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Investigation of the protective suitability of a dental fluorinated varnish by means of X Ray Fluorescence and Raman spectroscopy

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Investigation of the protective suitability of a dental fluorinated varnish by means of X Ray Fluorescence and Raman spectroscopy

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Abstract

Background and aim: Evaluating the protective effect in human enamel of a fluorinated varnish after enduring a citric acid erosive challenge.

Methods: An in vitro model was developed considering the intraoral environment, human saliva and acid erosive procedures. The evaluation of the enamel specimens was undertaken through the direct analysis of enamel by means of Raman spectroscopy and Energy Dispersive X Ray Fluorescence (EDXRF). Ten tooth specimens per group were analysed during three stages: 1- before treatment; 2- After varnish (treatment group) or toothpaste (control) application; 3- After citric acid cycle. Additionally, Particle Induced Gamma Ray emission (PIGE) was used to gauge the fluorine uptake by enamel after the application of the varnish (stage 2). Results were presented as mean and standard deviation with ANOVA and Tukey post hoc performed considering a significance level of 0.05.

Results: A significant ($p<0.05$) higher Ca levels were detected in treatment group at stage 2 (37.4±0.4 w/w%) and 3 (37.1±0.1) when compared to the control group. After varnish application in treatment group, depolarization ratios were significant lower ($p<0.05$) and anisotropy were significant higher ($p<0.05$), however no differences were detected in FWHM.

Conclusions: The use of a fluorinated dental varnish suggests a protective effect for human enamel against dental erosion demineralization process which was detectable in an in vitro model.

1. Introduction

Dental erosion is a multifactorial condition associated with chemical, biological, and behavioural factors whereby a non-bacterial chemical process leads to an irreversible loss of dental structure[1,2]. Based on the origin of the erosion-causing acids, a distinction between endogenous and

exogenous erosions is made. Intrinsic erosion most often affects the palatal and occlusal tooth surfaces, while extrinsically triggered erosion is initially localized on the labial surfaces of the anterior teeth. Consequences of this erosive process include painful sensitivity, susceptibility to further erosion, mechanical wear, changes in occlusion, exposure of dental pulp, and poor aesthetics [1,2]

Fluoride plays an important role in dental protection, as it strengthens teeth and prevents their decay by turning the surface enamel layers more resistant to the action of acids while accelerating the build-up of healthy minerals in the enamel. This protective role occurs by allowing the formation of hydroxy-fluorapatite or calcium fluoride, forms of hydroxyapatite more resistant to bacterial demineralization. According to Ismail et al[3] and Medjedovic et al.[4], the presence of fluoride in the oral environment and its subsequent incorporation can even prevent the already started tooth decay and demineralization process. Also, the fluorine available in the medium is highly anticariogenic since it causes a worthless and ineffective cycle of fluoride in main bacteria[5]

However, fluoride can only exert its protecting and remineralizing effect when it acts as a free and soluble agent in the aqueous surrounding oral environment (biofilm fluid or saliva). The appropriate use of fluoride will chemically reduce the mineral loss induced by the combination of biofilm accumulation and the production of acids from its exposure to sugars, through the precipitation of a fluoridated mineral on teeth [4–6].

Raman spectroscopy has proven to be a suitable method for a fast detection and identification of molecules and minerals[7,8]. Due to recent advancements in instrumentation that improved its sensitivity, along with non-destructive features, Raman has become one powerful tool for biomedical applications, namely Dentistry[9–11].

Raman spectroscopic technique has been employed for the examination of tooth samples to evaluate the demineralization effect of pharmaceutical products, such as whitening gels[12–14] and gustative stimulants[15] and to assess enamel mineralization degree and caries detection[16,17]. Sa et al.[18], who investigated the efficacy of micro-Raman spectroscopy on detecting mineral content change during the demineralization and de/remineralization cycling process, reported that phosphate peak intensity decreases dramatically during demineralization. It is known, however, that different teeth or even different locations on one tooth can have different Raman scattering efficiency due to bio-heterogeneity, therefore the measured Raman intensities could vary from person to person or even from tooth to tooth. In contrast, these parameters, as forms of peak ratios, respond to the underlying structure but not to the types of experimental variations[7]. By using polarized Raman measurements and comparing anisotropy and depolarization ratio instead of comparing actual peak intensities potential problems caused by instrumental variations and sampling heterogeneity are, hence, avoided. This way, Raman spectroscopy is more suitably employed in the determination of changes in composition and

