# Electron probe microanalysis of permanent human enamel and dentine. A methodological and quantitative study

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**Summary.** Sample preparation of dental tissues for quantitative electron microprobe analysis has not been critically examined because of the highly mineralized nature of these structures. The present study was designed to establish the most suitable method for the electron probe quantitative determination of calcium in human permanent enamel and dentine while preserving the morphological features. Comparisons of quantitative data obtained with air-drying and freeze-drying methods showed that calcium in enamel was more accurately measured in specimens prepared with cryopreservation and freeze-drying. No significant differences between the methods tested were found in dentine although cryopreservation and freeze-drying yielded less statistical variability. Moreover this approach did not modify morphological features of interest. We recommend this combination of processing techniques for human permanent teeth not only because it was found the most accurate and least variable in determining calcium concentration, but also because of its potential useful-ness in studies of alterations in tooth mineralization.

**Key words:** Enamel, Dentine, Microanalysis, Quantitative methods

## Introduction

The application of electron probe X-ray micro-analysis (EPMA) constitutes one of the most productive tools for the study of mineralization processes. This technique makes the chemical analysis of morphologically well-defined areas with the electron microscope possible, in which a sharply focused electron beam is directed to the sample (Landis, 1979; Engel and Hilding, 1984; Campos et al., 1994).

Enamel and dentine, two of the main calcificied tisssues in teeth, have been extensively studied with

EPMA. However, these studies were mostly carried out with a qualitative or semiquantitative approach, and used methods basically similar to those developed to prepare geological apatites (Crall et al., 1983; Hals et al., 1988; Sánchez Quevedo et al., 1989). A semiquantitative and quantitative aproach to EPMA initially developed by Hall et al. (1973) for thin specimens has been revised and applied to bulk specimens (Boekenstein et al., 1980, 1983, 1984; Gupta and Hall, 1982; Hook et al., 1986; Roomans, 1988a; Hall, 1989; Zs-Nagy and Casoli, 1990; Patak et al., 1993; Warley, 1993).

The quantitative analysis of bulk specimens can be performed more accurately using the peak-to-back-ground (P/B) ratio method (Statham and Pawley, 1978; Small et al., 1979). This method takes into account the background in the same energy range as the characteristic peak, and assumes that the absorption of the energy generated is the same for the characteristic and continuum radiation in the volume irradiated. The advantages of this method are that it is independent of variations in beam current intensities and specimen surface effects, and is suitable for quantitative analysis of rough surfaces in scannning electron microscopy (Boekestein et al., 1984; Armstrong, 1991). One of the problems with quantitative studies is the appropriate preparation of specimens, and the preparation of the adequate standards for mineralized tissues. Problems arise because the organic matrix is present in different proportions in hard tissues, ranging from less than 1% in the enamel to 20-35% in the dentine and bone (Avery, 1987).

Recently, bulk microcrystalline standards have been prepared to quantify hard tissues by EPMA. New information on calibration curves and electron beam sensitivity has demonstrated the suitability of these standards when applied to some mineralized tissues (Krefting et al., 1981; Campos et al., 1992; López-Escámez et al., 1992, 1993). Because of the presence of the organic matrix in hard tissue, specimen preparation is crucial. We were therefore interested in comparing air-drying versus freeze-drying of dental tissues as a model to evaluate the loss of calcium during the preparation of

calificied tissue specimens.

The purpose of the present study was to establish the most suitable method for the determination of calcium, and to provide a quantitative analysis of human enamel and dentine. Our aim was to preserve the redistribution of ions and the morphological features in these strongly mineralized tissues.

## Materials and methods

The material consisted of eight freshly extracted human permanent molars removed for periodontal reasons. All patients were being followed in the Stomatology Service of the University Hospital of Granada. All teeth were cut longitudinaly in four 1-mm slices with a diamond-impregnated disc (Accutom Struers, Denmark) at 700 rpm.

### EPMA sample preparation

Four different experimental groups were established to study the effect of tissue preparation on the elemental values of calcium in the enamel and dentine. One slice from each tooth was used in each experimental group.

### Group A

Eight slices were fixed in 2.5 glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 4 °C for 4 h, and freeze-dried at -80 °C for 48 h in a Polaron E 5350 freeze drying apparatus. Samples were left in the freeze-drying chamber to return slowly to room temperature.

### Group B

Another eight slices were fixed in glutaraldehyde as described for group A, but samples were air-dried at room temperature for 48 h on the specimen holders.

### Group C

Slices from the eight teeth were mounted on scanning electron microscope (SEM) holders and air-dried for 48 h.

