

Comparative Study Using Scanning Electron Microscopy and Energy Dispersive X-Ray Spectroscopy to Assess Morphological Modifications to the Enamel Surface Produced by Three Sodium Fluoride Solutions

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Abstract

Aims: The first aim of this *in vitro* study was the comparative assessment of enamel surface morphology and analytical assessment of calcium and fluoride ions after topical application of three sodium fluoride solutions: solutions A and B having the same concentration (0.05% sodium fluoride) and solution C having a higher concentration (0.1% sodium fluoride). The second aim was to assess the remineralisation capability of solution A, which has not previously been studied. **Methods:** Twelve sound premolars, extracted for orthodontic purposes from patients aged between 12-15 years, were used. Demineralisation areas were created by etching with 37% phosphoric acid gel for 60 seconds. Enamel sections were immersed in 100 ml of three solutions, twice daily for 30 days: solution A—Fluorostom (0.05% sodium fluoride), solution B—Colgate Total Protection (0.05% sodium fluoride), and solution C—Sensodyne ProNamel (0.1% sodium fluoride). Surface examination was performed using a scanning electron microscope. The calcium and fluoride concentrations in the enamel surfaces were assessed using energy-dispersive X-ray spectroscopy. **Results:** Mean remineralisation depths were: 5.73 μm [95% CI, 2.4-12 μm] for A, 5.63 μm [95% CI 2.5-12.7 μm] for B, and 8.28 μm [95% CI 2.91-15.54 μm] for C. There were significant differences between A versus C ($P=0.0006$) and B versus C ($P=0.003$) but no significant differences between A versus B ($P=0.83$). Morphological appearance of fluoride as globular precipitates was revealed in all treated specimens, regardless of ionic fluoride (F^-) content. The distribution of the deposits was more homogeneous and smaller in groups treated with higher fluoride concentrations and larger in groups treated with lower fluoride concentrations. The energy-dispersive x-ray spectroscopy revealed that the intensity of signals for calcium and fluoride varied significantly across the three groups ($P=0.0001$). **Conclusions:** In the teeth studied, the remineralisation depths depended on the fluoride concentration of the solutions. Globular structures of amorphous calcium fluoride precipitates, which act as a fluoride reservoir, were observed on the enamel surface after the action of all solutions. The differences between solutions A and B were not statistically significant.

Key Words: Sodium Fluoride Solution, Enamel, Scanning Electron Microscopy (SEM), Energy-Dispersive X-Ray Spectroscopy (EDS)

Introduction

The use of fluoridated mouth rinses has achieved a significant level of popularity among the public due to health care programmes, particularly those involving school-age children.

Topical applications of fluoride-containing products have demonstrated effectiveness in preventing and controlling dental caries [1,2]. A better post-eruptive effect and anti-caries effectiveness is obtained through frequent applications of low concentrations of fluoride [1]. The use of fluoridated

mouth rinses were and are recommended by the World Health Organization as alternative mechanism for caries prevention and as a treatment method to encourage remineralisation, which has an impact on public health [3]. Fluoride concentration in these solutions (mouth rinses) varies between 0.2% and 0.5% ionic fluoride (F^-), depending on the recommendation for a daily or weekly use [3].

After five years of studies, Ripa *et al.* (1983) concluded that the weekly use of mouth rinses with a neutral 0.2% sodium fluoride solution reduced the

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prevalence of caries in schoolchildren by 50% [2]. Previous studies have shown that after rinsing with solutions containing 0.2% and 0.05% sodium fluoride, even after a short period, deposits of calcium fluoride (CaF_2) can be observed on the enamel surface [1]. In Constanta (Romania), there was an average decrease in decayed/missing/filled teeth (DMFT) scores of 24% and in decayed/missing/filled surfaces (DMFS) by 20% after the first four years as a result of a dental caries prevention programme for children aged between 6 and 12 years [4]. In this programme, a fluoride (0.05%) mouth wash (Fluorostom) was used. After seven years of the fluoride mouth-rinsing programme, the mean DMFT score for 12-year-old children decreased by 12% in Iasi and by 29% in Constanta [5].

Several methods have been proposed for evaluating the anti-caries potential of commercial products containing fluoride. Among them, the most commonly used *in vitro* was simulation of the physicochemical effect of fluoridated products [6,7]. Lately, it has been suggested that calcium fluoride or a calcium fluoride-like material is deposited on enamel after exposure to fluoride solutions and that it is responsible for the cariostatic mechanism of topical fluoride [8]; therefore, the assessment of fluoride action should focus on the capacity of the solution used to form this type of compound and to provide a reservoir of fluoride [9,10].

Gerould (1945) [11], using scanning electron microscopy (SEM), was the first to report the presence of calcium fluoride deposits on human tooth enamel surfaces after the application of topical fluoride. Subsequently, this has also been reported by other authors [7,12]. Furthermore, it has been suggested that calcium fluoride may serve as a source of ionic fluoride whenever the pH falls to very low levels and that it plays an important role in the demineralisation and remineralisation processes of enamel. This is because, during the cariogenic challenge, calcium fluoride releases fluoride ions that are subsequently incorporated into enamel as fluorhydroxyapatite (FHAP) or fluorapatite (FAP) [13].

The morphological appearance of fluoride deposits on the surface of tooth enamel, when viewed using a scanning electron microscope, has been variously described as micro-crystals or as amorphous coatings. Most frequently, spherical globular deposits have been described [13]. Some authors have speculated that phosphate is responsible for the globular structure because pure calcium fluoride is cubical in shape [14].

