

**Original**

## Analysis of the molecular structure of human enamel with fluorosis using micro-Raman spectroscopy

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**Abstract:** The aim of this study was to analyze the molecular structure of enamel with fluorosis using micro-Raman spectroscopy and compare it with that of healthy enamel. Eighty extracted human molars were classified into four fluorosis groups according to the Thylstrup-Fejerskov Index (TFI) [TFI: 0, Healthy enamel; 1-3, mild; 4-5, moderate; 6-9, severe fluorosis]. All samples were analyzed by micro-Raman spectroscopy. The integral areas of  $\nu_1$  ( $960\text{ cm}^{-1}$ ) phosphate peak as well as B-type carbonate peak ( $1070\text{ cm}^{-1}$ ) were obtained to analyze structural differences among the specimens. Although the differences were not statistically significant ( $P > 0.05$ ), the mean of integral areas of  $\nu_1$  phosphate peak among groups indicated greater mineralization in the severe fluorosis group. However, there were statistically significant differences in the intensities, and the integral areas of B-type carbonate peak among groups ( $P < 0.05$ ). Therefore, mineralization of the carbonate peak at  $1070\text{ cm}^{-1}$  decreased significantly in fluorotic groups, suggesting that carbonate ions are easily dissolved in the presence of fluoride. Although structurally fluorotic teeth are not more susceptible to dental caries, serious alteration in its surface topography may cause retention of bacterial plaque and formation of enamel

caries. Micro-Raman spectroscopy is a useful tool for analyzing the molecular structure of healthy and fluorotic human enamel. (J Oral Sci 54, 93-98, 2012)

Keywords: healthy enamel; fluorosis enamel; micro-Raman spectroscopy.

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### Introduction

Despite improvements in the oral health status of populations across the world, problems persist particularly among disadvantaged groups in both developed and developing countries. According to the World Oral Health Report 2003, dental caries remain a major public health problem in most industrialized countries, affecting 60-90% of schoolchildren and the vast majority of adults (1). Water fluoridation, where technically feasible and culturally acceptable, remains a relevant and valid choice as a population measure for the prevention of dental caries; however, it is well-known that porosity and hypomineralization may develop due to retention of amelogenin proteins by fluoride (2). As a result, teeth are more susceptible to the effects of normally occurring chemical and mechanical injuries (3,4). In addition, several studies have suggested that severe fluorosis might increase the susceptibility to dental caries (5-7).

The prevalence of dental fluorosis (DF) has increased throughout the world (8), ranging from 7.7% to 80.9% in areas with fluoridated water and from 2.9% to 42% in areas without fluoridated water (9-12). In San Luis Potosí, Mexico, the prevalence of fluorosis was reported

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as high as 69% although the levels of water fluorine were less than 0.7 ppm. The fluorosis level increased to 98% at a fluorine level of 2 ppm (13). DF is likely the most well-studied condition and possibly has the most adverse effects on the development of enamel. However, little information exists on the organic and inorganic composition of enamel in pathological conditions and on the structure of fluorotic enamel.

Micro-Raman spectroscopy has been widely used to investigate the molecular structure of various materials, cells and tissues (14-17) because of its many advantages including minimal specimen preparation and non-invasive sampling capability. As a result, it has been introduced into biophysics to obtain molecular structure information in both *in vivo* (18,19) and *in vitro* studies (20,21). The high spectral resolution (up to  $1\text{ cm}^{-1}$ ) with Raman microscopy makes it an excellent tool for analyzing the components of human enamel specimens at the micrometer level. The aim of this study was to compare and contrast the molecular structure of healthy and fluorosed enamel using micro-Raman spectroscopy.

## Materials and Methods

### Subjects and sample preparation

Patients undergoing extraction of third molars at a local hospital and private clinics were asked to donate their extracted teeth, and informed patient consent was obtained. Erupted third molars were collected from three different locations 1. Ciudad Valles (San Luis Potosí, México), which has a water fluoride level between 0.1 and 0.6 ppm F; 2. San Luis Potosi City (Mexico) with a natural fluoride level between 0.7 and 2 ppm F; and 3. Salitral de Carrera (San Luis Potosí, México) with a natural fluoride level between 2 and 5 ppm. All samples were cleaned and disinfected in an ultrasonic bath, then washed in running water, dried, and analyzed for fluorosis severity according to the Thylstrup-Fejerskov Index (TFI) (22). The TFI is the only index that correlates the clinical appearance of fluorosis with the pathologic changes in enamel and is normally the index of choice for evaluation of fluorosis severity (23). It uses a 10-point scale, where zero represents the non-affected tooth and 9 the most severely affected tooth. Individuals with fluorosis can be classified as follows: Mild (TFI = 1-3), Moderate (TFI = 4-5) and Severe (TFI = 6-9).