### Group D

Eight slices from the teeth were plunge-frozen in liquid nitrogen-cooled Freon 22. The samples were transferred to the freeze-drying apparatus and dried as in Group A.

All specimens were mounted on a carbon stub and sputter-coated with a thin layer of carbon in an argon atmosphere (at 0.1 Torr) for 30 s.

### Electron probe X-ray microanalysis

Tooth slices prepared as indicated above were studied in a Phillips X-L 30 scanning electron

microscope (SEM) (operating voltage: 15 kV; spot size: 500 nm; tilt angle: 35°; take-off angle: 61.34). An energy dispersive spectrometer (EDAX DX-4) was used for quantitative analysis (count rate: 1200 cps; live time: 50 s). Spectra were collected by pin-point electron beam at x40,000. The number of analyses was 640 (10 for emamel and 10 for dentine in each slice, in each of the four groups). Elemental peaks on the X-ray emission spectra were considered significant when $P-b > 2(P+b)^{1/2}$, where P is the total peak integral and b the estimated continuum component of the peak integral according to Chandler (1977).

Microcrystalline salt standards used for calcium quantification (Campos et al., 1992; López-Escámez and Campos, 1994) were $Ca_2P_2O_7$, $C_6H_{11}O_7.1/2Ca$, $Ca_2P_2O_7$, $Po_4HCa$, $Ca(H_2PO_4)_2$, $PO_4HCa.2H_2O$, $C_{12}H_{21}O_{12}. 1/2Ca$, $CaH_4O_8P_2.H_2O$, $CaSO_4.2H_2O$.

Microcrystalline standards were mounted on 200 mesh nickel grids fixed with adhesive graphite lamina to SEM holders. The standards were analyzed in the SEM immediately after preparation to avoid contamination or chemical modification. The elemental weight percent (WP) of each salt standard was directly proportional to peak to local background, i.e., Cs = k (Ps/Bs) where Cs is the element WP of standards (known), (Ps/Bs) is the element peak-to-local continuum X-ray intensity ratio from the analysis of salt standards (determined during the analyses), and k is the characteristic calibration constant for each element, calculated from the above equation. The WP was determined by the direct proportion method based on the following relationship between the tooth (enamel and dentine) and the salt standard: Ce or Cd/Cs = (Pe/Be or Pd/Bd)/(Ps/Bs), where Ce or Cd is the element WP of a given tooth (enamel or dentine) (unknown), (Pe/Be or Pd/Bd) is the element peak-to-local continuum X-ray intensity ratio from the analysis of a given tooth (enamel or dentine), and Cs and Ps/Bs are the data from the standards.

### Morphological study

Carbon-coated specimens and specimens that were gold coated after EPMA analysis were examined in a Philip XL-30 SEM and photographed.

### Statistical study

Data for enamel and dentine were analyzed with nested ANOVA using one fixed factor (method) and two random factors (tooth and analytical results). The analytical results factor was nested within tooth-method. We used Levene's test to study the variability of each method. All statistical analyses were done with the BMDP statistical package.

## Results

The calcium standards used here were stable under the beam and yielded reproducible results within the

range used to determine mineralization in mineralized tissues. The regression coefficient was r = 0.9836. The quantitative results in enamel and dentine in WP are shown in Table 1 and illustrated in Figure 1.

Mean calcium concentrations in the enamel differed significantly depending on the method of sample preparation used ($F_{exp}$ = 3.52 (3;288 df) P = 0.0330). The highest values were obtained in Group D (P < 0.05). Dentine calcium concentrations did not differ significantly between the four methods tested ($F_{exp}$ = 1.77 (3;288 df) P = 0.1828). The results of Levene's test showed that in the analyses of enamel, the degree of variability was similar in each of the four methods ($F_{exp}$ = 1.89 (3;316 df) P = 0.1310). The results for dentine showed significantly less variability with method D ($F_{exp}$ = 5.09 (3;316 df) P = 0.0019).

Morphological features were well preserved in all methods regardless of whether the specimens were carbon-coated and examined before EPMA, or gold-coated and studied after EPMA (Fig. 2).