Aims

The first aim of this *in vitro* study was the comparative assessment of enamel surface morphology and analytical assessment of calcium and fluoride ions after topical application of three sodium fluoride solutions: solutions A and B having the same concentration (0.05% sodium fluoride) and solution C having a higher concentration (0.1% sodium fluoride). The second aim was to assess the remineralisation capability of solution A, which has not previously been studied.

Methods

The samples used in this investigation were produced from 12 caries-free premolars that had been extracted for orthodontic purposes from patients aged between 12-15 years. The parents of these patients agreed that the teeth could be used in this study and gave their written consent.

The solutions used in the study were:

- A: Fluorostom (National Institute of Chemical-Pharmaceutical Research, ICCF Bucharest, Romania): 0.05% sodium fluoride (220 ppm F^-).
- B: Colgate Total Protection (Colgate-Palmolive Co., New York, USA): 0.05% sodium fluoride (220 ppm F^-).
- C: Sensodyne® Pronamel™ (GlaxoSmith-Kline, Weybridge, UK): 0.1% sodium fluoride (450 ppm F^-).

The enamel surfaces of the samples were analysed using a stereomicroscope (Olympus SZX7; Olympus Corporation, Tokyo, Japan). Energy-dispersive x-ray spectroscopy (EDS) was used for the quantitative analysis of the chemical characterisation of surface deposits (Inspect S; FEI Company, Eindhoven, The Netherlands).

Ethical approval

Ethical permission to conduct the study was given by the Professional Ethical Committee of Ovidius University of Constanta and by the Ethical Committee of the Medical College of Constanta District.

Sample preparation

All the extracted teeth were kept in a sterile container containing isotonic sodium chloride (NaCl) solution of 154 mEq/l (saline), followed by the removal of the organic material from their surface by immersion in a 10% solution of sodium

hypochlorite (NaOCl). Then, they were brushed for 60 seconds with a brush on a dental handpiece using an abrasive, fluoride-free, prophylaxis paste (Clean Polish; KerrHawe, Bioggio, Switzerland) and water.

Samples were sectioned using diamond discs mounted in a handpiece. Sections were cut longitudinally in the bucco-palatal or bucco-lingual plane of the teeth. Each tooth yielded four sections of about 4-5 mm (*Figure 1*). After sectioning, all samples were rinsed with distilled water.

Demineralisation

One-third of each sample surface was covered with acid-resistant colourless nail varnish, so that this part of the enamel remained intact (*Figure 2*). The remaining uncovered two-thirds were subjected to demineralisation by etching with 37% phosphoric acid gel for 60 seconds (*Figure 3*).

At the end of the 60 seconds, all sections were thoroughly washed with distilled water and dried with the air spray from the dental unit. The demineralised external third was covered with red nail varnish to protect the initial demineralisation, leav-

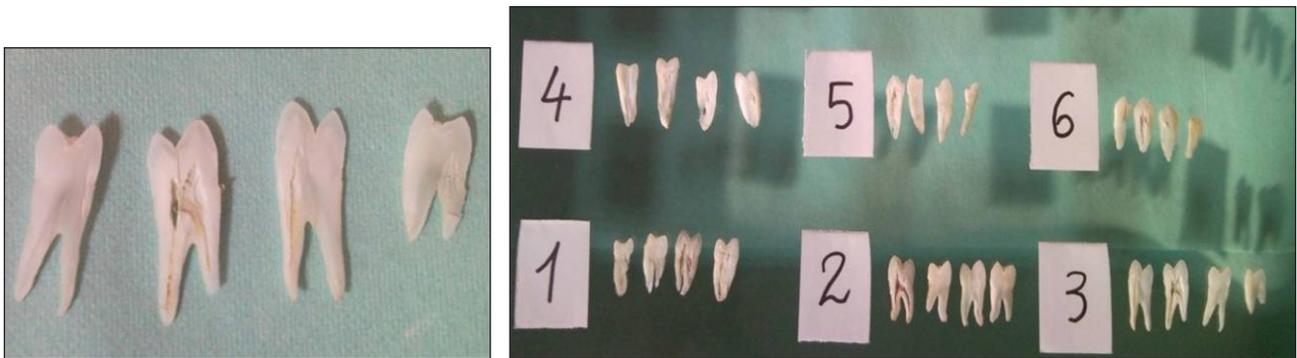


Figure 1. Bucco-palatal or bucco-lingual sections.