structure of human enamel by means of specific parameters such as full width at half maximum (FWHM), anisotropy and depolarization ratio of the symmetric stretching band of phosphate[14,16].
This technique has also proven valuable when used concomitantly with Energy Dispersive X Ray Fluorescence (EDXRF). EDXRF is a quantitative elemental technique suitable for the analysis of human dental tissues since these contain a variety of minerals, essential trace elements that are present as catalytic or structural components of large biochemical molecules[19]. Regarding human enamel samples, a decrease in Ca and/or P represent a clear indicator of demineralization[20–22].
In this work, the main objectives were to assess, in vitro, the protective effect of a dental varnish against the demineralizing effect of an erosive attack cycle, by evaluating the mineral content and distribution in human dental enamel. The studied parameters were obtained by means of Raman spectroscopy (polarized spectra acquisition) and EDXRF. Additionally, PIGE technique was used to analyse the F uptake in enamel, and it was adopted a similar approach of an erosive protocol used in a prior in-vitro study[15].

Materials and Methods

1. Specimen Selection and Sample Preparation

Twenty healthy (non-carious and non-restored) human teeth (incisive, canine, pre-molar and molar) previously extracted for periodontal or orthodontic reasons and preserved in a 0.5% (w/w) chloramine T3 – H_2O solution, at 4 °C, were carefully selected and evaluated by research dentists having as exclusion criteria the presence of superficial lesions observed employing a stereomicroscope (Meiji Techno EMZ 8RT, Japan).
Afterwards, teeth were cut with a precision diamond saw (Buehler Isomet 1000, USA) to obtain enamel specimens approximately 8x2 mm. Samples were stored in properly identified storage vials in a new chloramine solution until the beginning of measurements. Before measurements, each sample was brushed with a non-fluorinated toothpaste Couto (Pasta Dentifrica Medicinal Couto, Couto S.A., Portugal) rinsed with distilled water, cleaned and air dried. Fig. 1 depicts the EDXRF spectrum of the toothpaste.

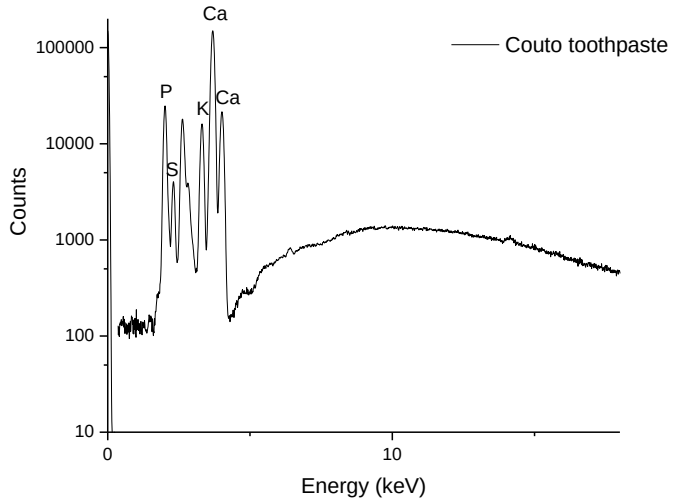

Fig.1 EDXRF spectrum obtained for the toothpaste. The presence of Ca and P, as well as other elements is highlighted. Unassigned bands correspond to the spectrometer.

2. Methodology and Experimental Groups

Figure 2 depicts the methodology of the study. Two experimental groups of human dental enamel samples, control (C) and treatment (T), each containing 10 tooth samples, were subjected to 5-immersion acidic cycle. Previously to the acidic attack, the T group was brushed with Couto toothpaste and treated with Fluoride containing dental varnish - VOCO Profluorid while group C was only brushed with Couto toothpaste. All sets of samples were analysed using Raman and Energy Dispersive X-Ray Fluorescence spectroscopies during three distinct stages: 1 - before any treatment, 2 - after application of dental varnish and/or toothpaste and 3 - after citric acid erosive attack procedure. Group T was also examined using PIGE and the fluorine content was evaluated. Raman spectra evaluation XRF methodology and F quantification using PIGE technique were previously described[14,20]. Products were applied according to manufacturer instructions. Between applications and treatment procedures, all teeth samples were brushed with a mild toothbrush and stored in renewed human saliva, to better simulate in vivo conditions[12].

