

Dentine surface modification and remineralization induced by bioactive toothpastes

Maria Giovanna Gandolfi¹ | Paola Taddei²  | Fausto Zamparini¹  | Livia Ottolenghi³ | Antonella Polimeni³ | Carlo Prati⁴

¹Laboratory of Green Biomaterials and Oral Pathology, School of Dentistry, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

²Biochemistry Unit, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

³Department of Oral and Maxillofacial Sciences, Sapienza University of Rome, Rome, Italy

⁴Endodontic Clinical Section, School of Dentistry, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

Correspondence

Maria Giovanna Gandolfi, Laboratory of Green Biomaterials and Oral Pathology, School of Dentistry, Department of Biomedical and Neuromotor Sciences, University of Bologna, 40125 Bologna, Italy.

Email: mgiovanna.gandolfi@unibo.it

Abstract

Objective: In this study, dentine surface was analysed through Environmental-scanning-electron-microscopy (ESEM) with energy-dispersive-X-ray-spectrometry (EDX) and Fourier-transform-infrared-spectroscopy (FTIR) with attenuated total-reflectance (ATR) to assess the morpho-chemical changes and variations in mineralization degree after demineralizing treatment, after five toothpastes application (HA & Citrate toothpaste, Zinc-HA toothpaste, Calcium Sodium Phosphosilicate toothpaste, Arginine & Calcium carbonate toothpaste, Colgate-Triple-Action, and Control toothpaste), after soaking in artificial saliva and after citric acid attack.

Methods: Ca/P, Ca/N and P/N ratios were calculated from EDX atomic data to evaluate the mineralization degree of dentine surface. The IR calcium phosphate (CaP)/collagen and carbonate/collagen ratios has been evaluated to assess the remineralization changes in dentine; the carbonate/collagen IR ratio was calculated to identify the nucleation of B-type-carbonated apatite and calcium carbonate.

Results: ESEM-EDX and ATR-FTIR showed residuals of toothpastes after the treatments in all cases, with a general increase in the mineralization degree after soaking in artificial saliva and a decrease after acid attack.

Treatment with Arginine & Calcium carbonate toothpaste showed the highest Ca/P value after treatment (Ca/P 1.62) and acid attack (Ca/P 1.5) in confirmation, IR showed the highest amount of carbonate after treatment and soaking in artificial saliva.

Arginine and calcium carbonate toothpaste and HA and citrate toothpaste remained to a higher extent on the dentine surface and revealed a higher remineralization activity. These formulations showed higher resistance to demineralization attack, as demonstrated by a higher $I_{CaP}/I_{Amide II}$ intensity ratio than those obtained after EDTA treatment.

Conclusions: Toothpastes that remained to a higher extent on dentine surface (arginine and calcium carbonate toothpaste in particular) were more able to promote remineralization. The formed calcium phosphate (CaPs) phase was intimately bound to dentine rather than a simple deposit.

KEYWORDS

Arginine, calcium sodium phosphosilicate, dentine, mineralization degree, sodium monofluorophosphate, Zinc nanohydroxyapatite

1 | INTRODUCTION

Dentinal tissue may be exposed to the oral environment for different reasons such as gingival recession, enamel wear, erosion (acid etching from dietary acids or gastroesophageal reflux), dissolution (acids produced by sugars metabolism of bacteria), abrasion or attrition (by tooth grinding or bruxism).^{1–3} Exposed dentine needs treatments able to avoid its demineralization and or to sustain its remineralization.^{4,5} In the last decades, different compounds and bioactive molecules have been introduced in therapeutical toothpaste for this purpose.^{6–8}

Hydroxyapatite and nanohydroxyapatite minerals have been synthesized as active ingredients for toothpastes due to their closeness to the mineral components of dentine and dental enamel.^{9–12} Toothpaste formulations containing hydroxyapatite,¹³ potassium citrate and hydroxyapatite,^{10–12} nanohydroxyapatite¹⁴ or zinc nanohydroxyapatite⁸ have been proposed in the last years.

Calcium sodium phosphosilicate (as 45S5 Bioglass)¹⁵ have been proposed for dentine treatment in the last decades showing protective effects due to bioactivity (apatite nucleation ability).^{16–18} A new calcium sodium phosphosilicate consisted of 45% SiO₂, 24.5% Na₂O, 24.5% CaPO and 6% P₂O₅ in weight,^{19,20} possessed controllable biodegradability^{19,20} and high reactivity, being able to immediately release sodium and calcium ions when come in contact with water or saliva.²¹

Arginine has been proposed as active ingredient inside a number of dentinal treatments^{22–27} such in formulations containing arginine 1.5%wt and calcium carbonate,^{22,23} in formulation containing 8%wt arginine²⁴ and in association to fluoride at low dosage.^{25,26} The latest formulation includes 8%wt arginine, natural calcium carbonate (particle size 3–7 microns), precipitated calcium carbonate (particle size 1–5 microns) and fluoride at low dosage.²⁷ Interestingly, arginine – found in saliva in micromolar concentrations²⁸ – represents a major source of alkali in the mouth: ammonia is produced by the arginine deiminase system (detected in several bacteria that colonize the teeth and oral soft tissues). The ammonia production increases the oral pH to a neutral or slightly basic pH that prevent cariogenic microflora growth²⁹ and favours the nucleation of apatite.^{30,31}

Environmental scanning electron microscopy (ESEM) with energy dispersive X-ray spectrometry (EDX) and Fourier-transform infrared spectroscopy (FTIR) with attenuated total reflectance (ATR) techniques have been used to analyse the changes in mineralization degree and the collagen modifications after chemical treatments.^{32–36}

ESEM enables the examination of soft, hydrated, unfixed and uncoated surfaces, i.e., bulk biological tissues in their “natural” state providing a great advantage for the studies in the biological field.^{37,38} EDX microprobe allowed to identify different mineralization degree areas.^{33–35,39} The evaluation of Ca/N and P/N ratios proved to be a useful method to assess the degree of mineralization of the organic matrix of dentine.^{33–35,39–41}

In mineralized biological systems, the IR spectrum gather the contribution from collagen and apatite phases. The intensities of IR absorption bands provide quantitative information about the sample composition, depending on the nature of the chemical bonds, their strength and environment.^{33,36,42} Previous investigations have used IR spectroscopy to study dentine collagen mineralization^{33,36,42–44} and useful spectroscopic markers have been identified to determine the changes in apatite/collagen ratio.^{33,36,41}

The aim of this study was to investigate surface modification and remineralization of demineralized dentine after treatment with toothpastes containing different active principles (namely, zinc hydroxyapatite, calcium sodium phosphosilicate, hydroxyapatite and potassium citrate, arginine and calcium carbonate), after soaking in artificial saliva and after citric acid attack.

2 | MATERIALS AND METHODS

The study has been carried out and prepared following the CRIS⁴⁵ and PRILE⁴⁶ guidelines for in vitro studies.

2.1 | Tested toothpastes

Toothpastes containing different mineral materials or active principles were tested (Table 1), including a toothpaste containing hydroxyapatite and citrates (HA and citrate toothpaste),¹² a toothpaste containing zinc hydroxyapatite (Zinc-HA toothpaste),⁹ a toothpaste containing calcium sodium phosphosilicate (calcium sodium phosphosilicate toothpaste)²⁰ and a toothpaste containing 8% arginine and calcium carbonate (arginine and calcium carbonate toothpaste)²⁷ and a toothpaste formulation with no stated active principles towards mineral content in dentine (control toothpaste).

2.2 | Measurement of the toothpastes pH

The pH of the toothpastes was measured using a selective temperature-compensated electrode (Sen Tix Sur WTW) connected to a multi-parameter laboratory meter (inoLab 750 WTW) that was previously calibrated with standard solutions.

2.3 | Dentine crown segments preparation

Human caries free molars, extracted for surgical reasons, were used to prepare crown segments (2.5 ± 0.5 mm thick). The segments were

TABLE 1 List of commercial names, LOT and composition of toothpastes used in the present study.

Name	LOT	Composition
Mentadent Maximum Protection Sensitive (Church and Dwight Trenton, New Jersey, USA)	41788CC	Water, hydrogenated starch hydrolysate, hydrated silica, potassium citrate, hydroxyapatite, ¹¹ PEG-32, zinc citrate, sodium lauryl sulfate, sodium monofluorophosphate (1450 ppm F), aroma, trisodium phosphate, cellulose gum, mica, sodium hydroxide, sodium saccharin, tocopheryl acetate, limonene, CI 73360 (red pigment), CI 77891 (TiO ₂ pigment)
Biorepair Fast Sensitive Repair (Coswell, Funo, Bologna, IT)	426,251,043	Water, zinc hydroxyapatite (microRepair®), ⁹ glycerin, sorbitol, silica, PEG-32, cellulose gum, aroma, sodium myristoyl sarcosinate, sodium methyl cocoyl taurate, sodium saccharin, citric acid, phenoxyethanol, benzyl alcohol, sodium benzoate
Sensodyne Repair and Protect (Glaxo SmithKline Brentford, London, GB)	242B G2	Glycerin, PEG-8, hydraulic silica, calcium sodium phosphosilicate (NOVAMIN®), ²⁰ cocamidopropyl betaine, sodium methyl cocoyl taurate, aroma, titanium dioxide, Carbomer, potassium acesulfame, limonene, sodium monofluorophosphate (1450 ppm F), sodium saccharin
Elmex sensitive professional (GABA International AG, Lorrach, Germany)	426,251,043	Arginine (ProArgin), ²⁵ calcium carbonate, water, sorbitol, sodium lauryl sulfate, sodium monofluorophosphate (1450 ppm F), aroma, sodium silicate, cellulose gum, sodium bicarbonate, titanium dioxide, potassium acesulfame, Xanthan gum, sucralose
Colgate Triple Action (Colgate-Palmolive Company New York, USA)	4140PL1136	Water, sorbitol, hydrated silica, glycerin, sodium lauryl sulfate, PEG-12, aroma, cellulose gum, sodium fluoride (1450 ppm F), saccharin, CI 74160 (blue pigment), CI 74260 (green pigment) and CI 77891 (TiO ₂ pigment)

obtained by a root-cutting 1 mm beneath the cementum enamel junction and by an occlusal enamel-cutting 1.0–1.5 mm above the pulp cornu using a low-speed water-cooled diamond saw (Remet) under constant water cooling.^{16,33,40}

The pulp tissue was removed with small forceps, without altering the pre-dentine surface or the inner part of the pulp chamber.

2.4 | Treatments application

The samples were randomly divided in six different groups (one for each toothpaste and one control group). Experiments were carried out in triplicate.

The upper dentine surface of each specimen was sanded with wet 600-grit SiC abrasive paper for 30s to create a standard flat dentine surface covered by a standardized smear layer.

Each material was applied on moist dentine surface (the surface appeared shiny and reflective to prevent dehydration) previously treated for 10min with EDTA 10% (Ogna) and then rinsed with 5 mL of distilled water.

Each toothpaste was applied on dentine surface by brushing 0.20g of toothpaste for 60s using a soft brush and then rinsed with 5 mL of distilled water.

In the control group no toothpaste was applied but only distilled water (water-treated dentine group).

The treated dentine samples were then immersed in artificial saliva (HBSS, Hank's Balanced Salt Solution without phenol red) for 24 h at 37°C. Artificial saliva composition was: CaCl₂ (0.7 mM), MgCl₂ · 6H₂O (0.2 mM), KH₂PO₄ (4.0 mM), KCl (30 mM), NaN₃ (0.3 mM) and HEPES buffer (20 mM).

As final step, samples were irrigated with 1 mL of 0.1% citric acid solution (1 irrigation every 30s) for 2 min and then rinsed with 5 mL of deionized water.

2.5 | Environmental scanning electron (ESEM) microscopy with energy dispersive X-ray (EDX) analysis

Dentine slices 1.0 ± 0.1 mm thick were prepared using a low-speed water-cooled diamond saw (Remet) and analysed using an environmental scanning electron microscope (ESEM; Zeiss EVO 50; Carl Zeiss) connected to a secondary electron detector for energy dispersive X-ray analysis (EDX; Oxford INCA 350 EDS) using computer-controlled software (Inca Energy Version 18).

