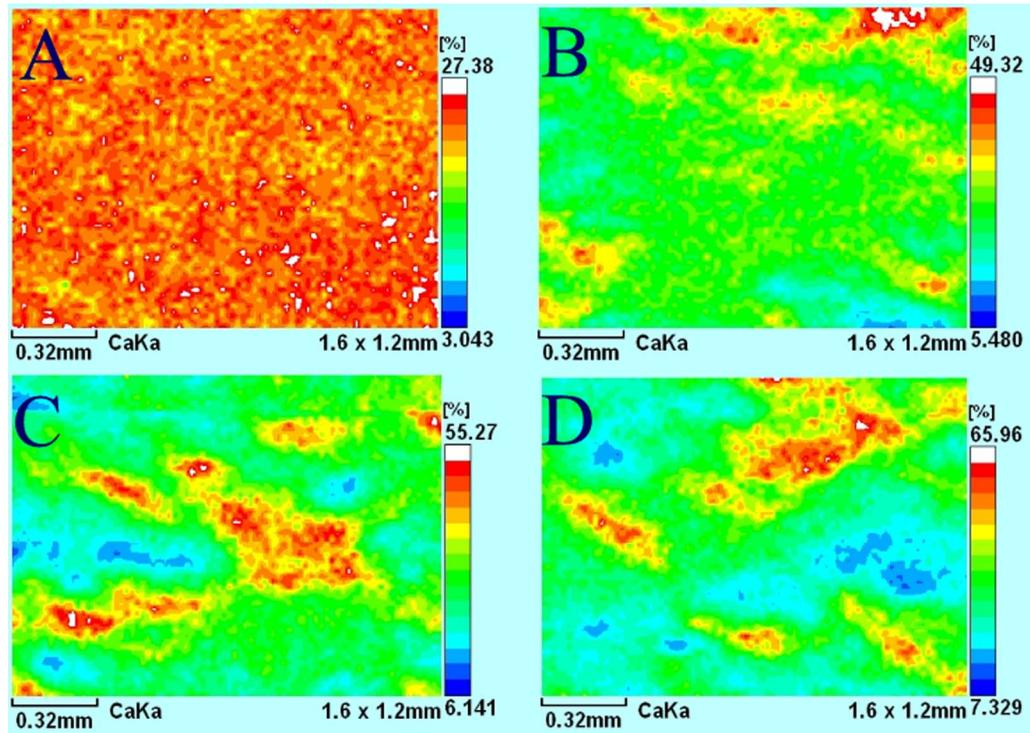


Fig. 1 Calcium mapping results obtained by X-ray fluorescence of specimens in group A with each subgroup of treatment: (a) CG, (b) GI, (c) GII, and (d) GIII.