2.1. Human saliva

Human saliva was collected from different non-smoking donors within the academic community. After collection, the saliva was frozen and maintained at -80º C to avoid any possible fluctuations in the pH and protein degradation with time[12] and thawed, mixed all together 30 minutes before each experimental use.

2.2 Citric acid solution

Figure 2 illustrates the erosive protocol. All dental enamel samples included in our study (n = 20) were kept and maintained in chloramine solution, and then carefully prepared and submitted to acidic erosive attack, as follows: acid erosive challenge (0.3% citric acid, pH 2.6,) during 5 minutes, human saliva (during 2 h), similarly to Zanatta et al. [23], and afterwards thoroughly washed, cleaned and maintained in chloramine solution.

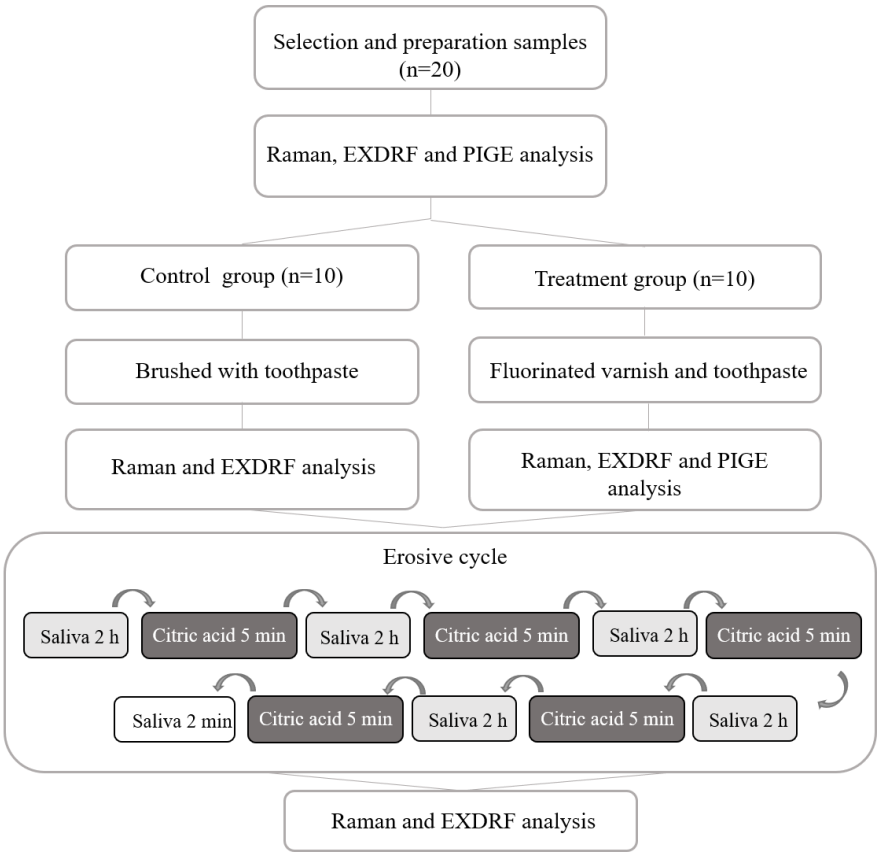

Figure 2 – Flowchart of the methodology employed in the surface enamel sample examination, during all study stages.

3. Experimental setup –

3.1. Micro energy dispersive X-ray fluorescence (µ-EDXRF)

The µ-EDXRF system employed in this study consisted in the M4 Tornado (Bruker, Germany). The X-ray tube was a micro-focus side-window Rh tube powered by a low-power HV generator and cooled by air. A polycapillary lens was used to obtain a spot size down to 25 µm for Mo-Kα. The filters used to reduce the background were a combination of 100 µm Al/ 50µm Ti/ 25µm Cu.