The samples were placed directly onto the ESEM stub and examined without previous coating or dehydration.

Dentine sections were examined at low vacuum (100 Pascal), accelerating voltage of 20 kV, working distance 8.5 mm, 0.5 wt% detection level, 133 eV resolution, amplification time 100 microseconds, measuring time: 600 s for element mapping and 60 s for spectra.

EDX microchemical analysis (elemental X-ray microanalysis) was carried out at random in areas of approx. 50 × 50 microns to evaluate the relative element content.

Microanalysis (weight % and atomic %) with ZAF correction method was performed in full frame and spot mode to analyse entire areas or specific points, respectively. EDX spectra refer to the whole image and the EDX elements percentages are an average over the whole image.

The Ca/P, Ca/N and P/N ratios were calculated from the obtained atomic data to evaluate the degree of mineralization of the dentine surface at the different experimental stages.^{34,35}

2.6 | ATR-FTIR spectroscopy

The same dentine samples analysed by EDX were submitted to attenuated total reflectance - Fourier transform infrared (ATR-FTIR) surface analyses by a Bruker Alpha (Bruker Optik GmbH) FT-IR spectrometer

in ATR mode with diamond inner reflection element (IRE). Three spectra at least were recorded on each sample at each step of the study. Under the used experimental conditions, the penetration into the sample thickness was about 2 microns. Average spectra were shown.

The calcium phosphate (CaP)/collagen IR ratio was evaluated through the $I_{\text{CaP}}/I_{\text{Amide II}}$ IR intensity ratio, where I_{CaP} and $I_{\text{Amide II}}$ were the intensities calculated as peak heights of the $\nu_3 \text{PO}_4^{3-}$ antisymmetric stretching band (around 1000 cm^{-1}) and Amide II of collagen (at about 1550 cm^{-1}), respectively.

The carbonate/collagen IR ratio was evaluated through the $I_{1410}/I_{\text{Amide II}}$ IR intensity ratio, where I_{1410} was the intensity calculated as peak height of the $\nu_3 \text{CO}_3^{2-}$ antisymmetric stretching at about 1410 cm^{-1} .^{47,48} $I_{\text{CaP}}/I_{\text{Amide II}}$ and $I_{1410}/I_{\text{Amide II}}$ values were reported as average \pm standard deviation.

The reduction of the $I_{\text{CaP}}/I_{\text{Amide II}}$ and $I_{1410}/I_{\text{Amide II}}$ ratios indicates the occurrence of demineralization, with the weakening of the apatite and carbonate bands compared to those of collagen, while an increase of the ratios indicates remineralization. The shifts of the collagen bands (Amide I at about 1635 cm^{-1} , Amide II, and Amide III at about 1240 cm^{-1}) allow to evaluate the collagen rearrangements.

The collagen $I_{\text{Amide II}}/I_{\text{Amide I}}$ IR ratio (where $I_{\text{Amide I}}$ was the intensity calculated as peak height of Amide I) was calculated, according to Du et al.,⁴⁹ to investigate the possible loss of interactions with Ca^{2+} ions and changes in hydrogen bonding.

ATR-FTIR analyses have been performed also on the tested toothpastes to have reference on their components and their IR bands.

2.7 | Statistical analysis

Ca/N, P/N, Ca/P atomic ratios and IR ratios were analysed using a two-way ANOVA and Student Newman Keuls *post-hoc* test to search for any statistical difference among treatments (toothpaste application) and times. In all cases, p value was previously set at 0.05.

3 | RESULTS

3.1 | EDTA treatment

Micromorphological ESEM-EDX analysis of dentine surfaces treated with EDTA showed the constitutive elements of dentine (Figures 1,3,5,7 and S1, S3). ESEM-EDX analyses of Water treated dentine (control group) is reported in Figure S3 A demineralization occurred, as revealed by the significant decrease ($p < 0.05$) of Ca/N and P/N ratios (Tables 2, 3). IR bands assignable to the B-type carbonated apatite phase⁴⁹ significantly decreased in intensity, but did not disappear (Figures 2,4,6,8 and S2). This qualitative finding is confirmed by the trend of the $I_{\text{CaP}}/I_{\text{Amide II}}$ and $I_{1410}/I_{\text{Amide II}}$ IR intensity ratios (Figures 9A,B), which significantly decreased upon EDTA treatment ($p < 0.05$).

Upon the partial loss of apatite phase, Amide I (at about 1635 cm^{-1}) and Amide III (at about 1240 cm^{-1}) did not undergo significant

wavenumber shifts, while the spectral profile of Amide II slightly changed in some samples. The relative intensity of the Amide II band increased with respect to that of Amide I and the $I_{\text{Amide II}}/I_{\text{Amide I}}$ increased ($p < 0.05$, Figure 9C). The $\nu_3 \text{PO}_4^{3-}$ band of the apatite phase underwent a narrowing and, in some samples, shifted to lower wavenumbers.

3.2 | HA and Citrate toothpaste

The pH of the toothpaste was 7.24 ± 0.03 .

After toothpaste application (T0), ESEM showed the presence of residual toothpaste with well-recognizable precipitates and some identifiable dentine tubules (Figure 1G). The mineralization degree showed a significant increase in Ca/N and P/N ratios ($p < 0.05$), while Ca/P remained nearly constant ($p > 0.05$) (Tables 2–4).

After 24h in artificial saliva (T24), few dentine tubules were detected on the surface, partially covered by residual toothpaste (Figure 1L). The Ca/N and P/N ratios underwent a significant decrease ($p < 0.05$) (Tables 2 and 3).

The acid attack induced the disappearance of the residual toothpaste and numerous well-visible dental tubules (Figure 1O). However, the Ca/N, P/N and Ca/P ratios did not significantly change ($p > 0.05$) (Tables 2–4), suggesting a potential protective action of the toothpaste.

Figure 2 shows the average ATR-FTIR spectra recorded on the dentine surfaces that underwent the application of HA & Citrate toothpaste. The spectrum of the toothpaste shows the bands assignable to silica, citrate, phosphate and carbonate ions.⁵⁰ The presence of the organic phase is confirmed by the detection of the bands at 2919 and 2851 cm^{-1} (range not shown). Upon treatment with HA and citrate toothpaste, several bands assignable to the toothpaste strengthened and the $I_{\text{CaP}}/I_{\text{Amide II}}$ and $I_{1410}/I_{\text{Amide II}}$ ratios increased, although not significantly ($p > 0.05$, Figures 9A,B and S4). The full-width at half maximum (FWHM) of the $\nu_3 \text{PO}_4^{3-}$ band increased from 141 to 171 cm^{-1} due to the contribution of the toothpaste bands.

The HA & Citrate toothpaste components other than calcium phosphate became undetectable on dentine surface after ageing for 24h in artificial saliva (Figure 2); the FWHM of the $\nu_3 \text{PO}_4^{3-}$ band decreased, and the $I_{\text{Amide II}}/I_{\text{Amide I}}$ ratio (Figure 9C) decreased towards the value observed in sound dentine. No significant changes in $I_{\text{CaP}}/I_{\text{Amide II}}$ were observed ($p > 0.05$, Figures 9A and S4), although the $\nu_3 \text{PO}_4^{3-}$ was found to strengthen (Figure 2). The average $I_{1410}/I_{\text{Amide II}}$ ratio did not significantly change ($p > 0.05$, Figure 9B); only sample 1 showed a significant change (i.e., a decrease, Figure S4).

Upon treatment with citric acid, both the ratios decreased but not significantly ($p > 0.05$).

3.3 | Zinc-HA toothpaste

The pH of the toothpaste was 8.46 ± 0.14 .

ESEM showed the presence of toothpaste residuals after treatment (Figure 3G). The significant increase of Ca/N and P/N ratios ($p < 0.05$) also indicate a significant presence of toothpaste

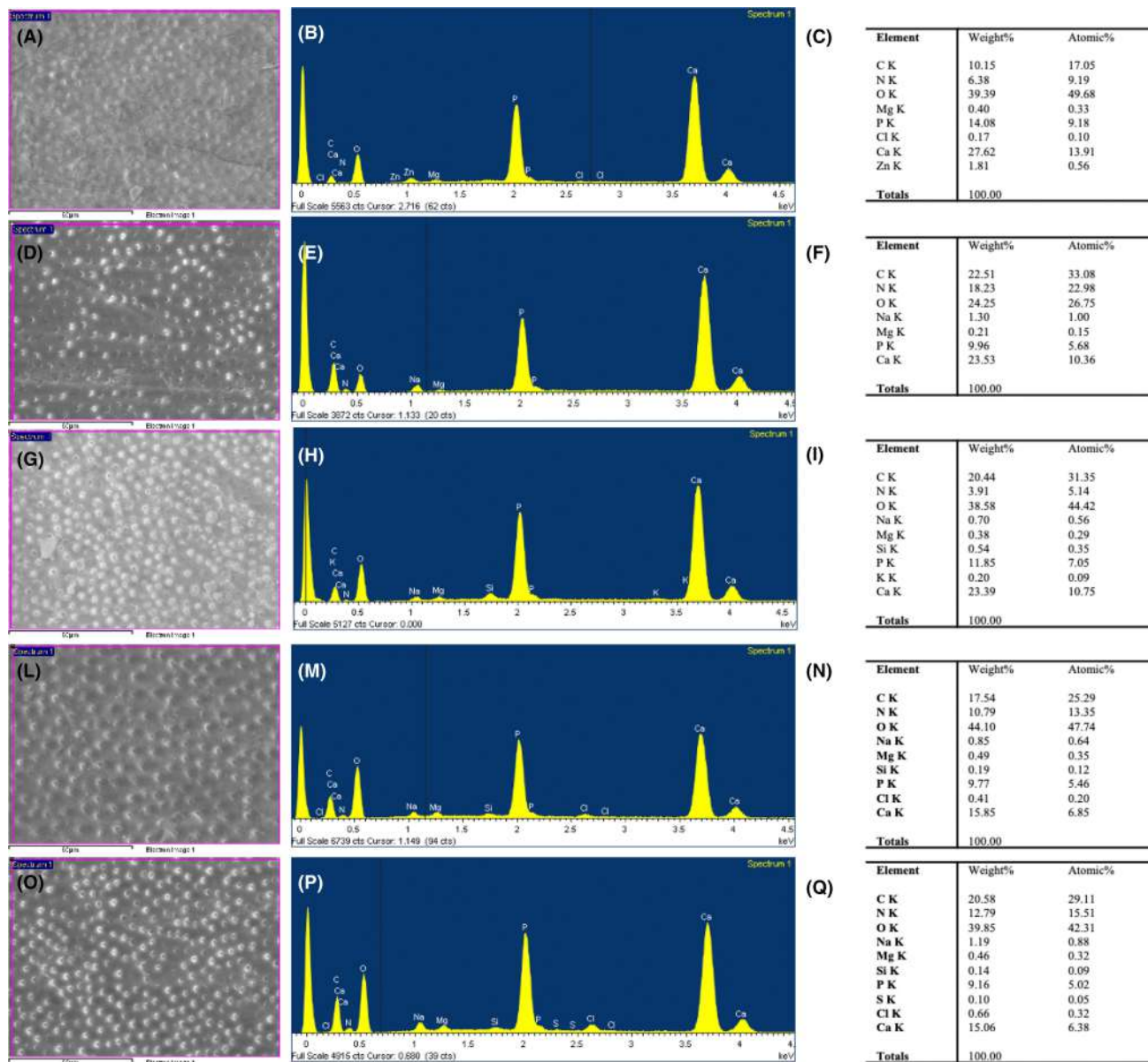


FIGURE 1 ESEM image, EDX spectra and table of elements detected by EDX of dentine with smear layer at 3000x magnification (A–D), after 10 min treatment with 10% EDTA solution (D–F), after treatment with HA and citrate toothpaste (G–I), after 24 h immersion in artificial saliva (L–N) and after 2 min irrigation with 0.1% citric acid solution (O–Q).

components in proximity to the dentinal tubules; Ca/P was quite constant (Tables 2–4).