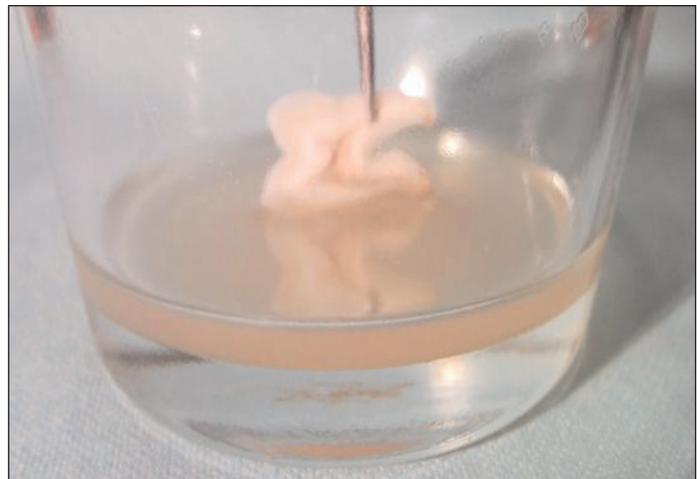
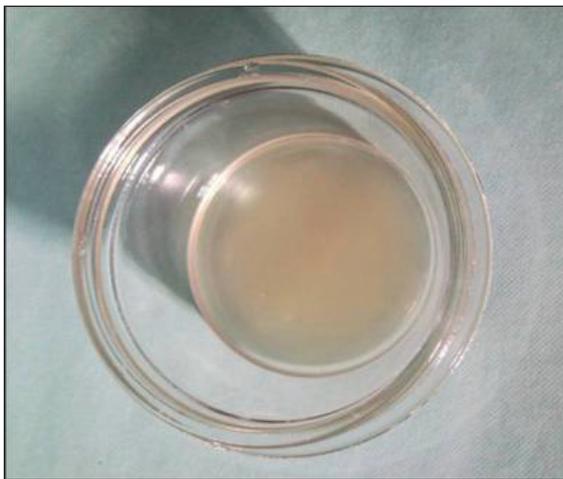


Figure 2. Nail varnish covering one-third of enamel that remained intact.



Figure 3. Demineralisation of the remaining uncovered two-thirds of the tooth.

ing the middle third uncovered for exposure to the fluoridated solution for remineralisation. The 48 sections were labelled and randomly allocated to four groups (A, B, C, M), so that a section from each tooth was present in each group.

Remineralisation

Each group was exposed to a different solution as follows (*Table 1*):

Group A: Fluorostom 0.05% sodium fluoride (220 ppm F⁻).

Group B: Colgate Total Protection 0.05% sodium fluoride (220 ppm F⁻).

Group C: Sensodyne® ProNameI™ 0.1% sodium fluoride (450 ppm F⁻).

Group M: Distilled water, forming the control group.

The enamel sections were placed in 100 ml of each solution twice a day, for 3 minutes, for 30 days. The fluoride solutions were renewed daily. In the interval between applications, all sections were kept in 100 ml distilled water. All procedures were performed at a room temperature of 26°C.

Specific preparation for the sample analysis

After remineralisation, the samples were embedded in self-polymerising acrylic resin to form a cylindrical shape. After the required 24 hours for self-polymerisation, the samples were sliced perpendicularly in the long axis of the cylinder. Each slice showed all three situations: first third with sound enamel, middle third with the rematerialised enamel, and last third with initially demineralised enamel.

The sections were then assessed using the stereomicroscope in order to observe any induced structural changes of the enamel surface. In this

manner, all the representative areas were produced and could be observed by SEM (for examination of possible morphological changes occurring on the tooth surface) and EDS analysis (for determination of the chemical elements of the surface deposits). Although EDS can assess a wide range of elements, we focused our attention on calcium and fluoride.

The depths of demineralisation and remineralisation were obtained after measurements made on the analysed images. To determine whether or not the remineralisation for each group was statistically significant, a student's *t*-test was used to compare the mean remineralisation depth of paired groups. The ANOVA test was used to compare the mean remineralisation depth. Data were analysed using statistical software (Statistical Package for Social Sciences version 13; SPSS Inc, Chicago, USA). Statistical tests were performed to a significant level of 95% confidence interval and $P < 0.05$.

Results

Measurements of initial demineralisation and remineralisation depth

The stereomicroscope highlighted areas of enamel with fluoride remineralisation depth (penetration). Remineralisation and initial demineralisation depth were then analysed by SEM at X2000 magnification operated at 25.0 kV (*Figure 4 a-c*) and EDS. Mean remineralisation depths were: A 5.73 µm (2.4-12 µm), B 5.63 µm (2.5-12.7 µm) and 8.28 µm (2.91-15.54 µm) for C, with a 95% confidence interval (*Table 2*). Student's *t*-test applied to the mean values showed significant differences between A versus C ($P=0.0006$) and B versus C ($P=0.003$) but no significant differences between A versus B ($P=0.83$) (*Table 3*).

Table 1. Sodium fluoride solutions used in the study

Products	Amount	Fluoride concentrations	Origin	Application frequency	Application mode
Solution A: Fluorostom	100 ml	0.05% sodium fluoride (220 ppm F ⁻)	ICCF, Bucharest, Romania	Twice daily for 30 days	Immersion in solution
Solution B: Colgate Total Protection	100 ml	0.05% sodium fluoride (220 ppm F ⁻)	Colgate-Palmolive, New York, USA	Twice daily for 30 days	Immersion in solution
Solution C: Sensodyne ProNameI	100 ml	0.1% sodium fluoride (450 ppm F ⁻)	GlaxoSmithKline, Weybridge, UK	Twice daily for 30 days	Immersion in solution

Table 2. Mean initial demineralisation, residual demineralisation and remineralisation by group

Group	Initial demineralisation (µm)	Residual demineralisation (µm)	Remineralisation (µm)
A. Fluorostom	21.15 (13.6-37.3)	15.53 (10.8-27.8)	5.73* (2.4-12.0)
B. Colgate Total Protection	19.33 (10.4-33.0)	13.79 (4.2-29.8)	5.63* (2.5-12.7)
C. Sensodyne ProNamel	31.25 (13.5-51.9)	21.87 (8.7-38.9)	8.28* (2.91-15.54)