Detection of fluorescence radiation was performed by an energy-dispersive silicon drift detector with 30 mm^2 sensitive area and energy resolution of 142 eV for Mn-Kα. Measurements were performed directly on the samples before and after each treatment with an average of 6 measured areas in each dental enamel sample. Acquisition parameters were 50 kV and 400 μA. Spectra deconvolution, fitting and quantification was performed using the in-built M4 Tornado software based on fundamental parameter method for bulk samples considering a matrix of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. Accuracy tests before starting the measurements were also performed, for establishing the detection limits for this method [20].

3.2. Raman spectroscopy

All acquired spectra of the examined samples in our study were obtained with a XploRA Raman Confocal Microscope (Horiba, France) employing a laser diode source operating at 785 nm, using a 1200 lines/mm grating. This way, the spectral range investigated was from 300 cm^{-1} to 1800 cm^{-1}, resulting in a spectral resolution of 4 cm^{-1}. Using an entrance slit of 200 μm, and a confocal hole of 300 μm, the scattered light collected by the objective was dispersed onto the air-cooled CCD array of an Andor iDus detector.

A 100x objective (N.A.= 0.9) was used to focus on the surface of enamel, as well as a 50% neutral density filter rendering an incident power on the sample of 5.0 ± 0.4 mW (lasercheck®, Edmund optics). For each sample, an average of 20 measurements (10 different points with parallel polarization between the incident radiation and the scattered one, always followed by other 10 with cross polarization) was performed. The exposure time for each measurement was 20 s with 4 accumulations. Fig. 3 compares two Raman spectra acquired with two different – parallel and cross polarization configurations. The parameters obtained and compared in each step of the study were depolarization ratio, anisotropy and band full width at half maximum of the symmetric stretching band of phosphate (υ_1 PO_4^{3-} ~ 959 cm^{-1})[14].

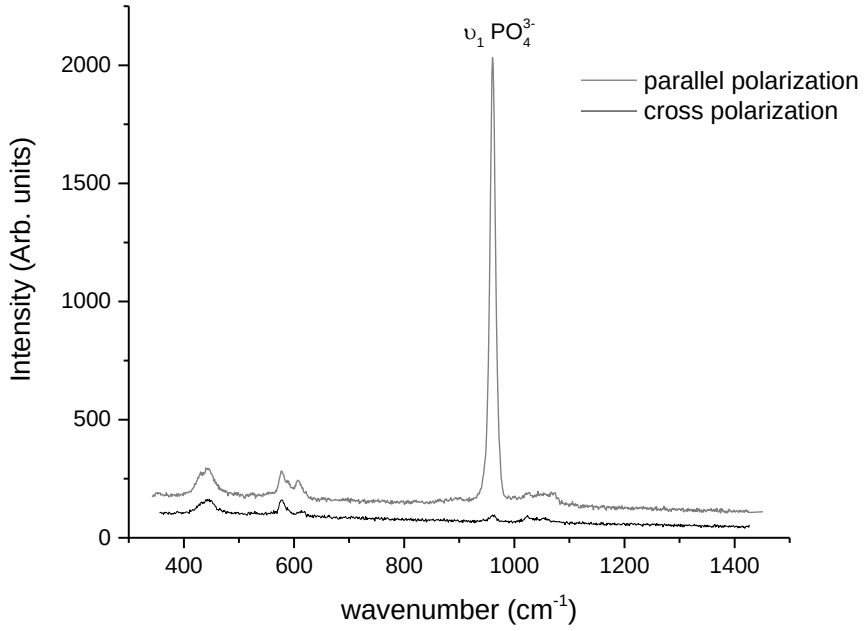

Figure 3– Comparison of two Raman spectra obtained for sound enamel with parallel polarization cross polarization before baseline subtraction.

4. Fluorine uptake assessment

In order to establish if the fluoride containing dental varnish - VOCO Profluorid, effectively incorporated fluorine in the treated samples, Particle Induced Gamma-ray Emission analysis were undertaken.