After 24 h in artificial saliva, the ESEM image showed that the dentinal tubules were hardly detectable and partially covered (Figure 3L). After the immersion in artificial saliva, there was a slight increase in Ca/N and P/N ratios ($p < 0.05$ only for Ca/N), suggesting a remineralization process; Ca/P showed a non-significant reduction ($p > 0.05$) (Tables 2–4).

The acid attack caused the disappearance of residual toothpaste and the tubules visible (Figure 3O). Both Ca/N and P/N underwent a significant reduction with respect to the previous step of the study ($p < 0.05$) and attained values similar to those after treatment with EDTA ($p > 0.05$). These trends suggest the occurrence of demineralization processes.

Figure 4 shows the average ATR-FTIR spectra recorded on the dentine surfaces that underwent the application of Zinc-HA toothpaste. The spectrum of Zinc-HA toothpaste appears dominated by the bands of the apatite phase, which was found to contain the carbonate ion, i.e., zinc carbonate-substituted apatite.⁵¹ The band at 470 cm^{-1} may be assigned to silica, which would contribute also to the 1090 cm^{-1} broad shoulder of phosphate ion. The organic component is revealed by the bands at 2929 and 2882 cm^{-1} (not shown) and contributes to several bands in the spectral range shown in Figure 4; in particular, the band at about 1600 cm^{-1} reveals the presence of a COO^- group.

After the application of the toothpaste (T0), the relative intensity of the bands above assigned to the zinc carbonate-substituted apatite increased, and the $I_{\text{CaP}}/I_{\text{Amide II}}$ and $I_{1410}/I_{\text{Amide II}}$ IR intensity ratios

TABLE 2 Ca/N (mean ± SD)

Toothpaste	Smear layer	EDTA	T0	T24	Citric acid
HA and citrate	1.29 ± 0.35Aa	0.34 ± 0.16Ba	1.90 ± 1.05Ca	0.64 ± 0.31Da	0.41 ± 0.14Ba
Zinc-HA	1.02 ± 0.08Ab	0.37 ± 0.03Ba	0.81 ± 0.35Cb	0.96 ± 0.54Ab	0.48 ± 0.26Ba
Calcium sodium phosphosilicate	1.04 ± 0.13Ab	0.34 ± 0.07Ba	0.28 ± 0.08Bc	0.92 ± 0.60Ab	0.45 ± 0.11Ba
Arginine and calcium carbonate	0.96 ± 0.11Ab	0.32 ± 0.08Ba	0.52 ± 0.29Cd	0.90 ± 0.49Ab	0.57 ± 0.48Ca
Control	1.31 ± 0.34Aa	0.38 ± 0.14Ba	0.55 ± 0.19Cd	0.82 ± 0.42Cb	0.59 ± 0.26Ca
Water	0.46 ± 0.04Ac	0.23 ± 0.01Bb	0.21 ± 0.01Bc	0.30 ± 0.09Bc	0.22 ± 0.02Bb

Note: EDTA = 10 min treatment with 10% EDTA solution; T0 = toothpaste application; T24 = 24 h immersion in artificial saliva; Citric acid = 2 min irrigation with 0.1% citric acid solution. Different letters represent significant differences ($p < 0.05$) among times (capital letters) or among toothpastes (small letter).

TABLE 3 P/N (mean ± SD)

Toothpaste	Smear layer	EDTA	T0	T24	Citric acid
HA and citrate	0.89 ± 0.20 Aa	0.21 ± 0.07 Ba	1.21 ± 0.73 Aa	0.51 ± 0.25Ba	0.32 ± 0.11 Ba
Zinc-HA	0.75 ± 0.05 Aa	0.25 ± 0.02 Ba	0.54 ± 0.22Cb	0.68 ± 0.36 Ca	0.34 ± 0.17 Ba
Calcium sodium phosphosilicate	0.76 ± 0.08 Aa	0.22 ± 0.05 Ba	0.19 ± 0.06 Bc	0.67 ± 0.45Aa	0.32 ± 0.07 Ba
Arginine and calcium carbonate	0.71 ± 0.08 Aa	0.23 ± 0.05 Ba	0.34 ± 0.22 Bd	0.60 ± 0.34Aa	0.39 ± 0.29 Ba
Control	0.80 ± 0.10 Aa	0.26 ± 0.11 Ba	0.41 ± 0.18 Bb	0.60 ± 0.30 Ba	0.42 ± 0.18 Ba
Water	0.35 ± 0.03 Ab	0.17 ± 0.01 Bb	0.15 ± 0.01 Bc	0.19 ± 0.06Bb	0.15 ± 0.01 Bb

Note: EDTA = 10 min treatment with 10% EDTA solution; T0 = toothpaste application; T24 = 24 h immersion in artificial saliva; Citric acid = 2 min irrigation with 0.1% citric acid solution. Different letters represent significant differences ($p < 0.05$) among times (capital letters) or among toothpastes (small letters).

(Figures 9A,B) increased accordingly, but not significantly ($p > 0.05$). The $\nu_3 \text{PO}_4^{3-}$ range (around 1000cm^{-1}) underwent significant wavenumber shifts and assumed a spectral profile more similar to the toothpaste.

Upon ageing in artificial saliva (Figure 4), the spectral profile became again more similar to that of dentine and gained further intensity with a corresponding increase in the $I_{\text{CaP}}/I_{\text{Amide II}}$ IR intensity ratio ($p > 0.05$, Figure 9A).

An opposite trend was observed upon acidic attack; the $I_{\text{CaP}}/I_{\text{Amide II}}$ IR intensity ratio decreased, although not significantly ($p > 0.05$), by becoming more similar to the value of EDTA-demineralized dentine ($p > 0.05$). The $I_{1410}/I_{\text{Amide II}}$ IR intensity ratio decreased both upon ageing in artificial saliva and acidic attack, although not significantly ($p > 0.05$, Figure 9B). This trend appears more evident in the single analysed samples (Figure 55). Upon ageing in artificial saliva, the $I_{\text{Amide II}}/I_{\text{Amide I}}$ ratio (Figure 9C) decreased attaining values similar to those of untreated dentine, suggesting the occurrence of remineralization processes.

3.4 | Calcium sodium phosphosilicate toothpaste

The pH of the toothpaste was 6.68 ± 0.09 .

After toothpaste application, ESEM showed the presence of some residual toothpaste components on the surface and on the intertubular dentine, the dentine tubules resulted clearly visible (Figure 5G). The Ca/P, Ca/N and P/N ratios did not undergo any significant change ($p > 0.05$) (Tables 2-4).

After 24 h in artificial saliva, few dentinal tubules were detectable, while residual toothpaste was still identified on the surface (Figure 5L). Both Ca/N and P/N increased ($p < 0.05$), indicating that a remineralization occurred (Tables 2-4).

The acid attack induced the disappearance of the residual toothpaste/mineral (Figure 3O). Citric acid induced the significant reduction of both Ca/N and P/N ratios ($p < 0.05$), the values were close to the ratios after EDTA treatment. The treatment with citric acid generated dentine demineralization (Tables 2-4).

Figure 6 shows the average ATR-FTIR spectra recorded on the dentine surfaces that underwent the application of Calcium sodium phosphosilicate toothpaste.

The spectrum of the toothpaste showed bands typical of calcium sodium phosphosilicate,^{52,53} which should also contain a carbonate component. The organic component was detected (bands at 2929 and 2877cm^{-1} and others in the $1750\text{--}400 \text{cm}^{-1}$ range), while the sodium monofluorophosphate was not evidenced probably due to

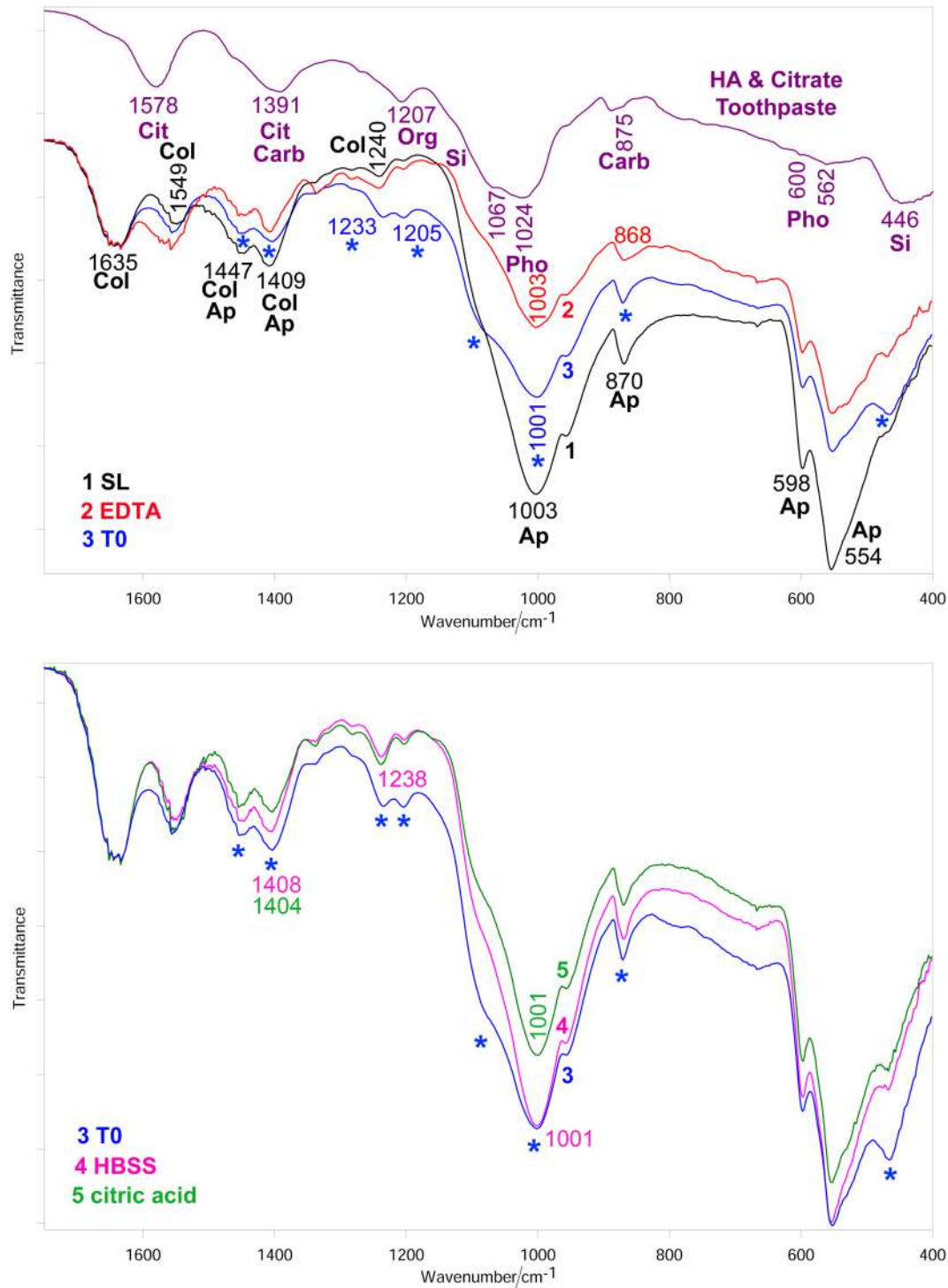


FIGURE 2 Average IR spectra recorded on dentine surfaces after smear layer (1, SL), treatment with EDTA (2, EDTA), application of HA and citrate toothpaste and rinsing (3, TO), immersion in artificial saliva for 24 h (4, HBSS), acidic attack with citric acid (5, citric acid). The spectrum of the toothpaste dried for 24 h under ambient conditions is reported for comparison. The bands assignable to silica (Si), citrate (Cit), carbonate (Carb), phosphate (Pho), organic phase (Org), collagen (Col), apatite (Ap) are indicated. The bands that at TO appeared strengthened due to the toothpaste are indicated with an asterisk.

its low content. Actually, the strongest band of this phase, reported at 1157 cm^{-1} ¹⁵⁴ was not observed in the spectrum of the toothpaste.