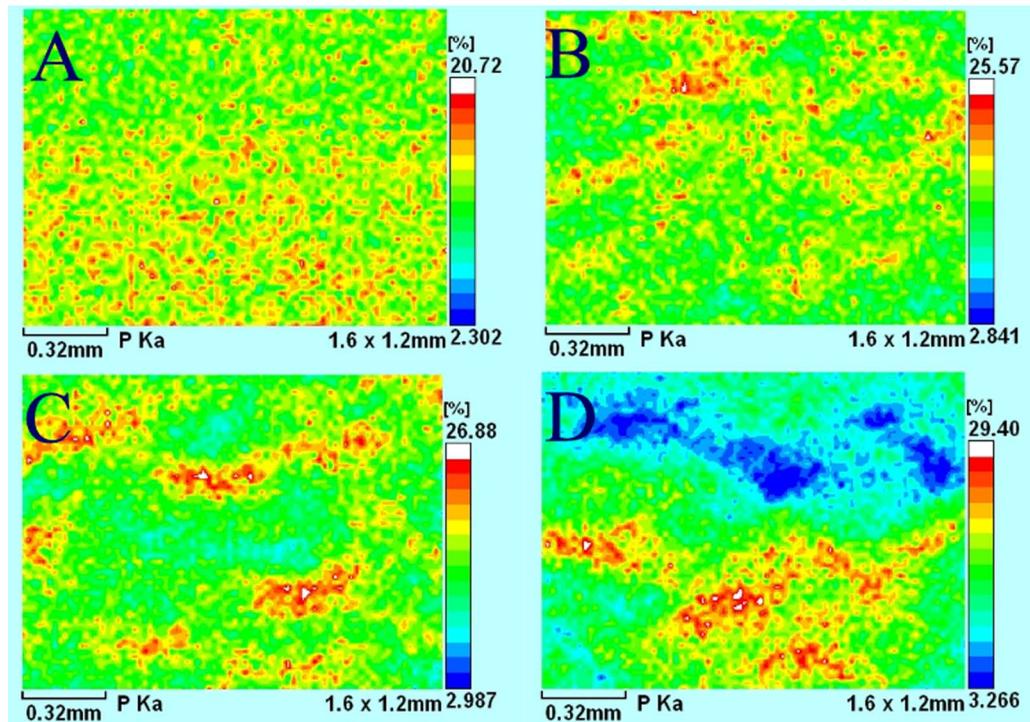


Fig. 2 Calcium mapping results obtained by X-ray fluorescence of specimens in group B with each subgroup of treatment: (a) CG, (b) GI, (c) GII, and (d) GIII.

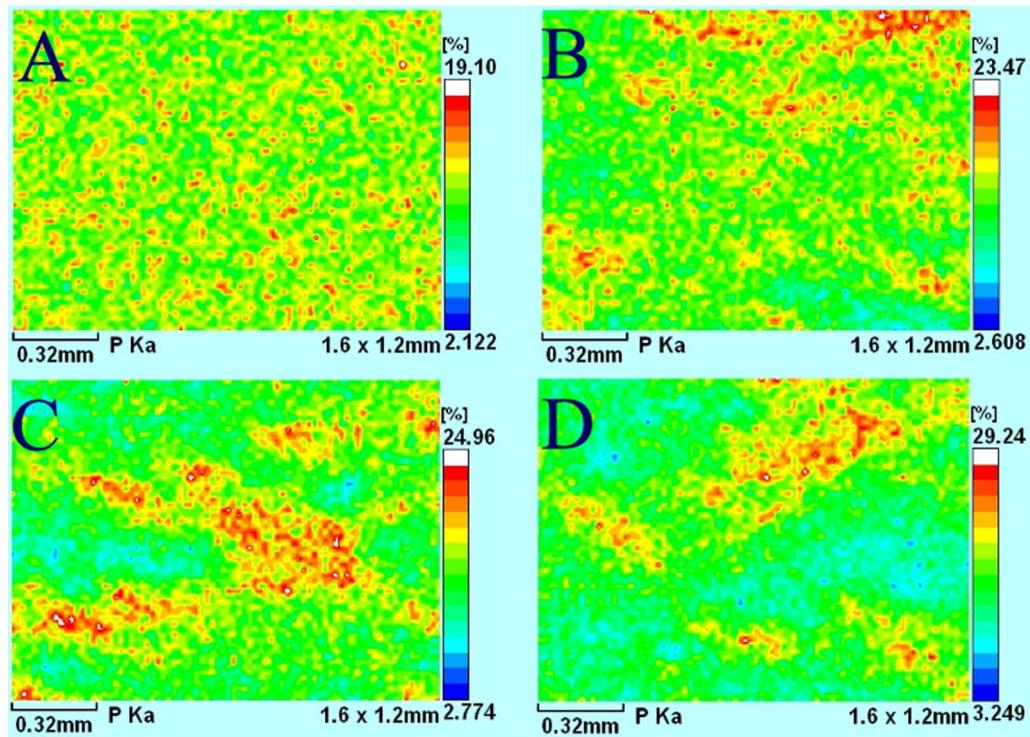


Fig. 3 Phosphorus mapping results obtained by X-ray fluorescence of specimens in group A with each subgroup of treatment: (a) CG, (b) GI, (c) GII, and (d) GIII.