4.1. Particle induced gamma-ray emission (PIGE) setup

The experimental work for PIGE was carried out at the IST/CTN 3 MV Tandem accelerator in Lisbon (Nuclear Technological Campus – IST), using proton beam energy of 3100 keV. Before entering the reaction chamber, the beam passes through a collimator which defines a beam spot of around 1 mm on the sample, allowing, in this case, two or three different spots per sample to be analysed. The fluorine gamma-rays from the $^{19}F(p,p'\gamma)^{19}F$ nuclear reaction were detected by a 45% HPGe (High-Purity Germanium) semiconductor detector located at 130° in relation to the incident beam direction and a distance of 55.5 mm with respect to the interaction point. The areas of the 197 keV gamma-ray peaks were obtained by an automatic script developed for Origin and ERYA software was used to quantify fluorine concentrations. The ERYA based quantification methodology was previously published [24,25].

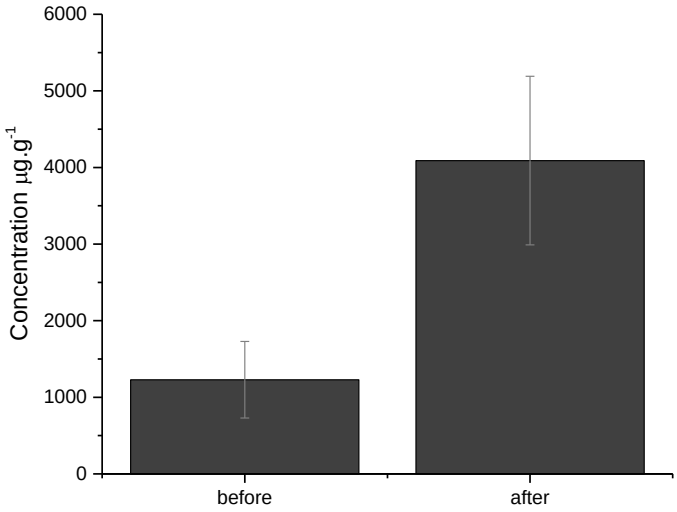

Figure 4 –Comparison of ^{19}F mean elemental concentration and standard deviation, obtained with PIGE technique between Stage 1 – before any treatment and Stage 2 – after dental varnish and toothpaste application.

In Figure 4 are presented the mean ^{19}F concentration values, determined for the samples in the treatment group. Error bars correspond to the standard deviation, relatively high due to the variability within samples. Regardless of this heterogeneity, there was an over 200% increase in ^{19}F concentration (1200 ± 700 µg/g to 4000 ± 1000 µg/g) after dental varnish application on the tooth enamel surface.

5. Statistical analysis

All data collected was analysed using IBM SPSS v25.0 software (IBM Statistics, Inc. Chicago, IL, USA). Parametric tests were used since the study had a sufficiently large sample size (all variables with 100 measurements for each sample except for EDXRF with 60 measurements) according to the central limit theorem[26]. ANOVA tests with Tukey post hoc were performed to analyse intragroup differences between study stages while paired t tests were performed to analyse intergroup differences at each study stage. It was considered a significance level of p=0.05.

6. Results

Ten teeth per study group were analysed without any drop-out during all proceedings. After analysis, all obtained results comprising the mean values and standard deviations are graphically represented in Figures 5-8. Results are presented for each group regarding the three treatment stages: Stage 1 – before

any treatment, Stage 2 – after dental varnish and/or toothpaste application and Stage 3 – after citric acid attack procedure.

EDXRF results for the elemental concentration of Ca during all three study phases, for both of groups, are presented in Fig. 5. The bar-charts show a slight, however statistically significant increase ($p< 0.05$) in Ca levels samples treated with fluorinated varnish, from 37.0 ± 0.8 to 37.4 ± 0.4 w/w%. After the acidic attack, the Ca concentration decreases in both study groups but presenting significant differences between groups ($p=0.05$). Indeed, there was a more prominent decreased for the control group 37 ± 2 w/w% ($p<0.01$) compared with the treatment group 37.1 ± 0.1 ($p=0.04$) concomitant with a decrease in the precision of the determined value.