Upon application of Calcium sodium phosphosilicate toothpaste, several bands ascribable to the toothpaste were detected in the spectrum of dentine, and the FWHM of the $\nu_3\text{ PO}_4^{3-}$ band

increased from 132 to 151 cm^{-1} due to the contribution of the calcium sodium phosphosilicate phase. The $I_{\text{CaP}}/I_{\text{Amide II}}$ IR intensity ratio (Figure 9A) increased, although the standard deviation associated to this value appeared very high and made this change not significant ($p=0.356$). The $I_{1410}/I_{\text{Amide II}}$ ratio only slightly increased

TABLE 4 Ca/P (mean ± SD)

Toothpaste	Smear layer	EDTA	T0	T24	Citric acid
HA & Citrate	1.43 ± 0.07Aa	1.50 ± 0.29Aa	1.62 ± 0.10Ba	1.27 ± 0.03Ca	1.31 ± 0.04Ca
Zinc-HA	1.35 ± 0.05 Aa	1.47 ± 0.10 Ba	1.49 ± 0.09 Bb	1.40 ± 0.11Aa	1.41 ± 0.12Ab
Calcium sodium phosphosilicate	1.37 ± 0.05 Aa	1.50 ± 0.08 Ba	1.48 ± 0.02 Bb	1.37 ± 0.06Ab	1.40 ± 0.09Ab
Arginine & Calcium carbonate	1.35 ± 0.01 Aa	1.41 ± 0.05Aa	1.67 ± 0.52Ba	1.50 ± 0.06Bc	1.50 ± 0.02Bc
Control	1.64 ± 0.39Ab	1.47 ± 0.11 Ba	1.41 ± 0.19 Bb	1.38 ± 0.07 Ba	1.41 ± 0.07 Bb
Water	1.31 ± 0.01 Aa	1.36 ± 0.05ABb	1.45 ± 0.04 Bb	1.54 ± 0.03Cc	1.40 ± 0.03 Bb

Note: EDTA= 10min treatment with 10% EDTA solution; T0=toothpaste application; T24= 24h immersion in artificial saliva; Citric acid= 2 min irrigation with 0.1% citric acid solution. Different letters represent significant differences ($p < 0.05$) among times (capital letters) or among toothpastes (small letters).

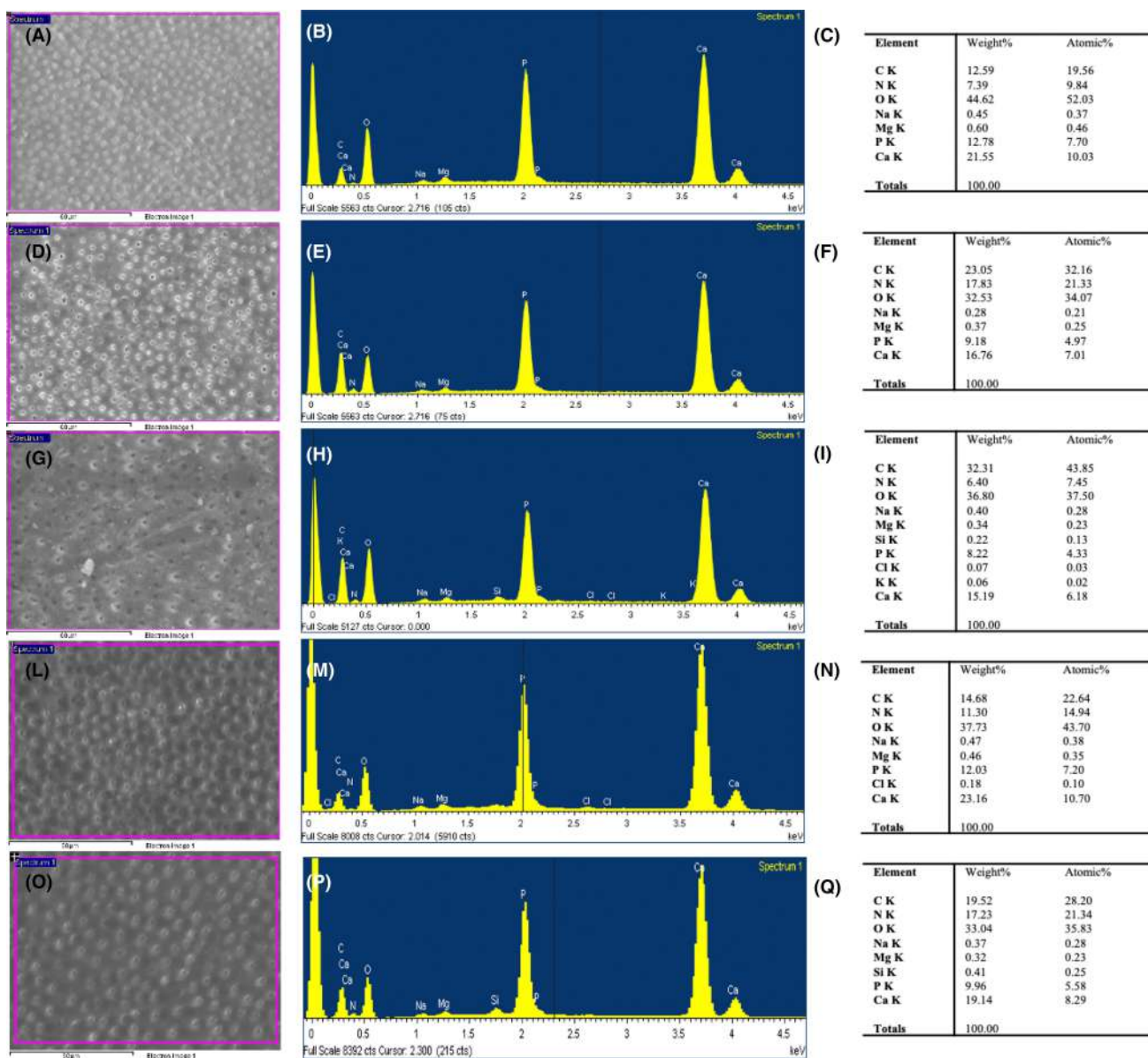


FIGURE 3 ESEM image, EDX spectra and table of elements detected by EDX of dentine with smear layer at 3000× magnification (A–C), after 10min treatment with 10% EDTA solution (D–F), after treatment with Zinc–HA toothpaste (G–I), after 24h immersion in artificial saliva (L–N) and after 2 min irrigation with 0.1% citric acid solution (O–Q).



($p=0.745$, Figure 9B) due to the contribution of the carbonate component present in the toothpaste. A further strengthening in the ν_3 PO_4^{3-} range was observed upon ageing in artificial saliva (Figure 6); however, the high standard deviation associated to the $I_{\text{CaP}}/I_{\text{Amide II}}$ values, i.e., to both the average (Figure 9A) and to the single analysed samples (Figure S6), made the change not significant ($p>0.05$). The $I_{1410}/I_{\text{Amide II}}$ ratio remained nearly constant ($p>0.05$, Figure S11B), while the $I_{\text{Amide II}}/I_{\text{Amide I}}$ ratio (Figure 9C) decreased attaining values similar to those of untreated dentine.

Upon treatment with citric acid, the intensity of the ν_3 PO_4^{3-} band decreased as well as the $I_{\text{CaP}}/I_{\text{Amide II}}$ ratio (change not significant, $p>0.05$), attaining values similar or slightly higher than EDTA-demineralized dentine ($p>0.05$, Figures 9 and S6). The $I_{1410}/I_{\text{Amide II}}$ ratio decreased attaining values similar to those of EDTA-demineralized dentine ($p>0.05$, Figures 9B and S6).

3.5 | Arginine and Calcium carbonate toothpaste

The pH of the toothpaste resulted 9.01 ± 0.06 .

After toothpaste application, ESEM showed the presence of sparse residual toothpaste covering few tubules (Figure 7G). The mineralization degree evaluated as atomic ratios showed an increase of Ca/N ($p<0.05$), P/N ($p>0.05$), and Ca/P ($p<0.05$) ratios compared to smear layer (Tables 2–4).

After 24 h in artificial saliva, ESEM image showed that the surface was still covered by some residual toothpaste, (Figure 7L). There was a significant increase in Ca/N and P/N ratios ($p<0.05$), suggesting a process of remineralization (Tables 2, 3).

The acid attack caused the disappearance of residual toothpaste (Figure 7O). Both Ca/N and P/N ratios reduced back to values similar to those at T0 ($p>0.05$), suggesting a demineralization process.

Figure 8 shows the average ATR-FTIR spectra recorded on the dentine surfaces that underwent the application of Arginine & Calcium carbonate toothpaste.

The spectrum of the toothpaste appears dominated by the bands of calcium carbonate (calcite).⁴⁸ The bands in the $1700\text{--}1500\text{ cm}^{-1}$ range are ascribable to the organic component, revealed also by the CH stretching bands at 2919 and 2851 cm^{-1} (not shown). The band at 1021 cm^{-1} has a contribution from sodium silicate⁵⁵ and bicarbonate⁵⁰ and possibly also from the organic component. On the contrary, sodium monofluorophosphate was not detected.

Upon application of arginine and calcium carbonate toothpaste (T0), the spectrum of the dentine surface underwent a significant strengthening in both the ν_3 PO_4^{3-} range (around 1000 cm^{-1}) and ν_3 CO_3^{2-} (around 1400 cm^{-1}) ranges, due to the contribution of the carbonate bands of the toothpaste. In particular, the ν_3 PO_4^{3-} band underwent a broadening (i.e. and increase in its FWHM from 126 to 152 cm^{-1}), due to the contribution of the calcite band at 1078 cm^{-1} . The $I_{\text{CaP}}/I_{\text{Amide II}}$ and $I_{1410}/I_{\text{Amide II}}$ IR intensity ratios (Figures 9A,B) significantly increased ($p<0.05$).

These findings agree with the ESEM data of dentine: granules of toothpastes remained on the surface of dentine despite the washing treatment. The spectral profile near 1550 cm^{-1} became similar to that of the toothpaste, suggesting that also the organic component should be present on the surface of dentine.

After immersion for 24 h in artificial saliva, the bands of calcite decreased in intensity with respect to T0, but were still detected (Figure 8); correspondingly, the $I_{1410}/I_{\text{Amide II}}$ IR intensity ratio (Figure 9B) decreased (although not significantly, $p=0.433$) and the ν_3 PO_4^{3-} band underwent a narrowing due to the lost contribution of the calcite band at 1078 cm^{-1} . At the same time, the ν_3 PO_4^{3-} band further strengthened (Figure 8) and the $I_{\text{CaP}}/I_{\text{Amide II}}$ IR intensity ratio (Figure 9A) increased accordingly, although not significantly ($p=0.075$). The spectral profile around 1550 cm^{-1} more closely resembled that of the Amide II of collagen and the $I_{\text{Amide II}}/I_{\text{Amide I}}$ ratio (Figure 9C) decreased towards the value observed in untreated dentine. Upon acidic attack, the bands of calcite were not still detected; the $I_{1410}/I_{\text{Amide II}}$ IR intensity ratio further decreased but not significantly (Figure 9B $p>0.05$) and the ν_3 PO_4^{3-} band further narrowed. The ν_3 PO_4^{3-} band weakened (Figure 8) but remained stronger than in the spectrum of the EDTA-demineralized sample, as also shown by the values of the $I_{\text{CaP}}/I_{\text{Amide II}}$ IR intensity ratio, which appeared significantly different ($p<0.05$, Figures 9A and S7). Upon acidic attack, slight shifts in Amide III and ν_3 CO_3^{2-} antisymmetric stretching bands were detected, in agreement with a previous study.⁴⁰

3.6 | Control toothpaste

The pH of the toothpaste used was 7.17 ± 0.04 .

After toothpaste application, ESEM showed the presence a toothpaste layer covering dentinal tubules (Figure S1G). A slight increase of Ca/N and P/N ratios was observed ($p<0.05$ only for Ca/N), while Ca/P remained constant (Tables 2–4).

After 24 h in artificial saliva, the surface presented less residual toothpaste but few dentinal tubules (Figure S1L). The Ca/N and P/N ratios underwent only a slight increase ($p>0.05$) after immersion in artificial saliva (Tables 2–4).