## Discussion

The application of EPMA to dental tissues has yielded valuable information on the participation of different chemical elements in proccesses of bio-mineralization (Engel, 1981; Engel and Hilding, 1984; Sánchez-Quevedo et al., 1989, 1992). The ability of EPMA to solve biological problems often depends on the use of a quantitative approach. EPMA allows the quantitative determination of chemical elements of biological materials by using reference standards which resemble the specimens. Naturally the specimens and the standards should be similar in terms of interaction with the electron beam. Although much has been published on the quantification of elements in biological thin specimens, experience with standards for X-ray micro-
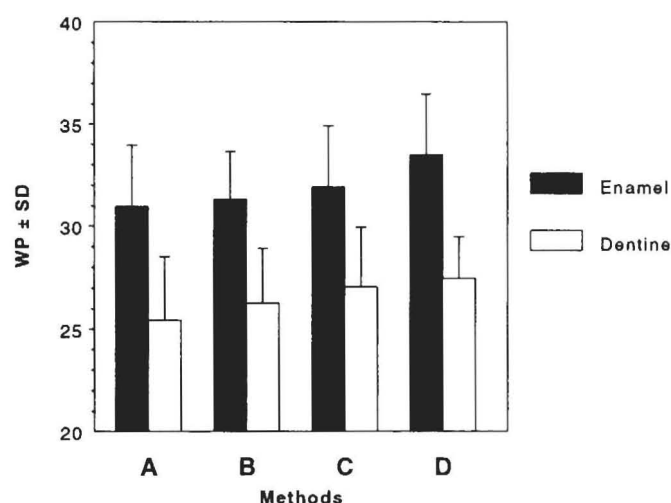
**Table 1.** Mean values and standard deviation of calcium concentrations in human enamel and dentine prepared for electron-probe microanalysis with four different methods.

| GROUPS | ENAMEL | DENTINE |
|--------|--------|---------|
| A | 30.961±3.001 | 25.448±3.072 |
| B | 31.311±2.336 | 26.262±2.662 |
| C | 31.915±3.004 | 27.061±2.895 |
| D | 33.494±2.991 | 27.473±2.031 |



**Fig. 1.** Mean values ± standard deviation of calcium concentration in human enamel and dentine.
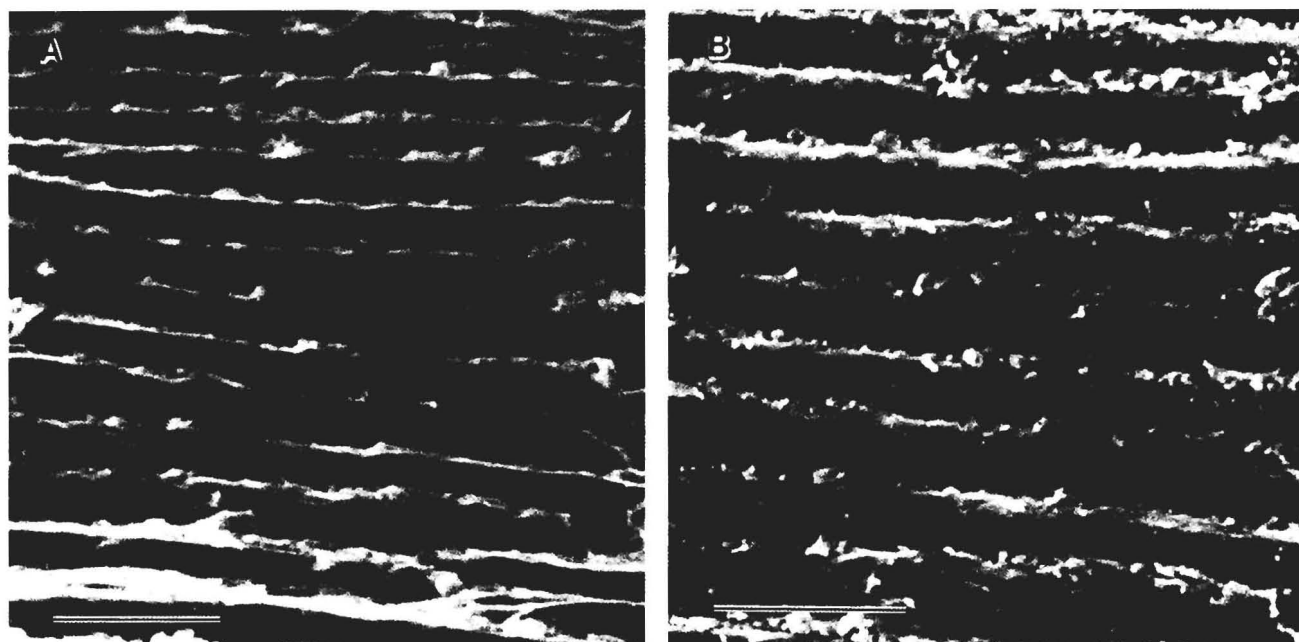


**Fig. 2.** Morphological features in human enamel prepared for SEM observation with: Freeze-drying and carbon coating only (**A**); and Freeze-drying and carbon- coating, EPMA, and subsequent gold-coating (**B**). Bar: 20 μm.

analysis of bulk specimens is limited, especially for calcified structrures, where the density of the specimen is difficult to estimate (López-Escámez and Campos, 1994).