The selected molars were divided into four groups: the healthy enamel group (H), the mildly fluorotic enamel group (MI), the moderately fluorotic enamel group (MO), and the severely fluorotic enamel group (S). All molars were stored in distilled water (Milli-Q, Millipore Co., Billerica, MA, USA) until experimental proce-

dures were performed. The molar cusps were sectioned perpendicular to the long axis of the tooth by means of a water-cooled low-speed diamond saw (# 7910, medium size grain; Brasseler, Savannah, GA, USA) later to be ground with a porcelain mortar and pestle to obtain enamel dust.

### Micro-Raman spectroscopy

An In Via Raman microscope (Renishaw Inc, 5277 Trillium Blvd Hoffman Estate, Illinois, IL, USA) UV laser (633 nm), and an excitation power of 20 mW were used to obtain Raman spectra. Spectra were calibrated for Raman shift frequency using known lines of silicon.

A small amount of enamel dust was placed on a micro-scope slide for micro-Raman analysis, and micro-Raman spectra were obtained using a 50 $\times$  objective focused on the sample area. A spectrum was obtained over the spectral region of 400 to 1,400  $\text{cm}^{-1}$  with 30 s of integration time and 50% laser power. Three Raman spectral acquisitions for each sample were obtained by means of WiRE 3.0 instrument control software (Renishaw Inc, 5277 Trillium Blvd Hoffman Estate, Illinois, IL, USA).

### Spectral data analysis

An Origin v 6.1 software (OriginLab Corporation, Northampton, MA, USA) and PeakFit v 4.0 software (Aspire Software International, Ashburn, VA, USA) were used to analyze the acquired Raman spectra. Without additional spectral smoothing, individual spectra were adjusted using multiple-point baseline correction. The ratios of  $\nu_1$  (960  $\text{cm}^{-1}$ ),  $\nu_2$  (432  $\text{cm}^{-1}$ ),  $\nu_3$  (593  $\text{cm}^{-1}$ ) and  $\nu_4$  (1043  $\text{cm}^{-1}$ ) phosphate peaks as well as a B-type carbonate peak at 1070  $\text{cm}^{-1}$  were obtained, but, only the ratios of  $\nu_1$  (960  $\text{cm}^{-1}$ ) phosphate peak and B-type carbonate peak at 1070  $\text{cm}^{-1}$  were used to analyze the differences in mineral composition among the specimens (16). The  $\nu_1$  vibration peak of phosphate at 960  $\text{cm}^{-1}$  was selected as the internal standard for normalization adjustment.

Examiners were calibrated by an expert in fluorosis using the intra-class correlation coefficient (ICC). All data are expressed as mean  $\pm$  standard deviation and range. The Shapiro-Wilks and Brown Forsythe methods were used to test the distribution of variables. One-way analysis of variance (ANOVA) was used to determine whether differences between means of variables were statistically significant. The JMP program (version 5.1) and Stat View (both from SAS Institute, Cary, NC, USA) were used for statistical analysis, and  $P < 0.05$  was considered statistically significant.