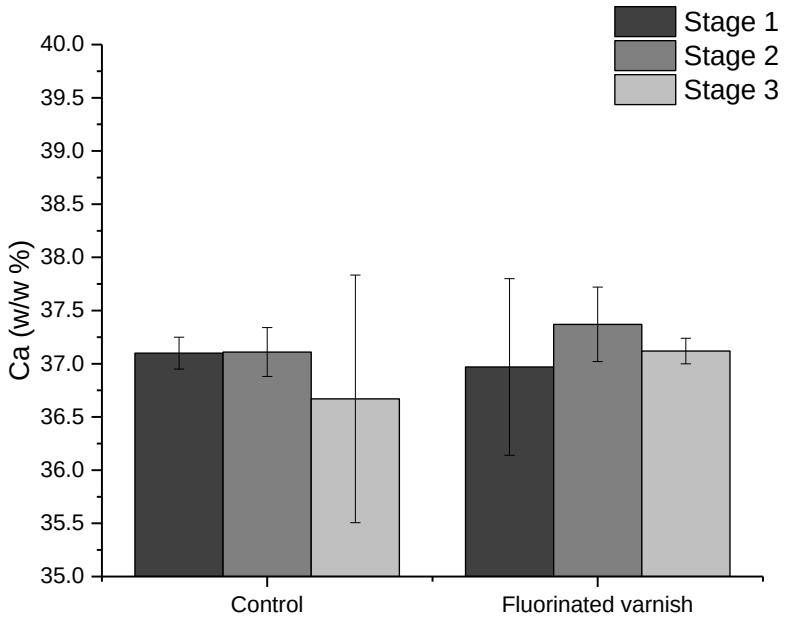

Figure 5 – Mean Ca concentration (w/w %) obtained for both groups during the different stages of the study (1 – no treatment, 2 – Dental varnish and/or toothpaste application, and 3 – Erosive cycle). Significant differences were found between stage 1 and 2 in the treatment group ($p<0.05$) and between stages 2 and 3 for both groups ($p=0.05$ and $p<0.01$ respectively for control and treatment groups).

Depolarization ratio (ρ959) values are presented in Figure 6 for control and treatment groups, during all study stages. Paired t test showed no significant differences between the two groups at Stage 1 of the study, however, significant differences were found between control and treatment group due to the application of the fluorinated varnish ($p<0.01$). In fact, there was a significant decrease of this parameter in the treatment group, from 0.07 ± 0.06 to 0.03 ± 0.02 ($p<0.01$ post hoc) and no significant change in the control group. Similarly, the depolarization ratio between control and treatment groups after acidic cycle of the study presented significant differences ($p<0.01$). However, although there was an increase of this parameter in both groups, it was not statistically different.

Regarding the anisotropy parameter (A959), there were also no significant differences among the 3 stages of the study for the control group (Fig. 7). This parameter was also similar between groups at stage 1 of the study. However, after the application of the fluorinated varnish there was a significant increase for the treatment group, from 0.9 ± 0.1 to 0.93 ± 0.04 ($p<0.01$ post hoc). After acidic attack, this parameter exhibits a significant decrease ($p<0.01$ post hoc) towards the initial values in Stage 1 0.90 ± 0.04.

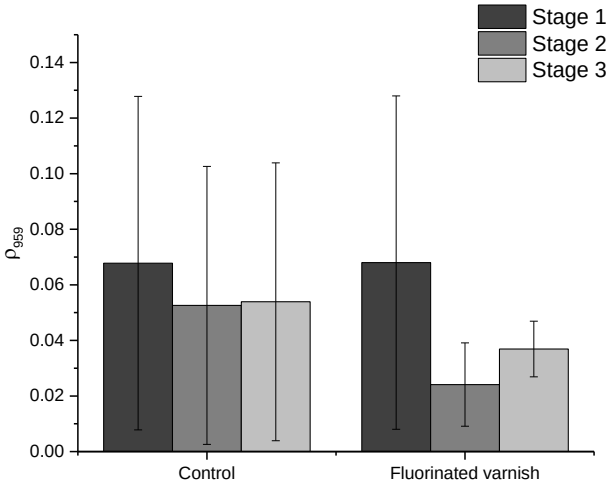

Figure 6 – Mean Depolarization Ratio obtained for both groups during the different stages of the study (1 – no treatment, 2 – Dental varnish and/or toothpaste application, and 3 – Erosive cycle).
Significant differences were found between stage 1 and 2 in the treatment group ($p<0.01$).