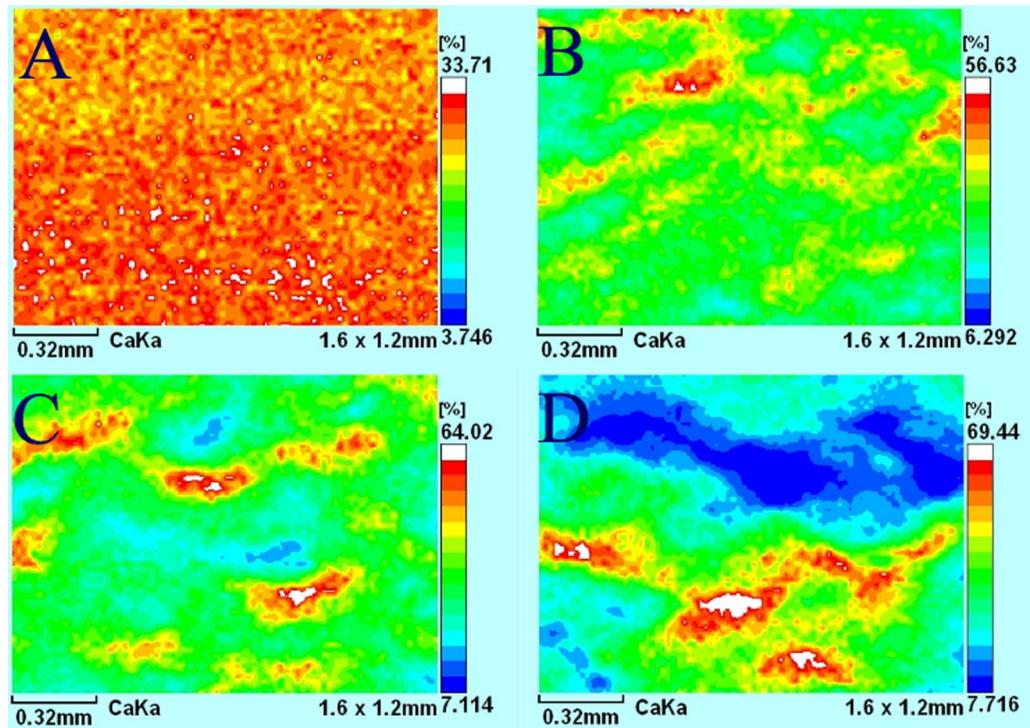


Fig. 4 Calcium mapping results obtained by X-ray fluorescence of specimens in group B with each subgroup of treatment: (a) CG, (b) GI, (c) GII, and (d) GIII.

highly irregular shape with partially opened dentin tubules. In contrast, acid-etched dentin retained a smoother surface with opened dentin tubules.^{3,5,8,9} Our results are in agreement with those previous reports; we found irregular patterns of elemental chemical distribution throughout the dentin surface after Er:YAG laser irradiation.

The surface produced by laser etching is also acid-resistant. According to Secilmis et al.,²⁵ laser irradiation of dental hard tissues modifies the Ca/P ratio and leads to the formation of a more stable and less acid-soluble structure. The alterations in chemical composition may affect the permeability and solubility characteristics of dentin, as well as the adhesion of dental materials to dentin.

In summary, EDXRF analysis proved an adequate research tool to study the changes that occurred in dentin following decontamination or storage and laser or chemical etching. Sample preparation is fairly simple, since it does not require a specific tooth size or dehydration procedure. The measurements can be performed under normal atmospheric conditions, without the need for high vacuum. Finally, because this technique is nondestructive, samples can be used in multiple analyses.

4 Conclusions

The results of EDXRF measurements showed that the amounts of Ca (Ca weight percent) and P (P weight percent) decreased significantly in the specimens that were autoclaved and treated with phosphoric acid (A₁CG). The results suggest that thymol storage treatment is advised for *in vitro* studies. Er:YAG laser etching at lower laser energies did not produce significant changes in dentin components. The EDXRF mapping data support the hypothesis that the acid etching on dentin produced a more chemically homogeneous surface, yielding a more favorable surface for the diffusion of adhesive monomers. These findings elucidate the chemical structure of dentin after laser etching, facilitating the development of effective guidelines for laser use.

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