The acid attack removed many residues and the tubules were more clearly visible, but in some areas a high number of tubules were still covered (Figure S1O). Both Ca/N and P/N ratios decreased, but not significantly ($p>0.05$) (Tables 2 and 3).

After treatment with Control toothpaste, the IR spectrum of the dentine surface showed the appearance/strengthening of bands ascribable to the toothpaste and also the $I_{\text{CaP}}/I_{\text{Amide II}}$ ratio significantly increased ($p<0.05$), both in the average (Figure 9A) and in the single analysed samples (Figure S8). The ν_3 PO_4^{3-} band was found to broaden as previously observed for other treatments.

After ageing in artificial saliva, most of the bands assignable to the toothpaste were lost, but the ν_3 PO_4^{3-} was found to strengthen (Figure S2) and the $I_{\text{CaP}}/I_{\text{Amide II}}$ ratio significantly increased, ($p=0.044$, Figures 9A and S8). The $I_{\text{Amide II}}/I_{\text{Amide I}}$ ratio (Figure 9C) decreased as previously observed for the other toothpastes.

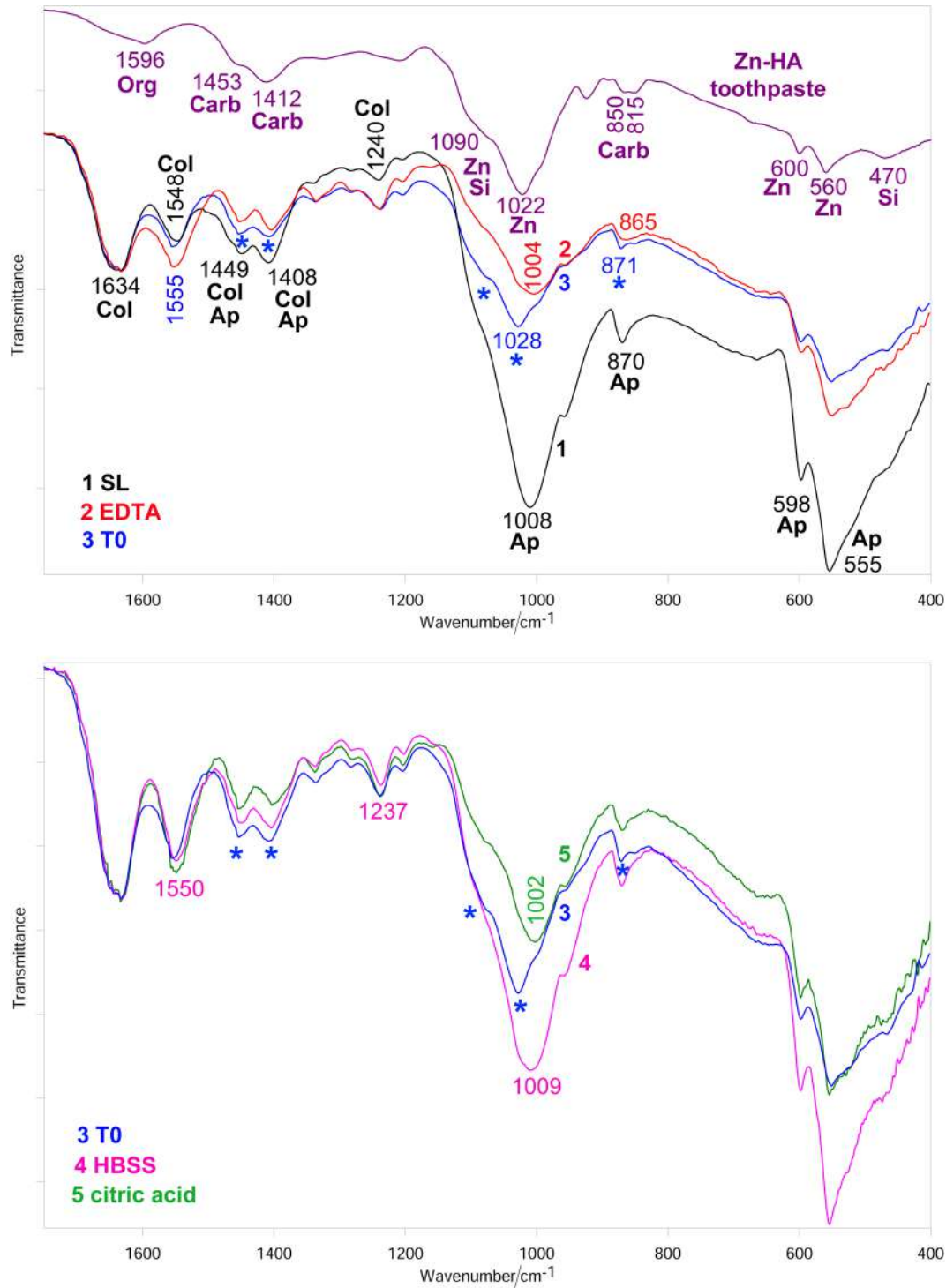


FIGURE 4 Average IR spectra recorded on dentine surfaces after smear layer (1, SL), treatment with EDTA (2, EDTA), application of Zinc-HA toothpaste and rinsing (3, TO), immersion in artificial saliva for 24 h (4, HBSS), acidic attack with citric acid (5, citric acid). The spectrum of the toothpaste dried for 24 h under ambient conditions is reported for comparison. The bands assignable to zinc hydroxyapatite (Zn) containing carbonate as substituting ion (Carb), silica (Si), organic phase (Org), collagen (Col), apatite (Ap) are indicated. The bands that at TO appeared strengthened due to the toothpaste are indicated with an asterisk.

Deminerzalization occurred after citric acid attack, although the $I_{CaP}/I_{Amide II}$ ratio remained higher than in the EDTA-demineralized dentine ($p < 0.05$, Figures 9A and S8). The $I_{1410}/I_{Amide II}$ ratio was

found to significantly decrease in all single analysed samples (Figure S8); the same trend was observed in the average (Figure 9B), with a higher standard deviation (change not significant $p = 0.345$).

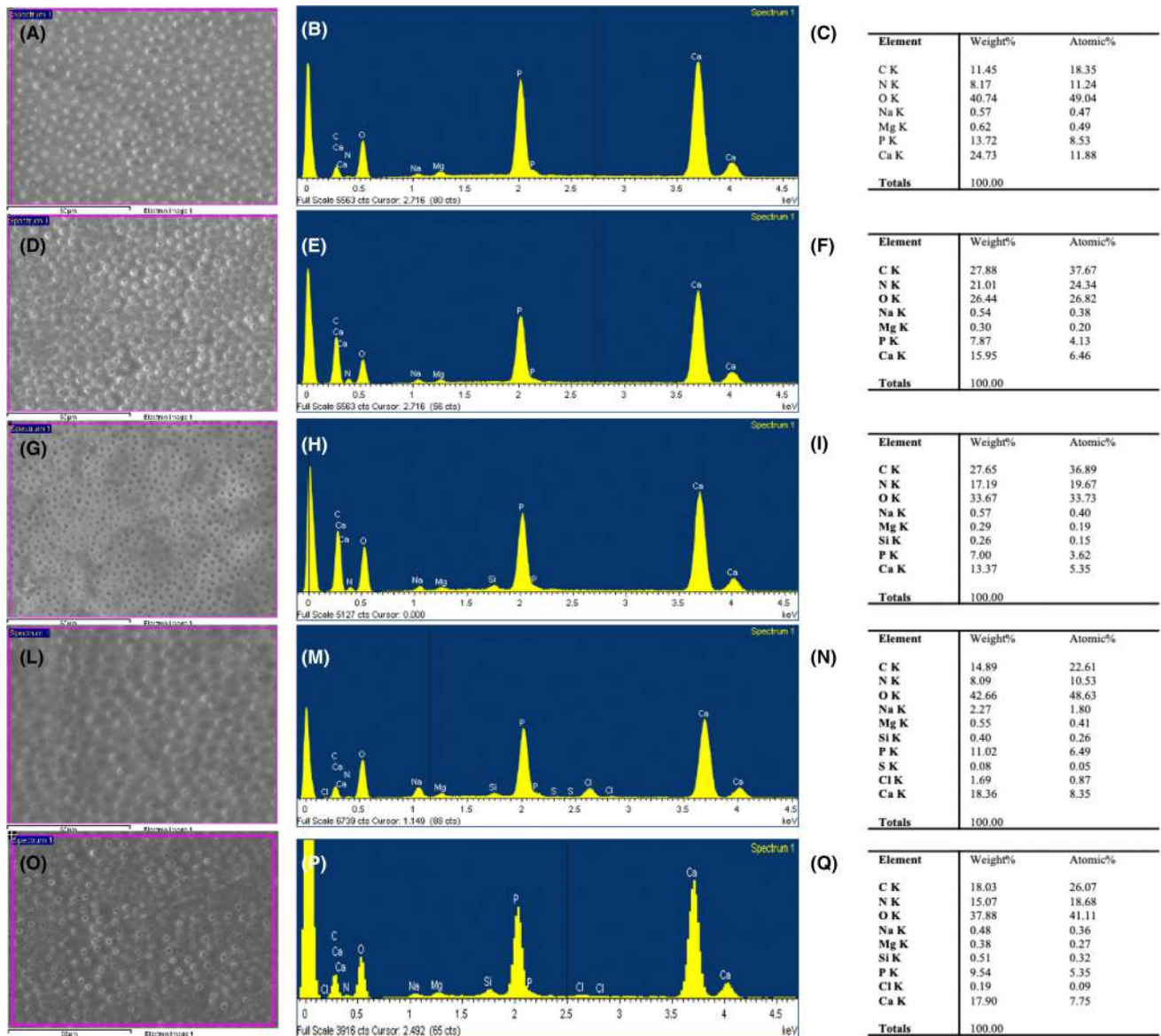


FIGURE 5 ESEM image, EDX spectra and table of elements detected by EDX of dentine with smear layer at 3000x magnification (A–C), after 10 min treatment with 10% EDTA solution (D–F), after treatment with Calcium sodium phosphosilicate toothpaste (G–I), after 24 h immersion in artificial saliva (L–N) and after 2 min irrigation with 0.1% citric acid solution (O–Q).

3.7 | Water-treated dentine (control)

ESEM images showed that tubules were visible at all the steps of the study (Figures S3G,L,O).

At the EDX analysis, elements were always the same, except for Cl that appeared after the immersion in artificial saliva and remained after citric acid attack (Figures S3M,N,P,Q). The Ca/N and P/N atomic ratios did not show any significant change ($p > 0.05$, Tables 2, 3).

Figure S9 shows the average ATR–FTIR spectra recorded on the dentine surfaces that were used as control samples. No significant differences ($p > 0.05$) were observed between the corresponding $I_{\text{CaP}}/I_{\text{Amide II}}$ values and the same result was obtained for the $I_{1410}/I_{\text{Amide II}}$ values (Figure 9A,B).

4 | DISCUSSION

An important requirement for oral health preventive dentistry is the development of materials (toothpastes) able to induce the remineralization of hypomineralized/demineralized (cariou) dentine.

The concept of remineralization is based on the reincorporation of mineral in dental tissues.^{56,57} Remineralization of demineralized/cariou dentine occurs by incorporation of mineral ions (calcium, phosphate, fluoride) from the oral fluid or from external sources, through the growth of existing apatite crystals (belonging to remnant crystallites in the subsurface).^{58,59} The precipitated mineral may act as a constant site for further nucleation of mineral promoting a continuous remineralization over time when in presence of environmental mineral ions.