The recent development of crystal salt standards for use in the analysis of mineralized structures such as the otoconia, which consist mainly of calcium carbonate, has made it possible to overcome some problems related with the quantification of hard tissues with EPMA (López-Escámez et al., 1993). It is particularly important to take into account electron beam sensitivity, defined by Hobbs (1987) as the alterations that affect the structure and chemical integrity of an irradiated specimen.

The calcium salt standards we used in our quantitative study were useful in reducing the effects of electron beam irradiation. Moreover, these standards produce reliable calibration curves (López-Escámez et al., 1993). This is important because in a study of human molar dentine, Edie and Glick (1980) reported that an increase in total electron dosage may modify the calcium concentration. The standards we used also offer the advantages of stability, homogeneity and known chemical composition. Although enamel and dentine are highly mineralized, they are dynamic structures that participate actively in the biology of the oral cavity (Avery, 1987; Sánchez-Quevedo et al., 1992). Sample processing for EPMA is, together with the preparation and selection of appropriate standards, a key step in quantitative electron microscopic studies. The techniques used to prepared samples of these structures are of prime importance to ensure accuracy in recording quantitative analytical data with EPMA, and to guarantee the validity of correlations with morphological features.

In the present study we compared four methods of sample preparations consisting of air-drying or freeze-drying, and chemical fixation or cryopreservation. Our results show that calcium losses from enamel were smaller when cryopreservation and freeze-drying were used. According to Müller and Moor (1984) cryofixation leads to the solidification of water and solutes in a microcrystalline state. As a result the microanalysis of enamel provides information on the calcium content relative to the small amount of non-mineralized components of this structures. Morgan (1983) found that freeze-drying avoided the transformation of amorphous mineral into apatitic mineral, a transformation which occurs when some fluids are used to prepare mineralized specimens. The values we obtained are consistent with the concentrations and percentages found with other chemical methods (Jenkins, 1978; LeGeros and LeGeros, 1984), although EPMA has the advantage of preserving the specimen and thus providing a suitable degree of morphological resolution. The data obtained with this approach can be used to establish quantitative patterns of calcium in hyper- or hypomineralization alterations of the enamel, which thus far has been studied with other methods (Wright et al., 1995). Levels of calcium in enamel can therefore be determined more accurately and correlated more reliably with morphological features.

In dentine our analysis failed to demonstrate significant differences in calcium levels measured with the four different methodological modifications. Regardless of the methodological factors that might account for this finding, there are two potential sources of variability that should be considered. Firstly, macromolecular aggregates in dentine, including glycosaminoglycans which are more abundant than in enamel, interact in a characteristic manner with calcium. Two general reactions were postulated by Engel and Hilding (1984): the formation of complexes in which calcium is bound, and a distribution and concentration of calcium in ionic form which approximates the conditions of the Gibbs-Donnan equilibrium. Secondly, although the standards we use are valid for otoconia and enamel, they might have been less reliable for dentine, because of its greater proportion of organic matrix. The accuracy of alternative standards such as those prepared by different authors (e.g. dextran and calcium salts) should be investigated to determine whether these are more useful than the microcrystalline salts used in the present study (Roomans, 1988b; Crespo et al., 1993; López-Escámez et al., 1993).

Our findings in dentine are compatible with results obtained with other chemical methods. In our hands cryopreservation and freeze-drying yielded significantly less variability in microanalytical determinations of calcium in human dentine. Therefore despite the absence of significant variations between the four methods, we recommend the use of cryopreservation and freeze-drying for quantitative EPMA because of the consistency in the resulting data. However, current improvements in the ability of EPMA to resolve the elemental composition of biological specimens in quantitative terms give rise to new methodological challenges. Although highly mineralized specimens such as enamel and dentine would not in principle have been expected to produce statistically detectable variations, our findings reveal some small differences which are important not only in determinations of the normal quantitative pattern of calcification, but also in studies of alterations in pathological states. Moreover, accurate information on the calcium levels obtained with quantitative EPMA in both healthy and abnormal teeth can potentially be correlated with morphological features observed with scanning electron microscopy.

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