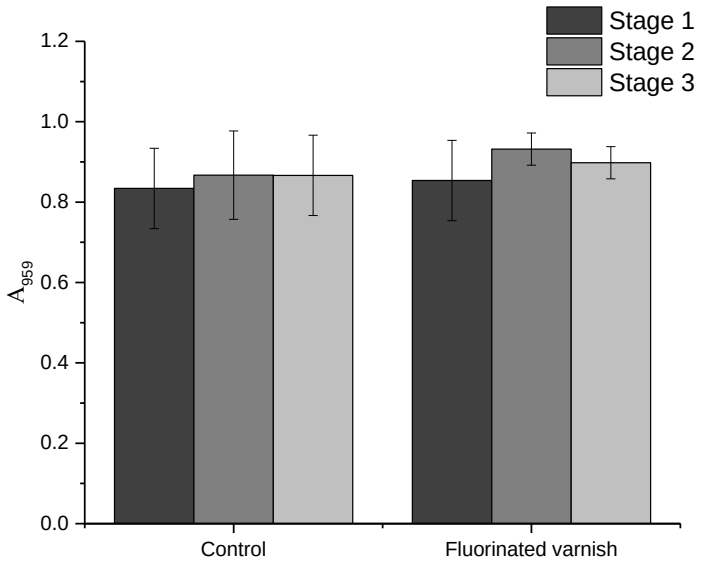

Figure 7 – Mean Polarization anisotropy obtained for both groups during the different stages of the study (1 – no treatment, 2 – Dental varnish and/or toothpaste application, and 3 – Erosive cycle).
Significant differences were found between stage 1 and 2 in the treatment group ($p<0.01$).

The evaluation of the full width at half maximum (FWHM) of the main phosphate Raman band (Fig. 8), rendered no significant differences between Control and Treatment in the 3 stages of the study. Also, no significant differences in this parameter were caused by the application of fluorinated varnish and/or toothpaste as well as the acidic attack.

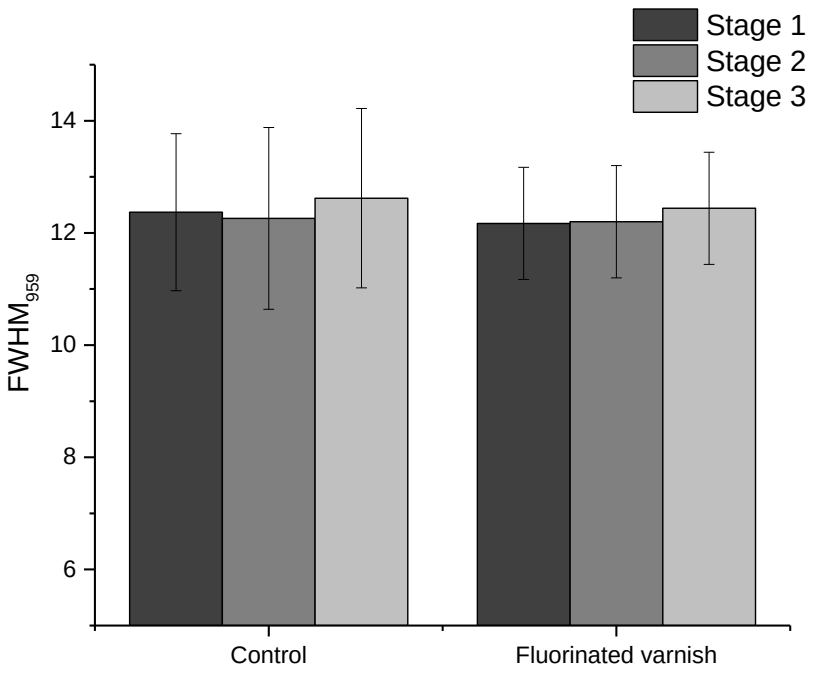

Figure 8 – Mean Full-Width-at-Half-Maximum (FWHM) for the symmetric stretching peak obtained for both groups during the different stages of the study (1 – no treatment, 2 – Dental varnish and/or toothpaste application, and 3 – Erosive cycle).