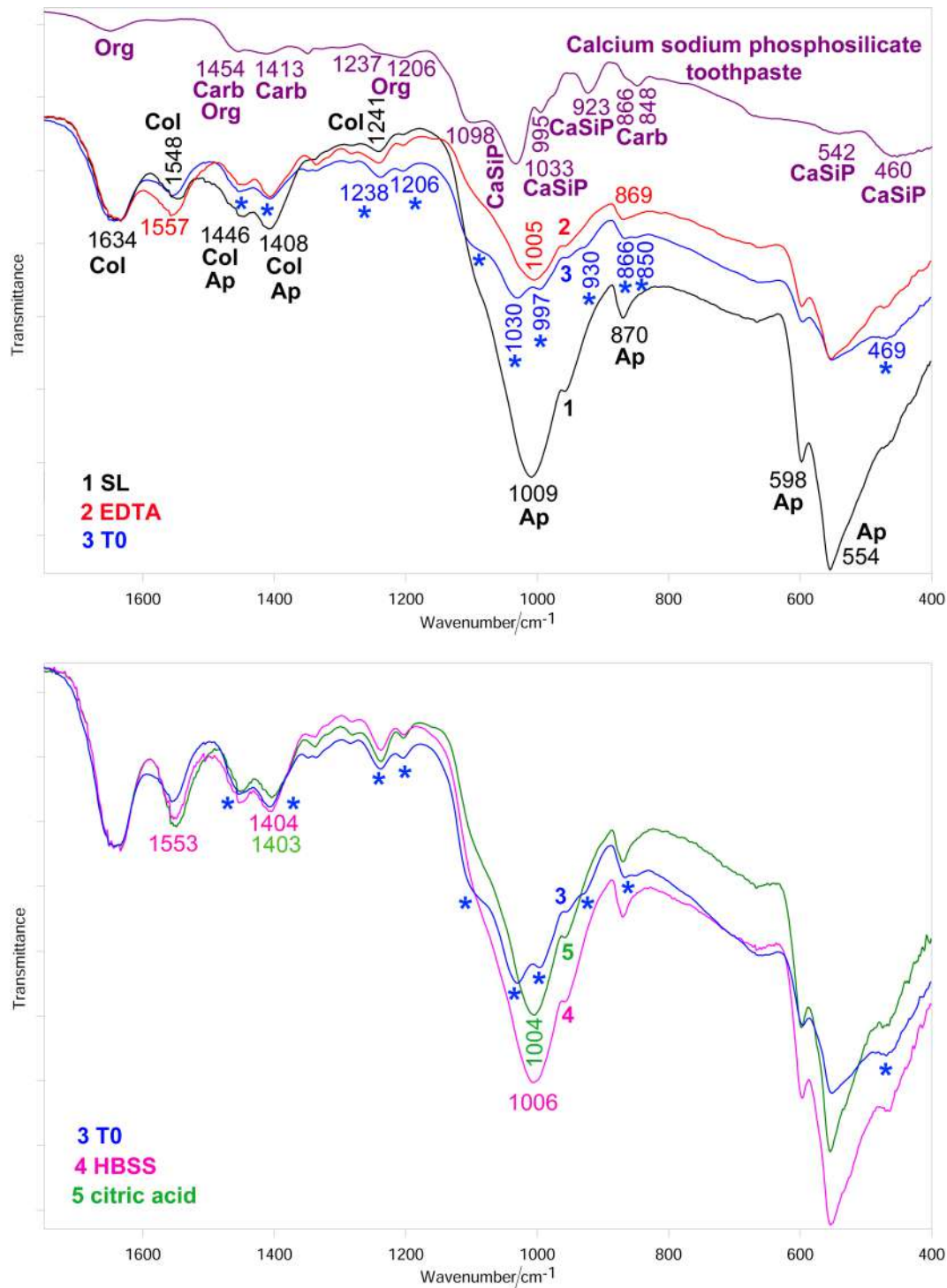


FIGURE 6 Average IR spectra recorded on dentine surfaces after smear layer (1, SL), treatment with EDTA (2, EDTA), application of Calcium sodium phosphosilicate toothpaste and rinsing (3, TO), immersion in artificial saliva for 24 h (4, HBSS), acidic attack with citric acid (5, citric acid). The spectrum of the toothpaste dried for 24h under ambient conditions is reported for comparison. The bands assignable to calcium sodium phosphosilicate (CaSiP), carbonate (Carb), organic phase (Org), collagen (Col), apatite (Ap) are indicated. The bands that at TO appeared strengthened due to the toothpaste are indicated with an asterisk.

The remineralization of demineralized dentine is the process of restoring minerals through the formation of inorganic mineral-like materials (bioremineralization)⁶⁰ and has been investigated through different methods using ion-containing solutions or

solutions mimicking the biological fluids (saliva, dentinal fluid, plasma).^{33,36,57,60-64}

The inclusion of an active/reactive component as filler in toothpastes may enhance (biocatalysis) the apatite formation.²⁴ In

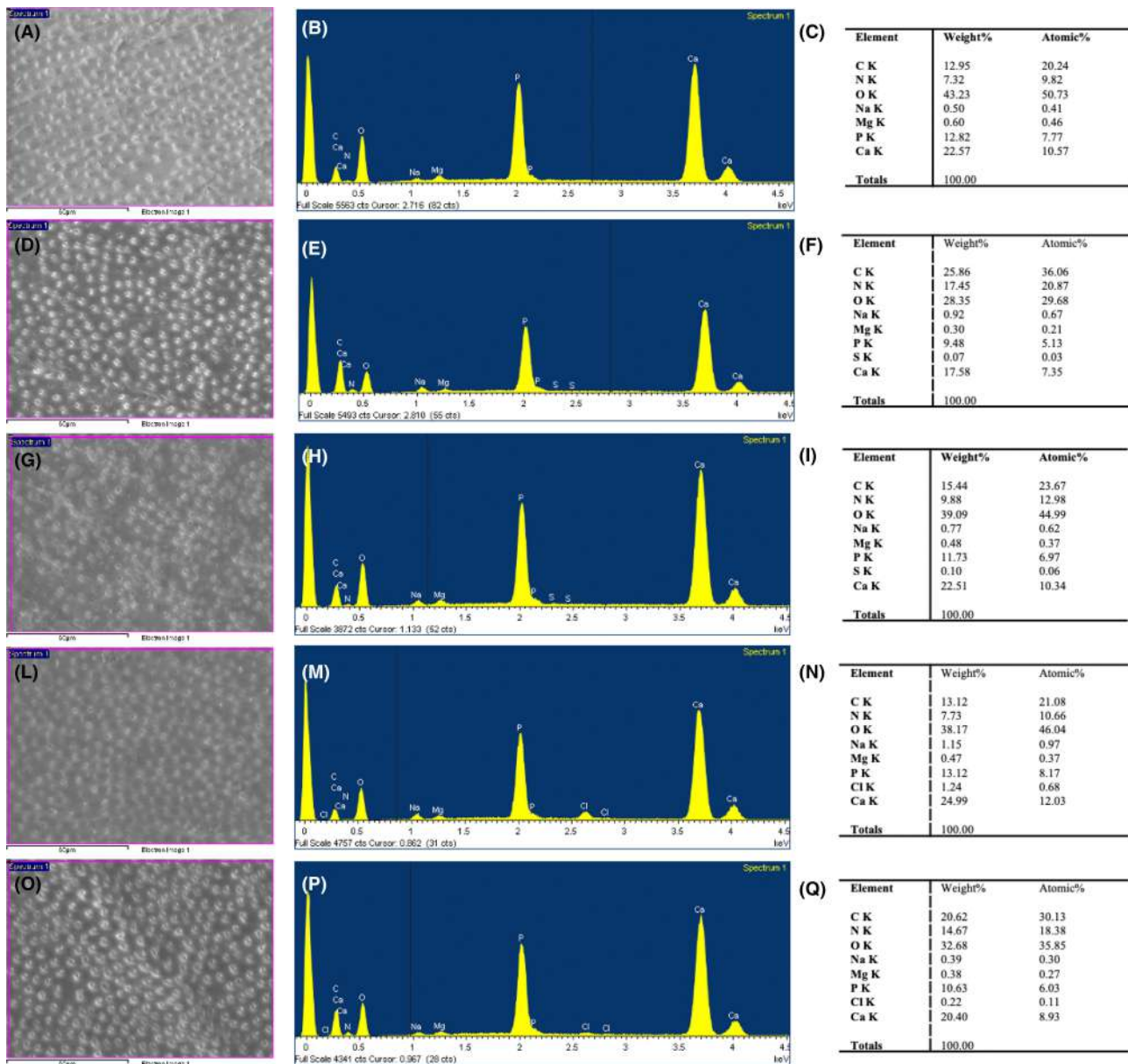


FIGURE 7 ESEM image, EDX spectra and table of elements detected by EDX of dentine with smear layer at 3000x magnification (A–C), after 10min treatment with 10% EDTA solution (D–F), after treatment with Arginine & Calcium carbonate toothpaste (G–I), after 24 h immersion in artificial saliva (L–N) and after 2 min irrigation with 0.1% citric acid solution (O–Q).

such remineralization process, the supply of mineral ions (Ca^{2+} , PO_4^{3-} , F^- , etc.) or molecules acting as nucleating sites is the basic requirement to enhance the apatite formation and the mineralization of the dentinal tissue in the presence of mineral saliva-like solutions.

To the best of our knowledge, bioremineralization of commercial toothpastes have been poorly investigated in a limited number of studies.^{65,66} Only micromorphological analyses by SEM after repeated toothpastes application were performed, the chemical composition and modifications of the layer were not investigated. A study performed using SEM and atomic force microscopy reported the remineralization ability of several commercial toothpastes containing Calcium sodium phosphosilicate, arginine 8% or zinc hydroxyapatite.⁶⁵ The

results showed that all formulation were able to induce a layer on the dentine but did not analyse the chemical conformation of the formed layer.⁶⁵ Another study analysed demineralized enamel blocks through nanoindentation, nanoscratch, scanning probe microscope SEM and XPS, revealing that 8% arginine and sodium monofluorophosphate toothpastes induced enamel remineralization with a marked improvement of enamel nanohardness and elastic modulus, but no information on the layer composition was provided.⁶⁶

In the present study, four toothpastes including different reactive components in their formulation and specifically designed for dentinal treatment, have been selected.

The addition of arginine in toothpastes formulation, in particular when also fluoride compounds are present (sodium

