

## CHAPTER 2

### MATERIALS AND METHODS

#### Materials

1. Samples:

Fifteen extracted sound premolars of patients aged 12-25 years.

2. Fluoride varnish:

The fluoride varnish used in this study was Duraphat® (Colgate Oral Pharmaceuticals; Cologne, Germany).

3. Artificial saliva (Macknight-Hane and Whitford; 1992)

The compositions of artificial saliva (grams per liter):

Methyl-p-hydroxybenzoate	2.00
Sodium Carboxymethyl Cellulose	10.00
KCl	0.625
MgCl <sub>2</sub> · 6H <sub>2</sub> O	0.059
CaCl <sub>2</sub> · 2 H <sub>2</sub> O	0.166
K <sub>2</sub> HPO <sub>4</sub>	0.804
KH <sub>2</sub> PO <sub>4</sub>	0.326

The pH of artificial saliva was adjusted to 6.75 with KOH.

4. Pumice

5. Acid resistant varnish (nail varnish)

6. Thin taper diamond bur

7. 0.05 mol/L glacial acetic acid prepared from 99% glacial acetic acid (Merck, Germany)

8. 10 N sodium hydroxide (NaOH), prepared from NaOH (Merck, Germany)

9. 10 N hydrochloric acid, prepared from 99% hydrochloric acid solution (Merck, Germany)
10. Standard buffer pH4 & pH7 for pH meter calibration (Scharlau, Spain)
11. 100 mg/L standard fluoride solution (Orion, USA)
12. 1000 mg/L standard calcium solution (Fluka, Switzerland)
13. 20  $\mu$ l micropipette
14. Scalpel



Figure 2. Fluoride varnish (Duraphat ®) used in this study.

## **Equipments**

1. pH meter (Precisa; model pH 900, Precisa Instruments AG, Dietikon, Switzerland)
2. Rotator shaking machine (Figure 3, Appendix 2)



Figure 3. A custom-made rotator shaking machine used in this study.

3. Magnetic stirrer (Corning; model PC 420, Corning Incorporated, New York, USA)
4. Inductively Coupled Plasma Optical Emission Spectrometer, ICP-OES (Perkin Elmer; model Optima 4300 DV, Connecticut, USA)
5. Scanning electron microscope (JEOL; model JSM 5800 LV, JEOL, Tokyo, Japan)

## **Methods**

### **Preparation**

The study samples were prepared according to the flow diagram in Figure 5. Fifteen extracted sound premolars for orthodontic reason of patients aged 12-25 years were collected. They were cleaned and polished with fluoride free pumice to remove residual soft tissue and stains. Hard deposits and calculus were removed with hand scalers. The teeth were stored in normal saline for 1 month at room temperature. Each tooth was longitudinally sectioned along the black line in Figure 4A with a thin taper diamond bur into three pieces, giving a total number of 45 samples for experiment (15

samples per group). Each sample was numbered on the root surface and systematically randomly assigned into three groups as follows:

Group A – Single fluoride varnish application group (2.26% Duraphat® applied day 1).

Group B – Single fluoride varnish application group (2.26% Duraphat® applied day 1)

Group C – Intensive fluoride varnish application group (2.26% Duraphat® applied day 1, 4 and 7).

All surfaces of the samples were coated with a nail varnish, except for a 2x2 mm windows of sound enamel at 1.5 mm above the cementoenamel junction (CEJ), as shown in Figure 4B

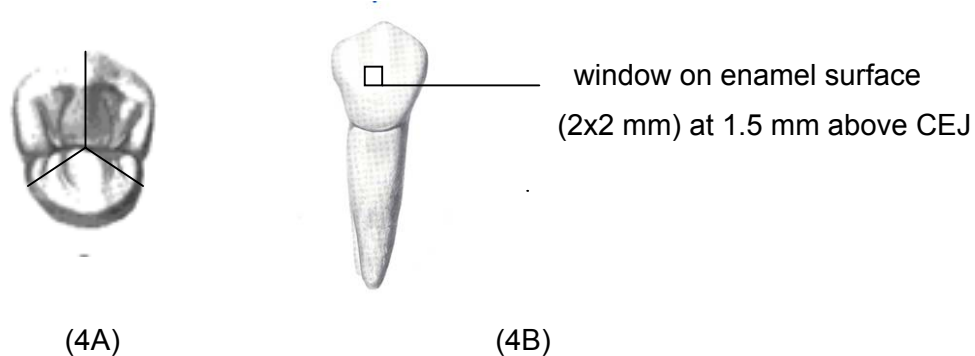


Figure 4. Sectional guide path of the teeth (4A) and a window on enamel surface sized 2x2 mm for fluoride varnish application (4B).