Value for depth in µm

* Paired difference *t*-test; *P*<0.05

95% confidence interval

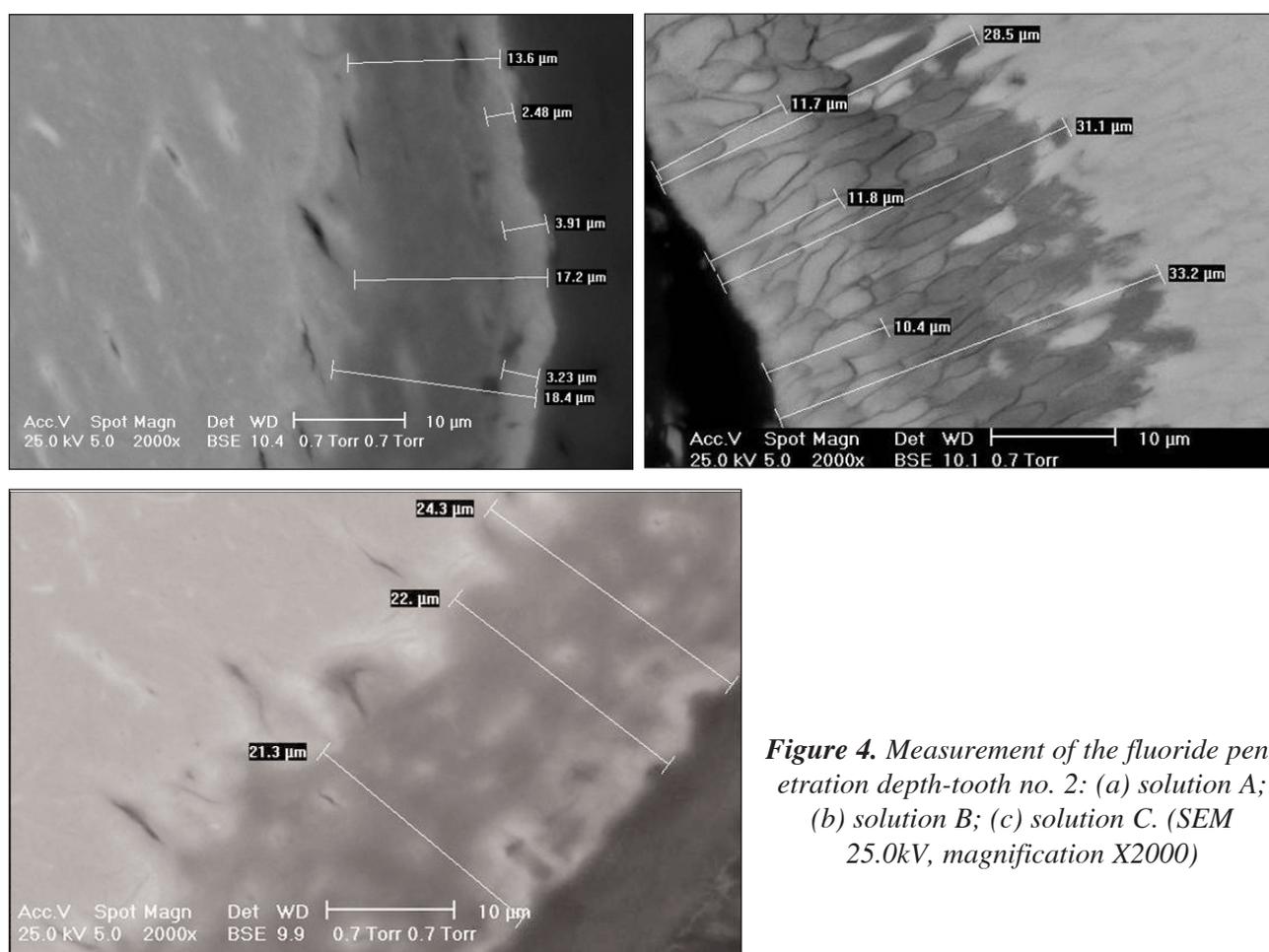


Figure 4. Measurement of the fluoride penetration depth-tooth no. 2: (a) solution A; (b) solution B; (c) solution C. (SEM 25.0kV, magnification X2000)

Table 3. Intergroup comparison of mean remineralisation

Group	Differences in means	<i>t</i> -test	Significance
GROUP A vs. GROUP B	21.15 vs. 15.53	0.20	0.88*
GROUP A vs. GROUP C	19.33 vs. 13.79	-3.76	0.0006*
GROUP B vs. GROUP C	31.25 vs. 21.87	-3.09	0.003*

Value for depth in µm

*Paired difference *t*-test; *P*<0.05

95% confidence interval

Because the control group was not subjected to the action of a fluoride solution, SEM enamel surface appearance was not modified, the image showing a similar situation with the demineralisation. Etching with 37% phosphoric acid for 60 seconds produced a type III etching pattern: irregular pattern of demineralisation, the surface structure of enamel was porous, with larger inter crystalline spaces.

Highlighting the presence of globular precipitate of calcium fluoride

Morphological changes on the enamel surface after application of fluoride in SEM revealed the presence of globular precipitate in all samples. The size and degree of agglomeration of the crystalline, amorphous, globular structures varied depending on the concentration of fluoride. These globular deposits did not completely cover the surface enamel in any of the observed samples. The density of the deposits on the enamel surface was higher when the fluoride concentration was higher, group C (Figure 5c). Deposits were spherical, regardless of the concentration of fluoride used. Globular deposits observed on enamel samples, treated with lower concentrations of fluoride, were fewer, but larger (Figure 5 a, b).