7. Discussion

This study intended to assess the protective effect of a dental varnish against the demineralizing effect of an erosive attack cycle. Data obtained indicate a significant loss of mineral content, namely calcium, after performing the tested erosive cycle. Moreover, this reduction in mineral content is more prominent in control group for which enamel samples were just brushed with fluorine-free toothpaste thus reinforcing the protective effect of topical fluoride application, with surface adherent remineralizing capacity. This effect was verified both by a lower mean Ca content for the control group samples as well as a higher standard deviation in the determined value, indication higher heterogeneity in the Ca content in the samples. The fluctuations in the Ca content throughout the study can also be accounted by another phenomenon. In fact, there is a significant increase in Stage 2 of the study that could be due to the topical application of toothpaste containing Ca and P (Fig.1), but it was not the case for the control group also brushed with the same toothpaste. X rays can penetrate considerable depths in matrices such as hydroxyapatite, meaning that the probed volume in a EDXRF measurement could be within tenths of micrometres for Ca[20], so the superficial and slight increase of this element might not be measurable. However, the significant increase in treatment group could mean that the incorporation of Ca (and P) in the enamel could have been enhanced by the concomitant application of the varnish.

Regarding the polarized Raman measurements, the gauging of protective effect of the varnish against acidic attack was overwhelmed by the effect that the application of the fluorinated varnish had on the enamel, namely, with the significant decrease of the depolarization ratio and increase of anisotropy in Stage 2. After application of the protective varnish and toothpaste, the decrease of $\rho 959$ is more significant, corresponding to a substantially greater alteration of the structure of enamel. Sound human enamel is composed of carbonated hydroxyapatite crystals bundled in a highly ordered structure, the majority of enamel rods have one orientation. However, the orientation of rod arrangement changes in a demineralized/remineralized region causing an increase/decrease of depolarization ratio and concomitant increase/decrease of anisotropy[7,27]. Mineralization of enamel after use of fluorinated whitening product was already determined by Bollineni et al. [28] after application of 10% carbamide peroxide gel with 0.463 % of F. Similar decrease of depolarization ratio, although in lesser extent, also was verified in the samples in the control group, brushed with Ca and P containing toothpaste. There is again evidence that the toothpaste plays a role in mineralizing enamel, here more evident because of

the reduced probed volume in a Raman measurement, within 1 micrometre, hence more sensitive to superficial changes.

Regarding the FWHM, a measurement of the crystallinity of the analysed material through the narrowness of the phosphate band, although there was a slight increase of this parameter after acidic attack, this was not significant. Pessanha et al. [14] determined significant broadening of the phosphate band after polishing enamel samples concomitant with the decrease of hardness obtained with Vickers test for the same samples after whitening with dentist-supervised nightguard bleaching product composed of 16% carbamide peroxide. The conservation of this parameter through our study might indicate that it is less sensitive to superficial changes caused in the enamel crystalline structure than depolarization ratio. Similarly, Buchwald et al.[29] found no significant correspondence between FWHM and the severity of carious lesion in enamel.

The robustness of our findings could only be improved by the analysis of an increased number of samples, as it has been shown that dental enamel mineralization and response to dental products and acid attacks varies substantially between individuals[30].

Conclusion

This study demonstrated that erosive acid procedures result in mineral loss, whilst control enamel samples treated only with dental toothpaste are more prone to demineralization and acid erosion. Dental varnish products used according to manufacturer instructions seem to preserve enamel integrity and structure of enamel surface layers, as well as the elemental Ca concentration, promoting remineralization and preventing the demineralization process. Dental fluorinated products efficiently incorporate fluorine in the surface aprismatic enamel crystallin structures.

Acknowledgments

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Disclosures

The authors of this research paper declare no conflict of interest.