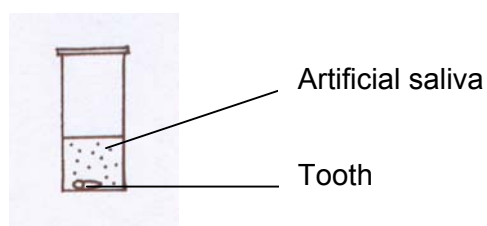
Each sample was placed in a bottle with 10 ml artificial saliva, pH 6.75 (Mcknight-Hanes and Whitford 1992). The bottles were rotated at 12-15 cycle/minute in a custom-made rotator shaking machine (Figure 5).



(5A)



(5B)

**BOTTLE**

(5C)

Figure 5. Custom-made rotator shaking machine, teeth were immersed in artificial saliva (5C).

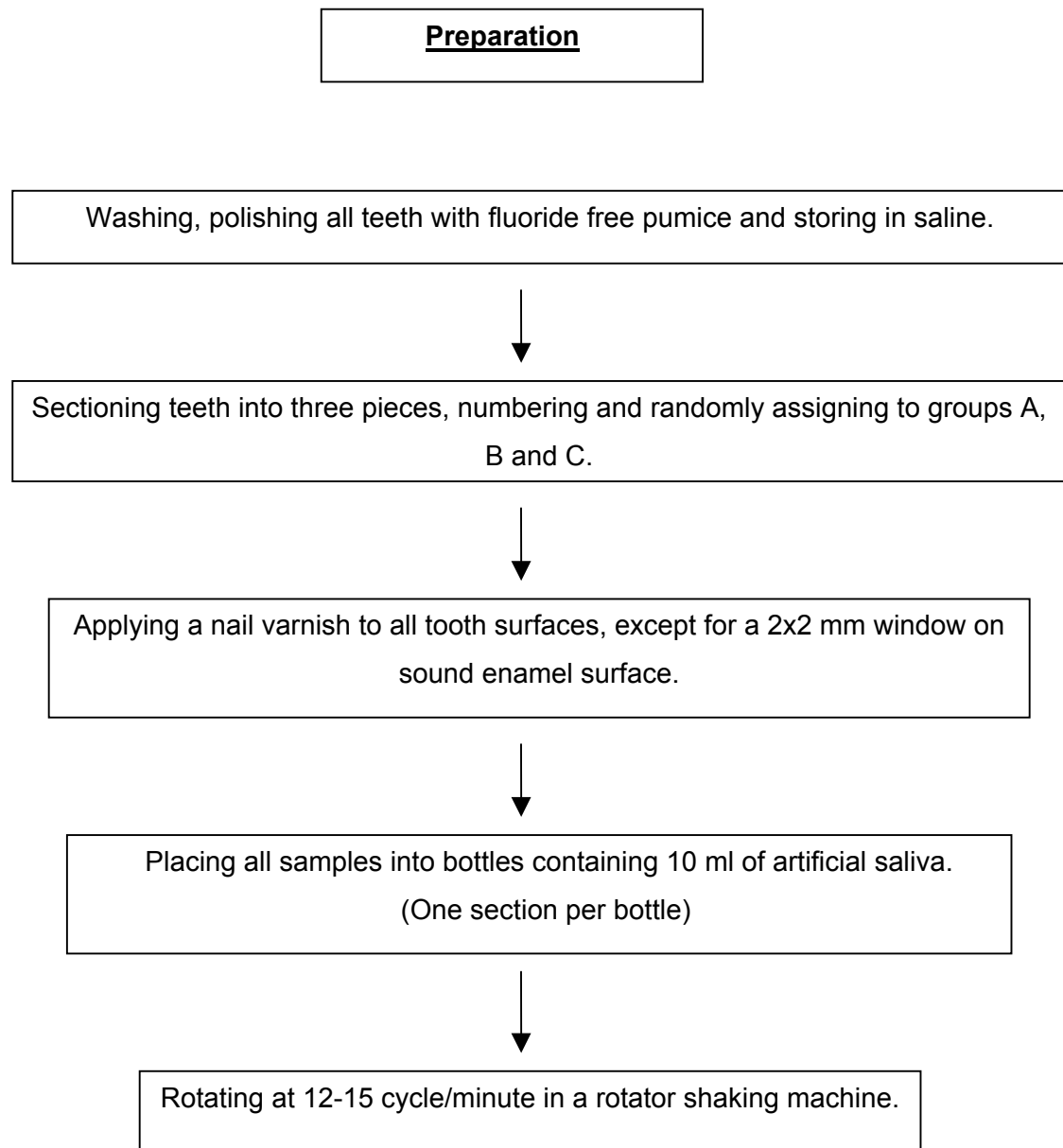


Figure 6. Preparation of the teeth for treatment.

## Treatment

This phase lasted 2 weeks and included application of fluoride varnish and pH cycling in artificial saliva (Figure 7).

### 1. Fluoride varnish application

Each sample was removed from the bottle, rinsed with distilled water and dried with air from rubber puffer and the fluoride varnish was applied to the window as:

Group A and B – Single fluoride varnish application (2.26% Duraphat® applied day 1)

Group C – Intensive fluoride varnish application (2.26% Duraphat® applied day 1, 4 and 7)

Approximately 10 µl of fluoride varnish was applied on each window area by micropipette, allowed to dry for 5 minutes and returned to the bottle. The fluoride varnish was removed after 24 hours by a scalpel, rinsed with distilled water and returned to the bottle.

### 2. pH cycling

To imitate the pH changes in the dental plaque when dietary carbohydrate is fermented by bacteria, pH was cycled by shifting the tooth specimens from the pH 6.75 artificial saliva to artificial saliva adjust to pH 5 with acetic acid. After 20 minutes, the specimens were removed from the acidic artificial saliva, rinsed with distilled water and returned into fresh artificial saliva pH 6.75. The pH was cycled 3 times per day, at 9 am, 12 pm and 5 pm for 2 weeks treatment phase.