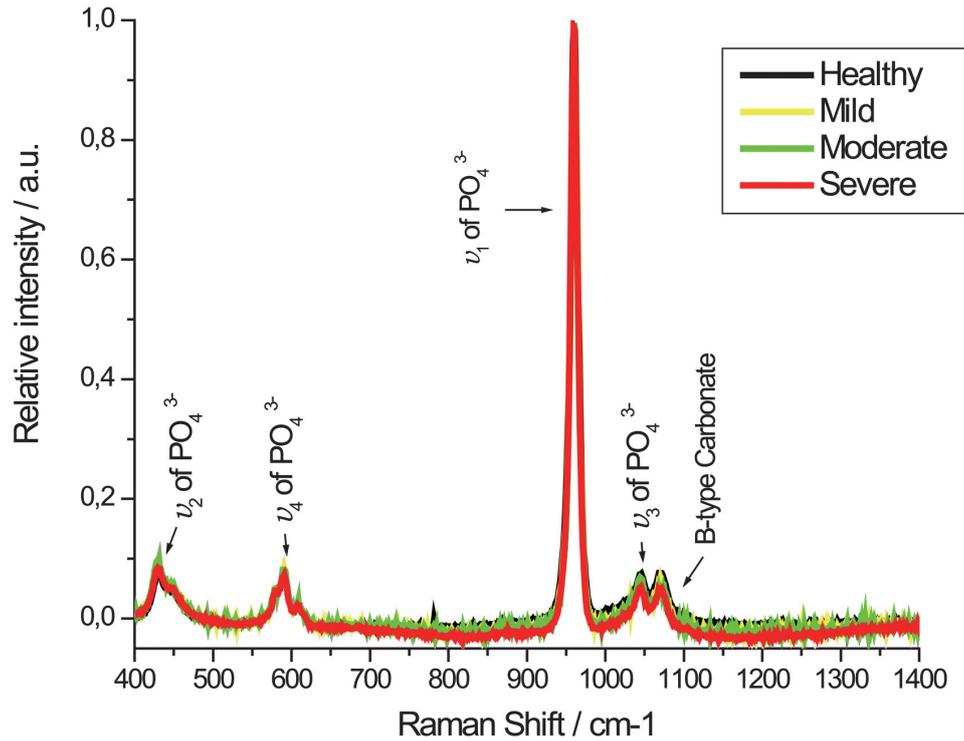


Fig. 1 Micro-Raman spectra for the four study groups.

Table 1 Descriptive statistics of phosphate and B-type carbonate peaks obtained by micro-Raman spectroscopy

Group	Raman shift (cm <sup>-1</sup> )	Integral area Mean $\pm$ SD
Healthy	960	46.72 $\pm$ 58.48
	1070	1.92 $\pm$ 1.53
Mild	960	34.84 $\pm$ 34.25
	1070	1.45 $\pm$ 0.73
Moderate	960	29.98 $\pm$ 29.54
	1070	0.98 $\pm$ 0.66
Severe	960	47.72 $\pm$ 44.88
	1070	0.90 $\pm$ 0.66

SD: Standard deviation; 960 represents phosphate peak; 1070 represents B-type Carbonate peak

## Results

From the 109 teeth collected, 80 erupted human third molars with complete roots and no enamel damage due to extraction were analyzed. A total of 20 teeth were obtained from Ciudad Valles (Healthy), 40 from San Luis Potosi City (Mild and Moderate), and 20 from Salitral de Carrera (Severe) all of which were from San Luis Potosi State, Mexico. The inter-observer reproducibility analysis for fluorosis revealed an intra-class correlation

coefficient of 0.99. The distribution of all variables was parametric.

Micro-Raman spectra for the four study groups in the region of 400-1,400 cm<sup>-1</sup> are shown in Fig. 1. All spectra were normalized based on the 960 cm<sup>-1</sup> phosphate peak, which was the most intense peak. The spectrum shows notable changes in peak intensity. The greatest peak intensities were associated with the  $\nu_2$  and  $\nu_4$  phosphate peaks in the moderate fluorosis group, followed by mild, severe and healthy groups. The B-type carbonate peak showed the greatest intensity in the healthy enamel group, followed by mild, severe and moderate fluorosis groups. The  $\nu_3$  phosphate peak exhibited major intensity in healthy enamel, followed by moderate, mild and severe fluorosis groups. Shown in Table 1 are the means, standard deviations and the ranges of integral area for phosphate peak at 960 cm<sup>-1</sup> and B-type carbonate at 1070 cm<sup>-1</sup> for each group.

The mean integral area and standard deviation for phosphate peak at 960 cm<sup>-1</sup> were as follows: Group H: 46.72  $\pm$  58.48; Group MI: 34.84  $\pm$  34.25; Group MO: 29.98  $\pm$  29.54 and Group S: 47.72  $\pm$  44.88.