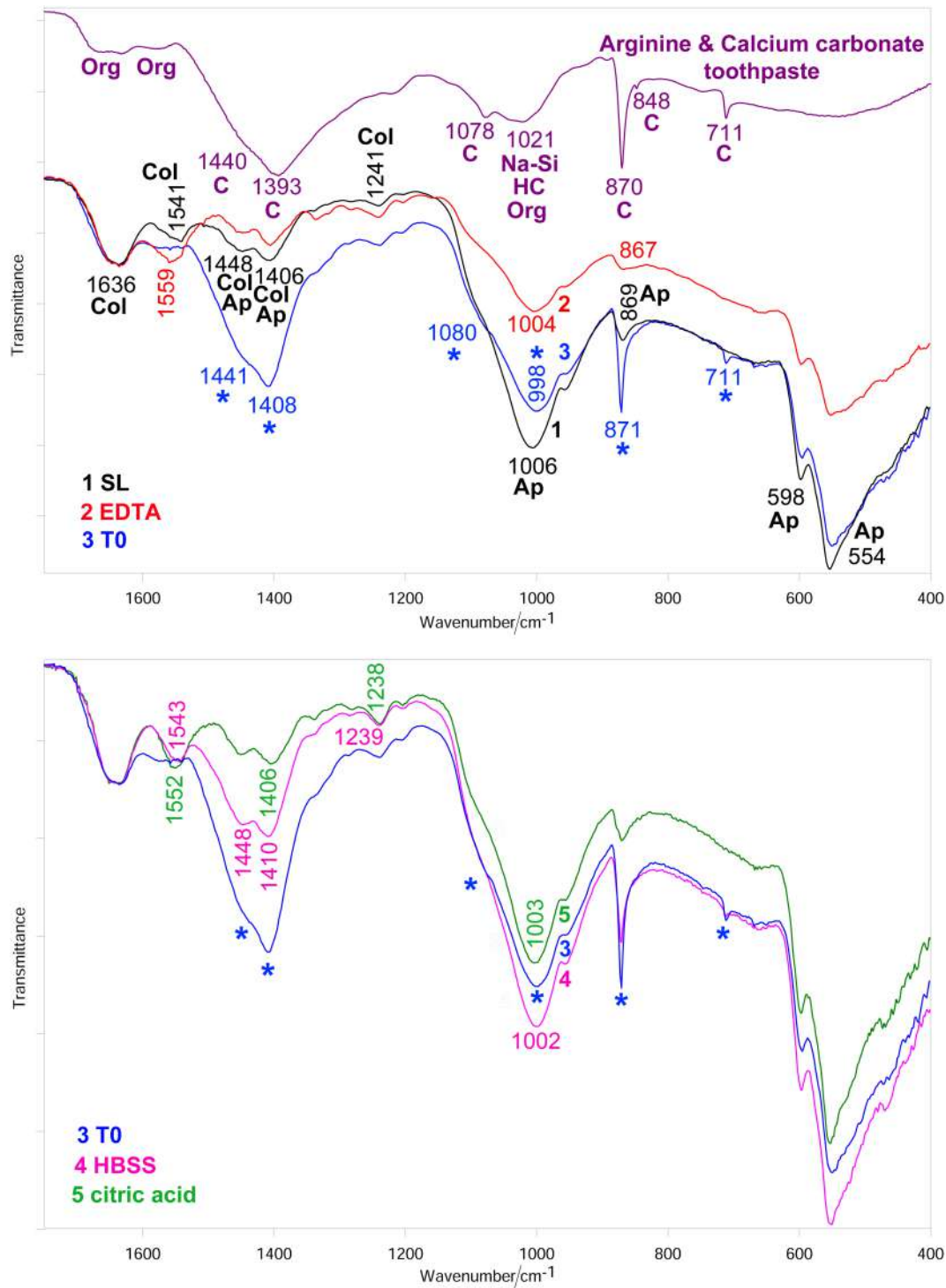
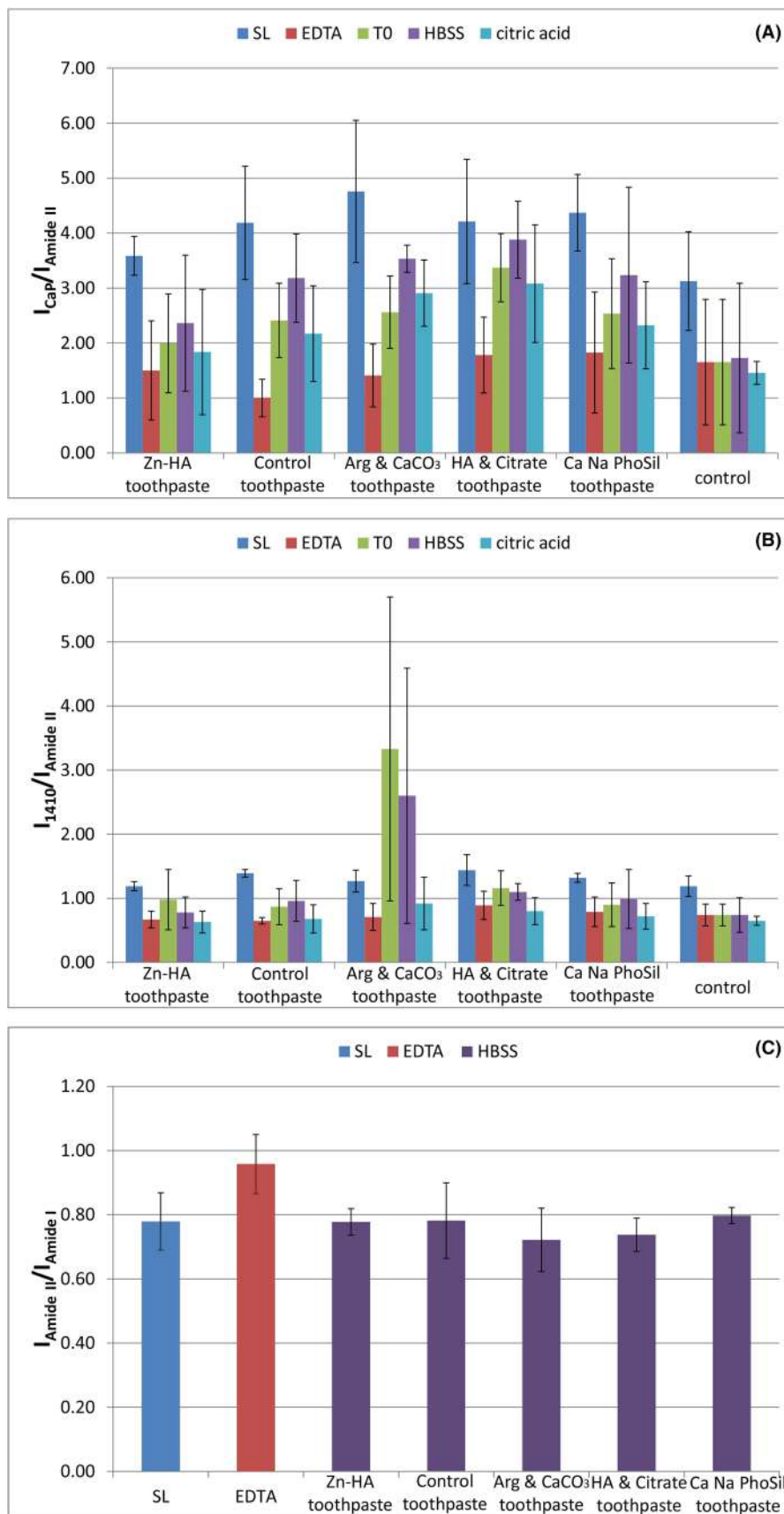


FIGURE 8 Average IR spectra recorded on dentine surfaces after smear layer (1, SL), treatment with EDTA (2, EDTA), application of arginine and calcium carbonate toothpaste and rinsing (3, TO), immersion in artificial saliva for 24 h (4, HBSS), acidic attack with citric acid (5, citric acid). The spectrum of the toothpaste dried for 24 h under ambient conditions is reported for comparison. The bands assignable to calcite (C), sodium silicate (Na-Si), bicarbonate (HC), organic phase (Org), collagen (Col), apatite (Ap) are indicated. The bands that at TO appeared strengthened due to the toothpaste are indicated with an asterisk.

monofluorophosphate in this case) has been considered attractive for oral health caries prevention and remineralization procedures, as arginine (a basic amino acid) raises pH and facilitates calcium ions

dissociation; their reaction with fluoride ions leads to the formation of insoluble calcium compounds.²⁵ Arginine attracts more phosphate and water in the lipid membranes and can form extensive H-bonding

FIGURE 9 $I_{CaP}/I_{Amide II}$ (A), $I_{1410}/I_{Amide II}$ (B) and $I_{Amide II}/I_{Amide I}$ (C) intensity ratios (average \pm standard deviation, calculated on all the analysed samples) as obtained from the IR spectra recorded on dentine surfaces after smear layer (SL), treatment with EDTA, application of the toothpastes and rinsing (T0), immersion in artificial saliva for 24 h (HBSS), acidic attack with citric acid.



with its five H-bond donors to stabilize Arg-phosphate clusters.⁶⁷ RGD (arginine-glycine-aspartic acid) peptides possess a collagen-binding domain. The arginine residues directly interact with the hydroxyl group of hydroxyapatites through electrostatic forces and hydrogen bonding.⁶⁸ The role of charged residues of collagen in binding Ca^{2+} and PO_4^{3-} ions has been demonstrated in particular for three specific aminoacidic sequences, such as Glu-Arg-(Gly)-Arg, Asp-Lys/Arg-(Gly)-Glu or Glu-(Ser/Ala)-(Gly)-Arg-Glu/Asp, which have the potential to sequester and bind Ca^{2+} and PO_4^{3-} ions.⁶⁹ Once Ca^{2+} and PO_4^{3-} ions are segregated in these collagen regions they can combine electrostatically to create a nucleus, leading subsequently to crystal growth and development and apatite formation. The growth of nuclei may occur through a series of additional events that include recruitment of other Ca^{2+} and PO_4^{3-} ions either by ion adsorption from the supersaturated extracellular milieu and/or by incorporation of the ions bound to charged amino acid residues in the neighbourhood of the growing crystal.⁷⁰ The binding of proteins to apatite depends on their isoelectric point.^{70,71} Clusters of ionized carboxyl groups (rather than total amount of negative charges) strengthen protein binding to hydroxyapatite.⁷¹ Calcium carbonate included in toothpaste formulation represents a low-hardness abrasive agent^{72,73} able to reduce dentine abrasion and damages. Calcium carbonate demonstrated low solubility at basic pH, the solubility at room temperature is determined by acid environments.^{74,75} Moreover, in simulated clinical conditions, human saliva is saturated by calcium ions, further reducing the solubility of calcium carbonate.^{74,75} Therefore, we may speculate that remineralization effect was mostly attributable to the arginine inclusion in the toothpaste formulation.

Calcium sodium phosphosilicate particles included in toothpaste formulation (Calcium sodium phosphosilicate toothpaste) showed to inhibit dentine demineralization and promote dentine remineralization via interfacial apatite precipitation.^{76,77} This apatite layer was reported tightly adherent to dentine and resistant to acid and brushing-abrasion wear challenge.⁷⁸ Furthermore, Bioglass has also been shown capable of inhibiting and reversing initial caries progression in enamel.^{79,80} Bioglass may form a protective layer on the enamel surface to inhibit demineralization when used before hydrogen peroxide bleaching or enhance remineralization when used after bleaching.

The mode of action of calcium sodium phosphosilicate has been investigated *in vitro* and was described to consist in the formation of a chemically and mechanically stable apatite-like calcium phosphate hydroxycarbonate layer.^{18,21,80} The initial reactivity of calcium sodium phosphosilicate particles was found to form a negative charge on the surface of exposed dentine *in vitro*, enabling establishment of covalent bindings of calcium sodium phosphosilicate to side groups of Type 1 dentinal collagen fibres.⁸¹ Local precipitation of apatite-like material was attributed to immediate release of sodium ions when calcium sodium phosphosilicate comes in contact with water or saliva. Also in this case, the rise of the local environmental pH subsequently facilitates release of calcium and phosphate ions.¹⁸

The rationale of apatite particles inclusion in HA-containing toothpastes, as hydroxyapatite (particle range 6–10 μm) (HA and citrate toothpaste) and Zinc Nanohydroxyapatite (particle range 20–200 nm)

(Zinc-HA toothpaste)^{9,12} is to remineralize hard dental tissue through the spreading of HA particles and nanoparticles onto the demineralized surface^{14,15} and/or the potential diffusion inside the dentinal tubules.^{9,12} However, Hydroxyapatite and nanohydroxyapatite minerals cannot provide a bioavailable biointeractive signalling (as calcium and phosphates ions)¹⁶ due to the negligible dissolution of hydroxyapatite at oral pH environment.^{74,82} Therefore, HA-containing toothpastes could not remineralize dentine through ions dissociation.

In the present study, IR spectroscopy in the ATR technique has been used to non-destructively verify the efficiency of the EDTA demineralizing procedure as well as the extent of the remineralization process and its stability after acid attack. EDTA treatment was performed to remove the smear layer generated by the cutting of dentine during the disc preparation and to simulate a partially demineralized dentin.^{16,83}

Upon treatment with EDTA, the IR bands assignable to the B-type carbonated apatite phase significantly decreased in intensity without disappearing (Figures 2,4,6,8 and S2). This result suggests that under the used experimental conditions, in the first 2 microns (i.e., the thickness which the recorded IR spectrum refers to) a certain amount of this phase remained. This qualitative finding is confirmed by the trend of the $I_{\text{CaP}}/I_{\text{Amide II}}$ and $I_{1410}/I_{\text{Amide II}}$ IR intensity ratios (Figure 9A,B), which significantly decreased upon EDTA treatment. The obtained results are consistent with those obtained in previous studies^{40,42}: the used experimental conditions (i.e., 10% EDTA for 10 min) were definitely less aggressive than upon treatment with 17% EDTA for 2 h after which no apatite phase was detected in the first two microns,^{36,42} but more aggressive than upon treatment with Tubuliclean (i.e. 10% EDTA) for 1 min (for which a lower apatite removal was obtained).⁴⁰ Under the EDTA treatment conditions used in the present study, the Amide I, Amide II and Amide III bands underwent only slight changes, which also in this case appeared consistent with the trends previously reported.^{40,42} This result suggests that in the first 2 microns, collagen underwent only slight (if any) conformational rearrangements upon the partial loss of the apatite phase. The most evident result appeared the strengthening of the Amide II band with respect to Amide I and the corresponding increase of the $I_{\text{Amide II}}/I_{\text{Amide I}}$ ratio (Figure 9C). This behaviour has been already observed³⁶ and has been interpreted according to previous studies⁸⁴ as a sign of the loss of interactions between collagen (in particular through its C=O groups) and Ca^{2+} ions upon demineralization.