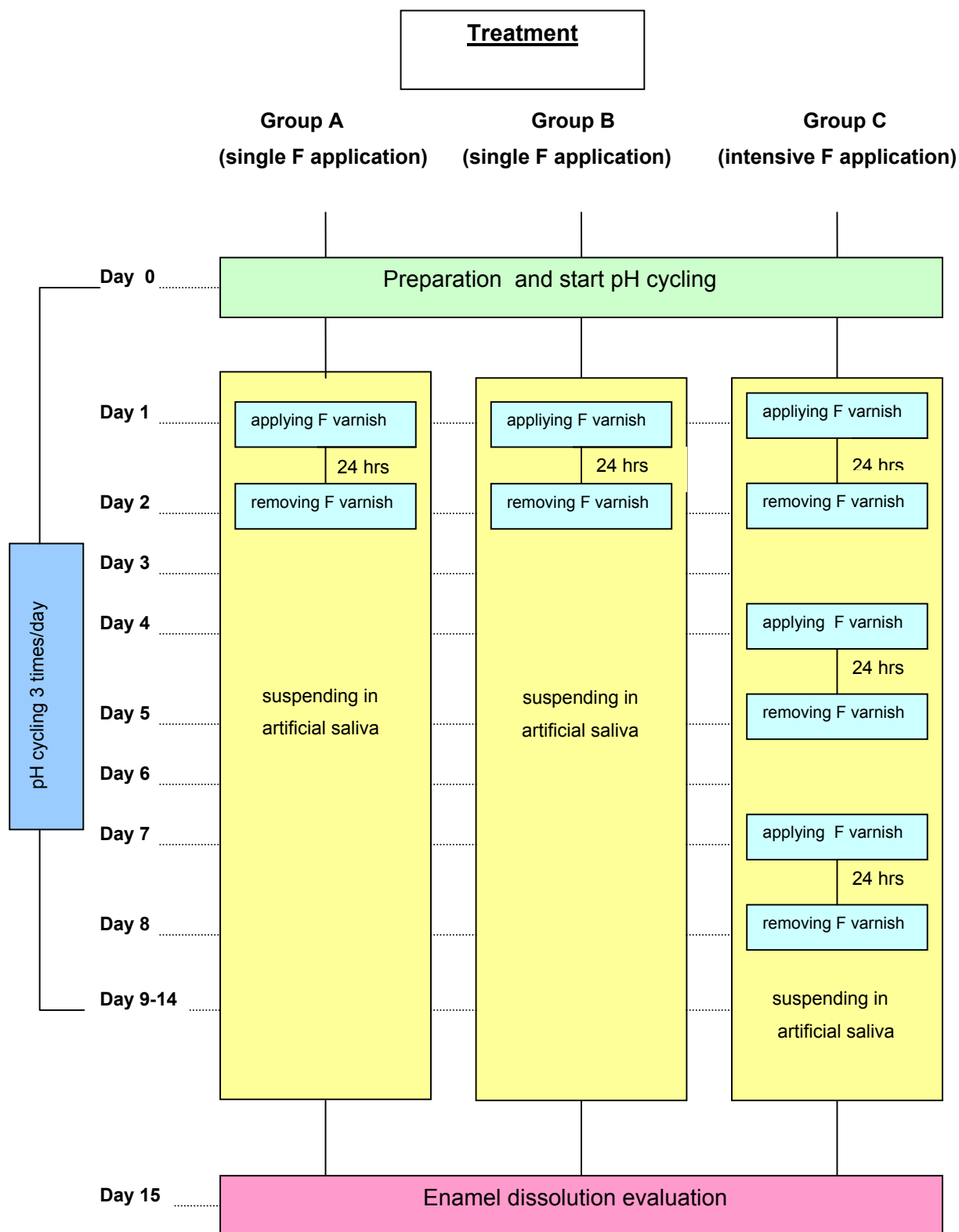


Figure 7. Treatment of the teeth.



### **Enamel dissolution**

At the end of the treatment, all specimens were removed from artificial saliva and carried on to the enamel dissolution test (Figure 8). The specimens were demineralised with 0.05 mol/L acetic acids containing two different levels of fluoride concentration, 1 and 10 mg/L. Each specimen was placed into each individual bottle with 6 ml of demineralising solution (pH 4) as follow:

Group A - 0.05 mol/L acetic acid with 1 mg/L fluoride.

Group B and C - 0.05 mol/L acetic acid with 10 mg/L fluoride.

The bottles were returned to the rotator shaking machine. After 8 hours, all samples were removed from the demineralising solution and rinsed with distilled water. The calcium concentration in solution was determined by ICP-OES.

### **Examination of enamel surface texture**

The surface texture of five dried specimens per group was examined by scanning electron microscope (SEM). Nail varnish at the right border of the window areas removed before the gold-palladium coating. The samples were examined at 500 and 7000 x magnification to see signs of enamel dissolution in the rod areas. All samples (five samples per group) were photographed in four areas as follows:

- marginal area between dissolved and non-dissolved area at 500 x of magnification
- non-dissolved are at 7000 x of magnification
- dissolved are at 500 x of magnification
- dissolved are at 7000 x of magnification