Elemental analysis of the sample by EDS

The EDS analysis highlighted the presence of calcium and fluoride ions in all analysed sections. After collecting EDS spectrum, an automatic identification of items was carried out, followed by per-item quantifying, so the letter that appears after each item is the energy level at the time of collection. The collection was made at three energy levels: K, L and M (weight-mass ratios, at-atomic percentages) (Figure 6 a-c).

Calcium was the predominant element in all sections studied. The fluoride signals showed an upward trend in the samples treated with solutions with higher concentration (group C) and signals for group A were similar to those in the group B. In the enamel of the control group, fluoride was below the limit of the limit of detection.

The results of the one-way ANOVA test allow the acceptance of the research hypothesis that the calcium and fluoride would vary significantly across the three groups ($P=0.0001$) (Table 4).

Discussion

The efficiency of fluoride ions present in mouth rinses has been demonstrated in recent *in vitro* studies [6]. These results have been supported by *in vivo* studies [3].

The scanning electron microscope has been used by several authors [11,15,16] to assess the demineralisation/remineralisation effect of fluoride-containing products. In most studies using a scanning electron microscope, samples have been coated with metals such as gold or palladium to improve image quality [11,15-17]. Harding *et al.* (1994) [17] observed samples using the scanning electron microscope without coating so that they could be observed again, if necessary, after the study ended. In the current study, this technique was used so that there would be an option of re-evaluation in the future.

Nelson *et al.* (1983) [16] suggested that the particle size of calcium fluoride crystallites can be an important factor in determining the effectiveness of a topical fluoride agent. It is well known that for particles in the nanometre range, the solubility of crystals decreases with increasing their size. Thus, calcium fluoride particles that are smaller would be more soluble than larger crystals. Therefore, it can be assumed that the higher globules observed in

Table 4. ANOVA TEST: Elements (Ca, F) composition of the enamel

		Sum of Squares	df	Mean Square	F-test	P-value
Ca	Between-groups	518.18	2	259.09	16.27	0.000 (<0.05)
	Within-groups	429.71	27	15.91		
	Total	947.90	29			
F	Between-groups	3.22	2	1.61	21.68	0.000 (<0.05)
	Within-groups	0.96	13	0.07		
	Total	4.18	15			