The mean integral area and standard deviation values for B-type carbonate peak at 1070 cm<sup>-1</sup> were as follows: Group H: 1.92  $\pm$  1.53; Group MI: 1.45  $\pm$  0.73; Group MO: 0.98  $\pm$  0.66 and Group S: 0.91  $\pm$  0.66. It is clear that

Table 2 Inter-group comparison of phosphate and B-type carbonate peaks obtained by micro-Raman spectroscopy

Groups	Raman shift (cm <sup>-1</sup> )	Integral area <i>P</i> value
H vs. MI	960	0.4379
	1070	0.2235
H vs. MO	960	0.2603
	1070	0.0369
H vs. S	960	0.9519
	1070	0.0172
MI vs. MO	960	0.6336
	1070	0.0154
MI vs. S	960	0.3140
	1070	0.0095
MO vs. S	960	0.1480
	1070	0.7234

H: Healthy enamel group; MI: mild fluorosis group; MO: moderate fluorosis group; S: severe fluorosis group; 960 represents phosphate peak; 1070 represents B-type Carbonate peak; *P* values are from the analysis of variance (ANOVA)

the B-type carbonate peak decreased with the severity of fluorosis.

In Table 2, the analysis of variance (ANOVA) did not reveal a significant difference ( $P > 0.05$ ) in the means of integral area for the  $\nu_1$  phosphate peak among the groups. On the other hand, for the B-type carbonate peak, significant differences ( $P < 0.05$ ) for integral area between H and S groups and between MI and S were observed, but there was no difference between the rest of the groups.

## Discussion

The analysis of the mineral components of healthy enamel revealed that calcium exists in hydrous calcium form, that is denoted by the formation of hydroxyapatite [Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>] (24). DF is a developmental enamel disturbance caused by sustained exposure to high concentrations of fluoride during tooth development, leading to enamel with a lower mineral content (hypomineralization) because of changes in the structure of external surfaces (25). Micro-Raman spectrometry allows a thorough molecular analysis of mineralized dental tissues. The information provided is in the form of curves representing the intensity of the signal according to the frequency, and mathematical analyses allow for any type of comparative and quantitative analysis (26). To our knowledge, this is the first study that has characterized and compared the molecular structure of healthy and fluorotic enamel by micro-Raman spectroscopy. The spectra were normalized based on the 960 cm<sup>-1</sup> phosphate

peak. Although the differences were not statistically significant, the mean of integral areas of  $\nu_1$  phosphate peak in the groups may indicate greater mineralization in the severe fluorosis group, presumably due to dissolution of hydroxyapatite with increased phosphate levels after the fluoride concentration in plasma reached the peak (27). However, the integral area of the B-type carbonate peak showed statistically significant differences among groups, which indicates that the mineralization of carbonate peak at 1070 cm<sup>-1</sup> decreased significantly in fluorotic groups, suggesting that carbonate ions are easily dissolved in the presence of fluoride. It has been reported that carbonate interferes with proper apatite crystallization and has a weakening effect on the bonds in the structure, as it increases the dissolution rate and solubility; thereby, contributing to the susceptibility of dental apatite containing carbonate to caries (28). The crystallinity increased with an increase in phosphate in samples with severe fluorosis. Moreover, a decrease in carbonates could indicate less susceptibility to caries in this degree of fluorosis, however epidemiological studies have described otherwise suggesting that severe fluorosis could increase susceptibility to the formation of dental caries (5-7). Although the present study as well as other studies found that structurally fluorotic teeth were not more susceptible to dental caries, serious alteration of the surface topography may cause retention of bacterial plaque and formation of enamel caries (27,28).

We conclude that the mean integral areas of  $\nu_1$  phosphate peak in the groups indicate greater mineralization in the severe fluorosis group, however, the intensities, and the integral areas of B-type carbonate peak, showed statistically significant differences among groups, which indicates that mineralization of carbonate peak at 1070 cm<sup>-1</sup> decreased significantly in fluorotic groups. In conclusion, the micro-Raman spectroscopy is a useful tool for analyzing the molecular structure of healthy and fluorotic human enamel. However, it is necessary to extend the analysis of enamel hypoplasias to different characterization techniques such as crystallography and scanning electron microscopy.

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