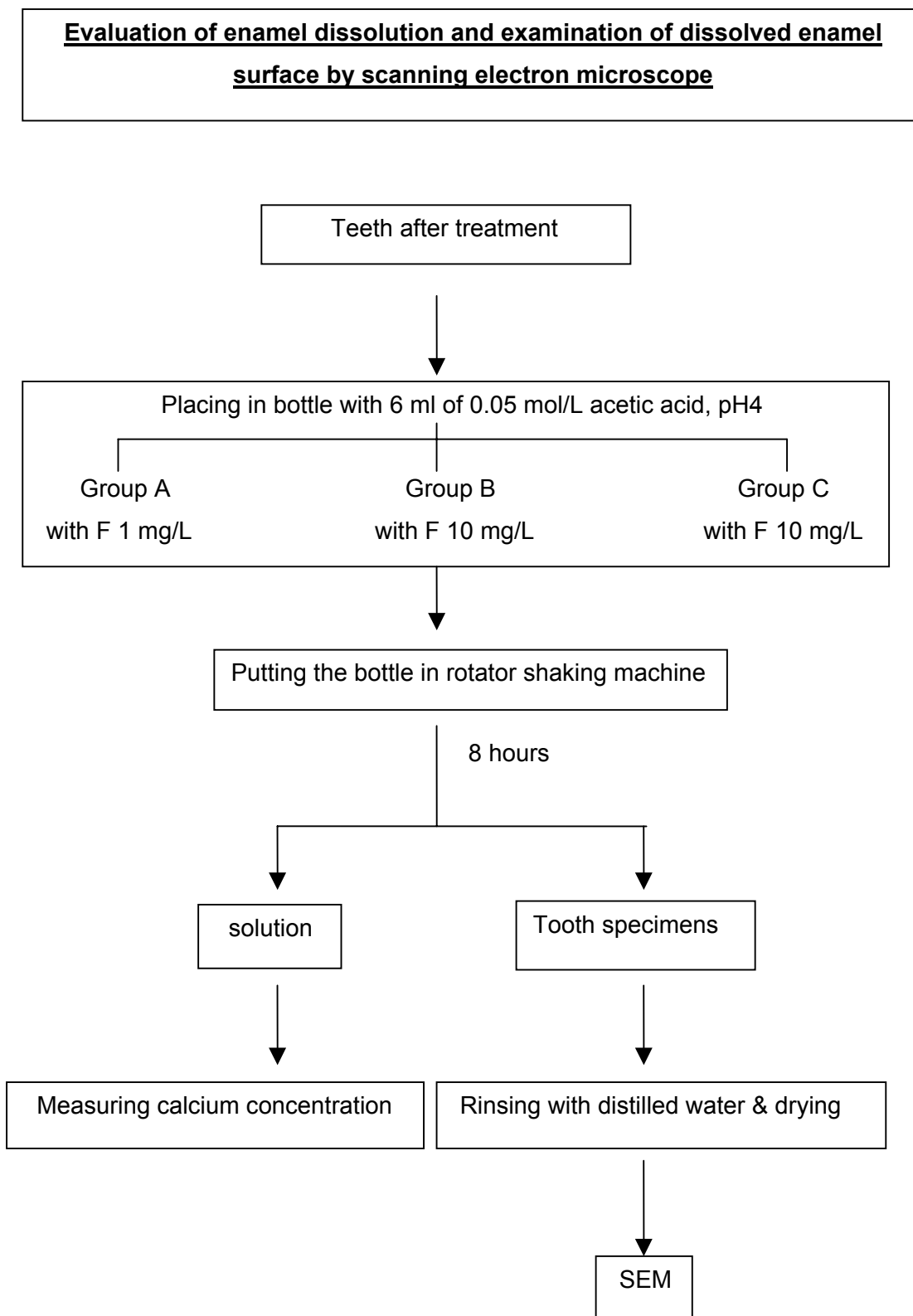


Figure 8. Enamel dissolution and examination of dissolved enamel surface by scanning electron microscope.

## **Statistical analysis and descriptive analysis of SEM**

### **1. Statistical analysis**

After data collection, all data were checked and entered into computer. The Kruskal-Wallis test of SPSS program was used to compare means of dissolved calcium ion of the samples in group A, B and C. Then Wilcoxon sign rank test was used to compare between group A-B and B-C.

### **2. Descriptive analysis of SEM**

The SEM photographs of group A, B and C were compared for any differences in dissolution of the rod areas. The photographs of the marginal areas were used to compare between dissolved and non-dissolved area as well as the depth of the lesion between groups.