One-way ANOVA test, $P<0.05$

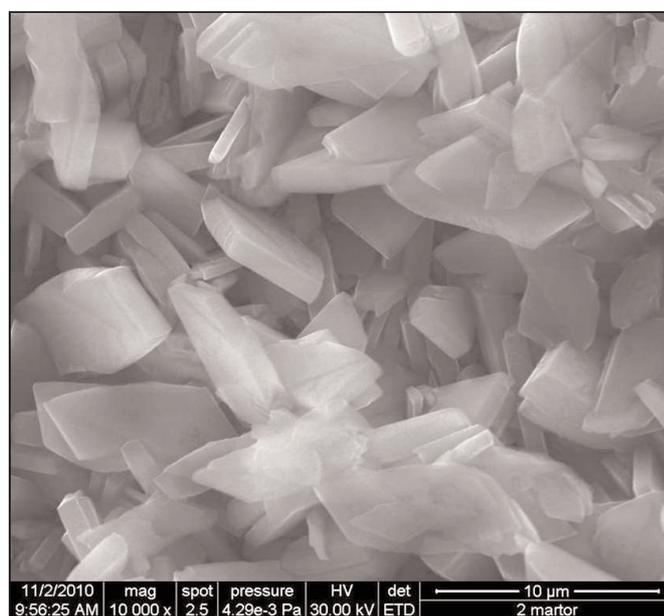
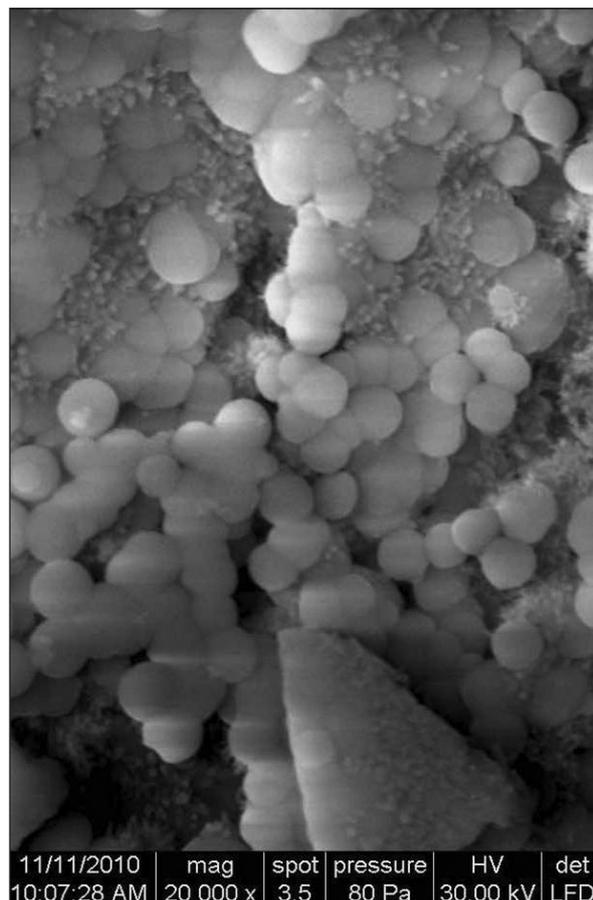
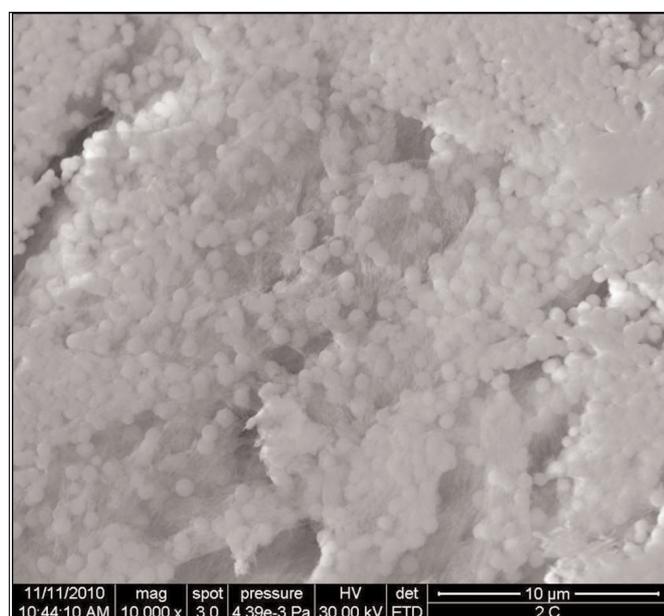
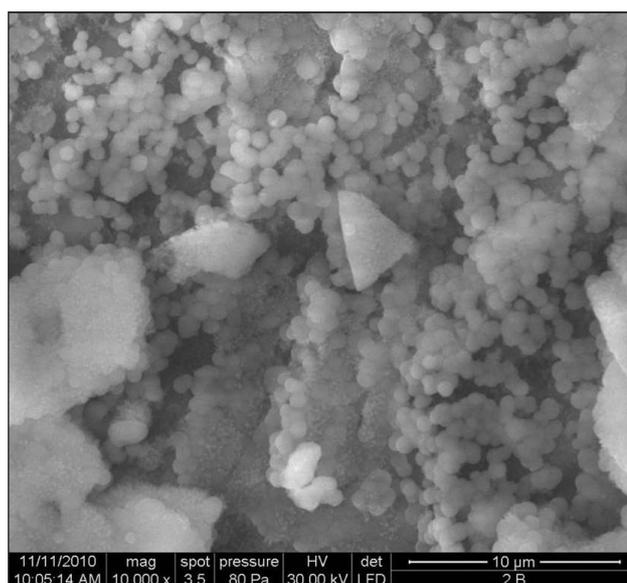
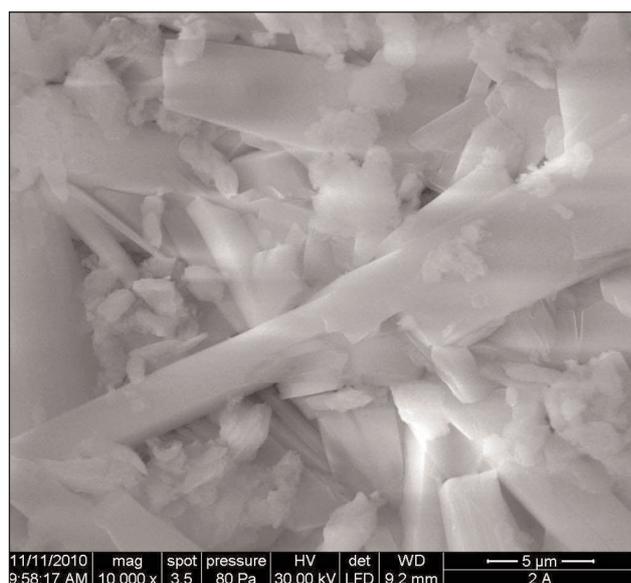
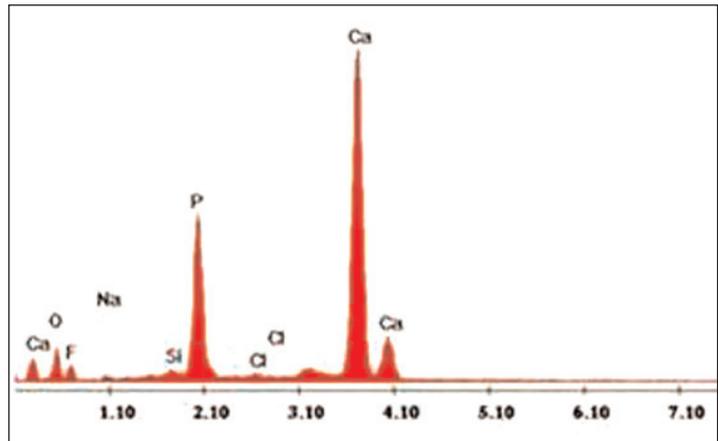
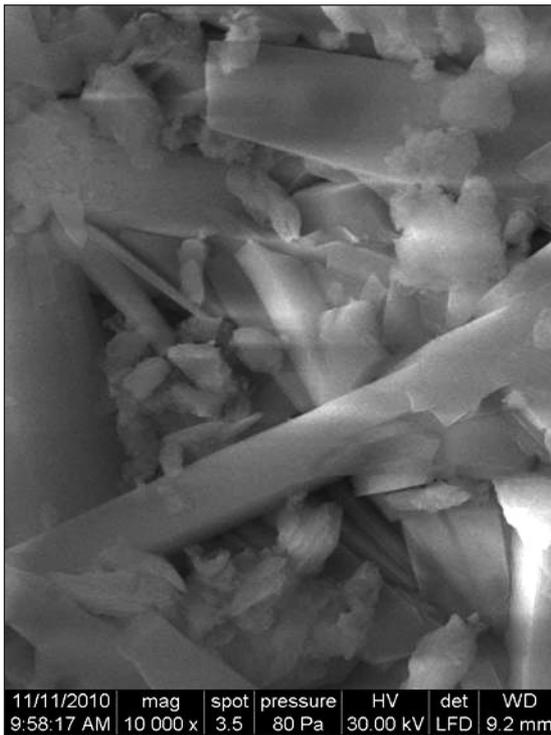