IR spectroscopy showed that in all cases, after the application of the toothpastes and rinsing with water (T0), a number of toothpastes granules remained on the surface on the dentine slices, in agreement with the ESEM results. For this reason, it is not surprising that the standard deviation associated to both IR and EDX ratios was often high. The inhomogeneous nature of the samples at this step of the study also accounts for the differences sometimes observed between IR and EDX data, due to the different sampling volumes of the two techniques.

Remineralization proceeded upon ageing in artificial saliva for 24 h. The standard deviation associated to the average $I_{\text{CaP}}/I_{\text{Amide II}}$

and $I_{1410}/I_{Amide II}$ IR intensity ratios (Figure 9A,B) as well as to Ca/N and P/N ratios (Tables 2 and 3) was high, indicating a certain variability from sample to sample.

With regards to the IR measurements, in most cases, lower standard deviations were observed in the single analysed samples (Figures S4-S8). The reported data showed that the $I_{CaP}/I_{Amide II}$ ratio measured after 24 h in artificial saliva was significantly higher than that measured after EDTA treatment in most cases, while the $I_{1410}/I_{Amide II}$ ratio showed less significant increases, with the exception of Arginine & Calcium carbonate-toothpaste. At this stage, the

$I_{1410}/I_{Amide II}$ ratio was significantly higher for Arginine & Calcium carbonate -toothpaste than for Zinc-HA toothpaste, Calcium sodium phosphosilicate toothpaste and Control toothpaste ($p < 0.05$), while no significant differences were observed between the samples with regards to $I_{CaP}/I_{Amide II}$. In spite of the high standard deviation associated to the measurements and the poorly statistically significant differences between treatments, Figure 10A shows that the average $I_{CaP}/I_{Amide II}$ IR intensity ratio measured upon treatment in HBSS well correlates with that measured after application of the toothpaste and rinsing (T0), and an analogous trend was obtained

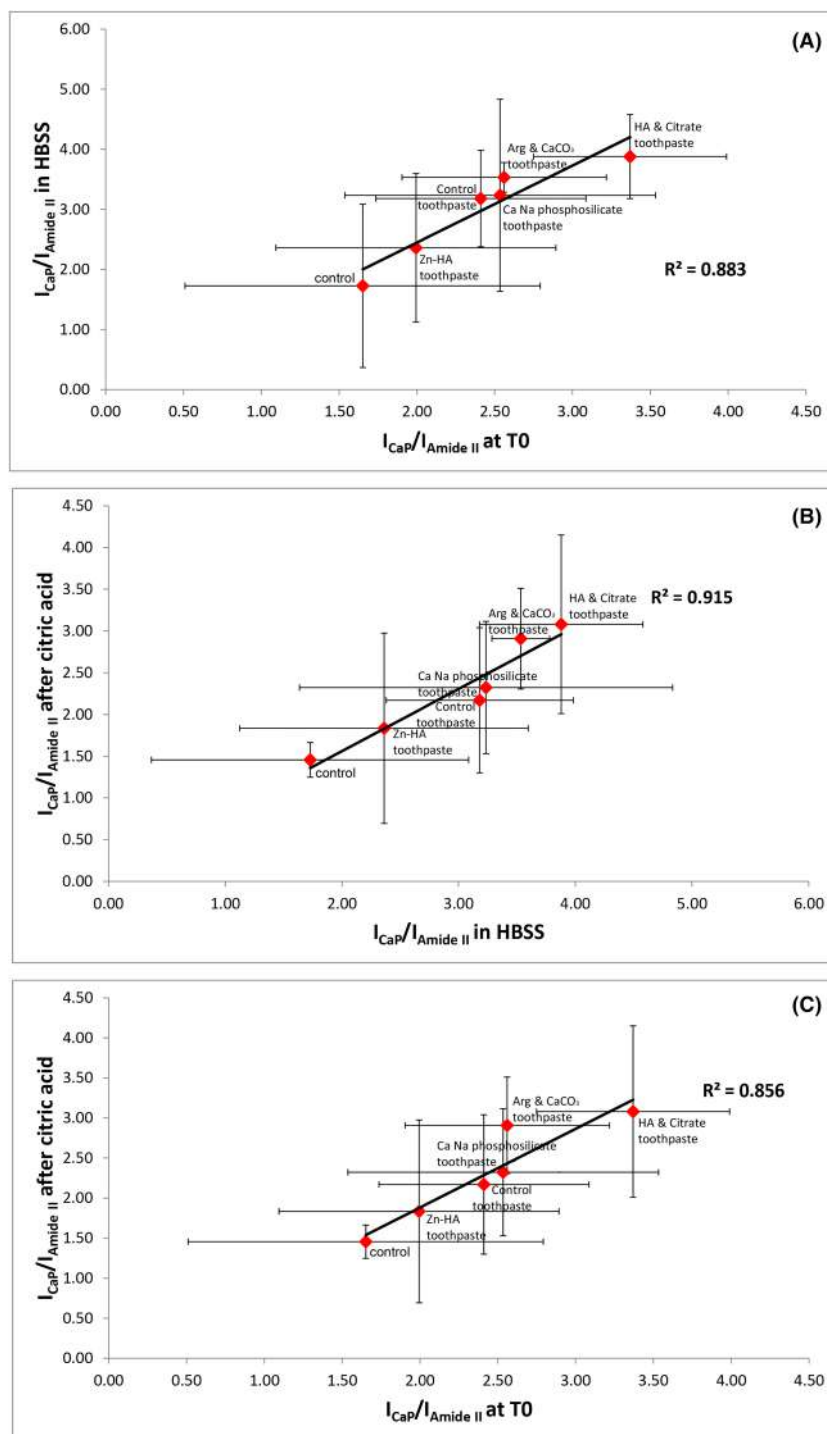


FIGURE 10 Trend of: (A) $I_{CaP}/I_{Amide II}$ measured after immersion in artificial saliva for 24 h (HBSS) as a function of the same ratio measured at T0; (B) $I_{CaP}/I_{Amide II}$ measured after acidic attack (citric acid) as a function of the same ratio measured after immersion in artificial saliva for 24 h (HBSS); (C) $I_{CaP}/I_{Amide II}$ measured after acidic attack (citric acid) as a function of the same ratio measured at T0. The data are average \pm standard deviation, calculated on all the analysed samples.

for the $I_{1410}/I_{Amide II}$ IR intensity ratio (Figure S10A). This trend would indicate that the toothpaste residuals remained on dentine surface played a not negligible role in remineralization. At the same time, it must be stressed that the control samples subjected to EDTA treatment were not able to remineralize upon immersion in HBSS under the same conditions (Figure 9A,B and S9), in agreement with previous studies.⁴⁰ These results allowed to deduce that arginine & Calcium carbonate toothpaste and HA & Citrate toothpaste, the toothpastes that remained to a higher extent on the surface of dentine samples (leading to higher values of $I_{CaP}/I_{Amide II}$ and $I_{1410}/I_{Amide II}$ at T0), were more able to promote remineralization and led to meanly higher $I_{CaP}/I_{Amide II}$ and $I_{1410}/I_{Amide II}$ after 24 h in artificial saliva. No analogous correlation was observed for the EDX Ca/N and P/N ratios, probably due to the above recalled differences in the sampling volumes between the IR and EDX techniques.

On the other hand, all the samples previously treated with the toothpastes in artificial saliva, the $I_{Amide II}/I_{Amide I}$ decreased to the values typical of untreated dentine (Figure 9C). This result shows that the interactions between collagen and Ca^{2+} that were lost upon EDTA treatment restored upon ageing, suggesting that the calcium phosphate phase formed on the dentine surface was intimately bound to it rather than a simple deposit.

Remineralization of dentine can occur either by the simple precipitation of mineral into the loose demineralized dentine matrix between collagen fibrils (net remineralization) or by the chemical tight association of mineral to the dentine matrix structure (functional remineralization). The simple precipitation of mineral generates an increased mineral content but may not necessarily provide an optimal interaction with the organic components of the dentin matrix.

It is interesting to note that the acidic attack did not remove completely the apatite phase formed upon immersion in artificial saliva. Actually, as shown in Figure 9A, the average values of the $I_{CaP}/I_{Amide II}$ IR intensity ratio measured for arginine &

Calcium carbonate toothpaste and HA & Citrate toothpaste application after citric acid treatment were significantly higher than those obtained after EDTA demineralization ($p < 0.05$). An analogous behaviour was observed for several single analysed samples, i.e., Arginine & Calcium carbonate toothpaste samples 1 and 2 (Figure S7), Zinc-HA toothpaste sample 3 (Figure S5 IR), HA & Citrate toothpaste sample 3 (Figure S4), Control toothpaste samples 1, 2 and 3 (Figure S8). Although no significant differences between the different treatments were observed at this cstep of the study either in $I_{CaP}/I_{Amide II}$ and $I_{1410}/I_{Amide II}$ or in the EDX ratios, some interesting trends may be observed. Figure 10 shows that the average $I_{CaP}/I_{Amide II}$ IR intensity ratio measured after citric acid treatment well correlates with the same ratio measured after immersion in artificial saliva (part B) and at T0 (part C). These trends (which were observed also for $I_{1410}/I_{Amide II}$, Figure S10, B and C) indicate that the samples that after acidic attack remained the most mineralized, being characterized by the meanly highest $I_{CaP}/I_{Amide II}$ and $I_{1410}/I_{Amide II}$ values, were those that at toothpaste application and after 24 h in artificial saliva had the highest $I_{CaP}/I_{Amide II}$ and $I_{1410}/I_{Amide II}$ values (i.e., were the

most mineralized). A poorer correlation was observed for the EDX data only for Ca/N and P/N ratios after citric acid treatment versus the same ratio measured after immersion in artificial saliva (Figure S11).

5 | CONCLUSIONS

The study demonstrated that:

1. All toothpastes induced the remineralization of dentine in artificial saliva.
2. Toothpastes containing arginine and calcium carbonate (arginine and calcium carbonate toothpaste) and hydroxyapatite (HA and citrate toothpaste) remained to a higher extent on the dentine surface and revealed a higher remineralization activity after immersion in artificial saliva, as demonstrated by higher $I_{CaP}/I_{Amide II}$ and $I_{1410}/I_{Amide I}$ intensity ratios. Both formulations also showed higher resistance to demineralization attack when compared to the other toothpastes.
3. The remineralization of activity of arginine and calcium carbonate toothpaste (arginine and calcium carbonate toothpaste) was attributed to direct interaction with the hydroxyl group of hydroxyapatites and to the alkalizing activity that favours the apatite nucleation.
4. The remineralization activity of hydroxyapatite-containing toothpaste (HA and citrate toothpaste) was attributed to the ions supplied from simulated body fluids rather than from hydroxyapatite ion dissociation, due to the basic environment.

6 | CLINICAL RELEVANCE

6.1 | Scientific rationale for study

An important requirement for oral health preventive dentistry is the development of materials able to induce the remineralization of hypomineralized dentine. Remineralization of demineralized/carious dentine occurs by incorporation of mineral ions from the oral fluid or from external sources.

6.2 | Principal findings

Tested toothpastes induced remineralization of dentine in artificial saliva. The formulations that remained to a higher extent promoted a higher mineralization.

6.3 | Practical implications

The mineral layer formed after toothpaste application may act as a constant site for apatite nucleation, promoting a continuous

remineralization over time and providing additional protection from food derived or bacteria acid attack.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

ORCID

Paola Taddei  <https://orcid.org/0000-0001-8478-4508>

Fausto Zamparini  <https://orcid.org/0000-0002-0071-4463>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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