Figure 5. Deposits of CaF_2 on the enamel surface-tooth no. 2: (a) solution A; (b) solution B; (c) solution C; (d) control (SEM 30.0kV, magnification X10 000); (e) higher magnification of (a) tooth no. 2, solution A (SEM 30.0kV, magnification X20 000).



EDAX ZAF Quantification (Standardless)
 Element Normalized
 SEC Table : Default

Elem	Wt %	At %	K-Ratio	Z	A	F
F K	10.90	14.40	0.0118	0.9814	0.1099	1.0005
CaK	41.31	25.87	0.3644	0.9801	0.8999	1.0000

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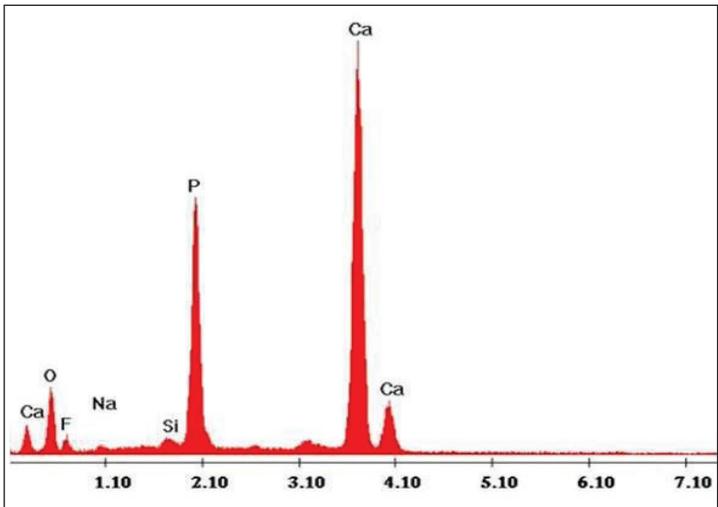
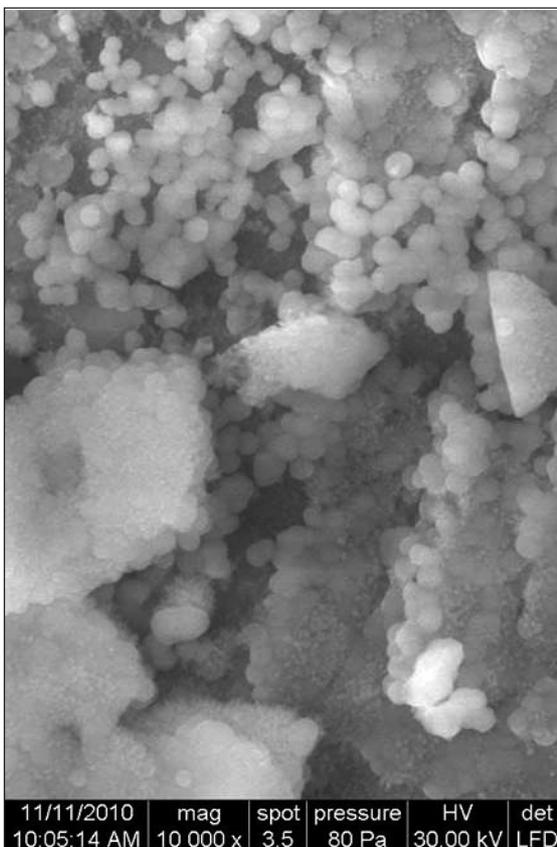
Label :2 A zona 1

Acquisition Time : 11:14:53 Date : 11-Nov-2010

kV: 29.98 Tilt: 0.00 Take-off: 34.79 AmpT: 51.2

Det Type:SUTW, Sapphire Res: 134.00 Lsec: 42

Figure 6. EDS analysis: intensity signals for Ca and F⁻ tooth no. 2: (a) sample 2A-zone 1



EDAX ZAF Quantification (Standardless)
 Element Normalized
 SEC Table : Default

Elem	Wt %	At %	K-Ratio	Z	A	F
F K	7.44	9.21	0.0078	0.9760	0.1071	1.0006
CaK	34.15	20.03	0.2988	0.9736	0.8986	1.0000

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Label :2 B zone 2

Acquisition Time : 11:16:59 Date : 11-Nov-2010

kV: 29.98 Tilt: 0.00 Take-off: 34.93 AmpT: 51.2

Det Type:SUTW, Sapphire Res: 134.00 Lsec: 43

Figure 6. EDS analysis: intensity signals for Ca and F⁻ tooth no. 2: (b) sample 2B-zone 2

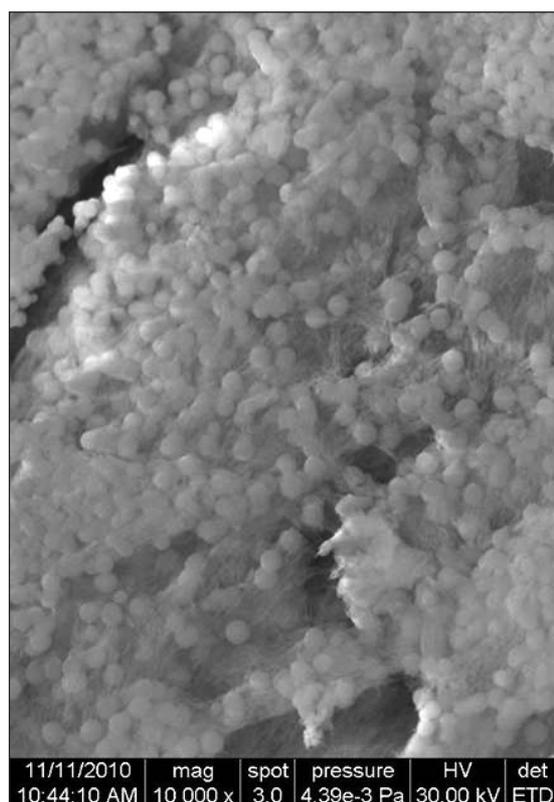
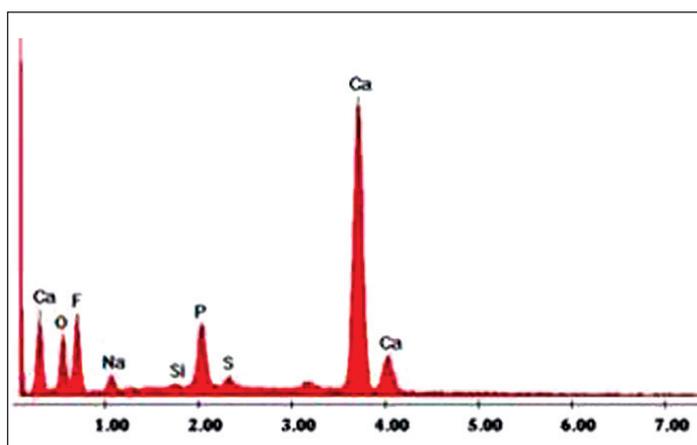


Figure 6. EDS analysis: intensity signals for Ca and F⁻ tooth no. 2: (c) sample 2C-zone 1.



EDAX ZAF Quantification (Standardless)

Element Normalized

SEC Table : Default

Elem	Wt %	At %	K-Ratio	Z	A	F
F K	25.77	29.18	0.0319	0.9760	0.1268	1.0004
CaK	25.29	13.57	0.2297	0.9718	0.9344	1.0000

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Label :2 C zone 1

Acquisition Time : 12:21:38 Date : 11-Nov-2010

kV: 29.98 Tilt: 0.00 Take-off: 35.11 AmpT: 51.2

Det Type:SUTW, Sapphire Res: 134.00 Lsec: 94

Group A (a product that has not previously been studied) and Group B will break down more slowly after the action of solutions with low concentrations of fluoride and will serve as a reservoir of fluoride for longer periods of time and thus contribute to the caries-preventive effect.

The EDS qualitative analysis showed that the intensity of fluoride signals increased with the increasing concentration of fluoride, whereas calcium signals decreased. In the current study, intensity of fluoride signals was higher in group C, where the concentration of ionic fluoride was greater.

Etching with 37% phosphoric acid creates a porous surface due to selective dissolution of apatite crystals. Depending on the orientation of the crystals dissolved, several acid-etching patterns are described: type I involves crystals of enamel prism belonging to the centre, type II causes dissolution of crystals at the periphery of prisms, and type III is associated with an irregular demineralisation pattern [18]. In the current study, after demineralisation with 37% phosphoric acid for 60 seconds, a type III etching pattern was mainly achieved. The results are similar to those of Legler *et al.* (1990) [19].

Conclusions

In the current study:

1. The remineralisation depth, determined by solutions containing sodium fluoride, depends on fluoride concentration and there were no statistically significant differences between samples treated with the same concentration of sodium fluoride, and a statistically significant difference when a higher concentration was used.
2. After the action of Fluorostom on the enamel surface amorphous globular structures of calcium fluoride were produced similar to those observed in samples treated with Colgate Total, which contains the same amount of sodium fluoride. Thus both products can be recommended because they produce a calcium fluoride globular structure formation.
3. EDS qualitative analysis highlighted the presence of the fluoride signals in all samples treated with sodium fluoride solutions.
4. The results of this study suggest that the Romanian product Fluorostom is effective for maintaining constant levels of fluoride

in the enamel surface and has beneficial effects on reducing the solubility and promoting the remineralisation of enamel. Unsurprisingly, as it contains the same concentration of sodium fluoride, the remineralisation potential of Fluorostom was the same as that produced by Colgate Total.

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Contributions of each author

- CSN planned the conceptual model for the study and its design, analysed the results, drafted and redrafted the paper, and approved the final version.
- CIA planned and supervised the study, critically reviewed its drafts, and approved the final version.

Statement of conflict of interests

As far as the authors are aware, there are no conflicts of interest.

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