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STANDARDS FOR X-RAY MICROANALYSIS OF CALCIFIED STRUCTURES

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Abstract

The ability of electron probe X-ray microanalysis (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 specimen in the mode of interaction with the electron beam. Although there is a large experience in the quantification of elements in biological thin specimens, experience with standards for X-ray microanalysis of bulk specimens is limited, especially for calcified structures where the density of the specimen is difficult to estimate. The quality of the results in EPMA depends on obtaining accurate calibration curves which allow the establishment of the relationship between the signal measured and the concentration of the element of interest. The different methods for specimen preparation and the thickness of the specimen will also determine the precise nature of the standardization technique to be adopted. The physics of the electron beam-specimen interactions impose limitations upon the accuracy of calibration, and the choice of an unstable standard can result in large errors in the quantification of elements. We have reviewed the different types of compounds that have been used as standards for biological EPMA of thin and bulk specimens and discuss their potential use for quantitative analysis of mineralized tissues, with special reference to otoconia, the calcified structures of the vestibular system.

Key Words: Electron probe X-ray microanalysis, standard, calcium, quantification, biomineralization, otoconia.

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Introduction

Living organisms have more than forty different minerals in their constitution. Among these, the most frequently found are the phosphate and carbonate salts of Ca in association with organic polymers, such as collagen or chitin, which give structural support to bone and shells (Mann, 1988). Salts of Ba, Sr, Si and Fe are also found. These minerals, especially those formed by salts of Ca, may be present in membrane-bound vesicles within the eukaryotic cells, in the mucilaginous layers of cell walls in bacteria, or impregnated in biopolymers in the extracellular space. This latter situation occurs in calcified tissue, where salts of Ca are laid down in orderly arrays in association with a matrix of organic macromolecules. As living structures, they undergo active formation and remodelling, reflecting the ability of Ca salts to modify their mineral phase (Nancollas *et al.*, 1989). The types and function of the main Ca salts found in biological systems are presented in Table 1.

Electron probe X-ray microanalysis (EPMA) is a technique which allows the chemical analysis of microvolumes of material by using the X-radiation generated during the interaction between electron beam and specimen (Morgan, 1985; Roomans, 1988a).

The ability of EPMA to solve biological problems often depends on the use of a quantitative approach in which the preparation of suitable standards is required (Roomans, 1988b; Hall, 1989). Although different methods of quantification of elements in biological specimens have been described (Chandler, 1977; Boekestein *et al.*, 1980, 1984; Roomans, 1981, 1988b, 1990; Morgan, 1985; Hall, 1989; Warley and Gupta., 1990), the subject of standards for biological X-ray microanalysis is covered only briefly, especially for calcified tissues, since the density of the specimen is difficult to estimate.

The present paper summarizes the different types of compounds that have been used as standards for biological EPMA of thin and bulk specimens. We discuss their use for quantitative analysis of calcified structures found in biological systems with a high density matrix.

Calibration Standards and Quantitative EPMA

The theory of quantitative EPMA for biological thin specimens was developed by Dr. T.A. Hall (Marshall and Hall, 1968; Hall, 1968; Gupta, 1991). The absolute mass fraction $C_{x,sp}$ of the element x (i.e., element mass per unit mass of specimen) can be determined if specimen and standard are measured under identical analytical conditions by the Hall equation (Hall, 1971; Hall *et al.*, 1973):

$$Cx_{sp} = Ax \frac{(Ic,x/W)_{sp}}{(Ic,x/W)_{std}} (Nx/NZ^2)_{std} (Z^2/A)_{sp} \qquad (1)$$

where A_x is the atomic weight of the element x, $I_{c,x}$ is the characteristic X-ray intensity, W is the continuum or white radiation intensity, N_x is the number of atoms of element x, NZ^2 is the total number of atoms in the same volume weighted by the square of the atomic number (Z) and

$$Z^2/A = \Sigma(f_x Z^2/A^x) \qquad (2)$$

where f_x is the mass fraction of element x expressed as elemental mass/total mass (Hall and Gupta, 1986).

Other methods have been used for absolute quantification of elements in biological bulk specimens, i.e., the net peak intensities with ZAF (Z= atomic number, A= atomic weight, F= fluorescence) corrections (Boekestein *et al.*, 1980, 1983; Roomans, 1981). Nevertheless, quantitative analysis of bulk specimens can be performed more accurately using the peak-to-background (P/B) ratio method (Statham and Pawley, 1978; Small *et al.*, 1979), which was originally designed for particle analysis. This method is based on correcting for particle size and shape effects by measurement of both peak intensities and background radiation in the same energy range. This procedure assumes that emitted characteristic X-rays and the continuum radiation in the same energy region are influenced by absorption to the same extent, which requires that the relative depth and lateral distributions of characteristic and continuum X-ray production are the same (Armstrong, 1991). The principal advantages of the P/B ratio method are that it is independent of variations in beam current intensities and irregularities of the specimen surface (Boekestein *et al.*, 1984).

Quantification of biological tissues in an analytical electron microscope depends on the choice of suitable standards (Hall, 1971). Methods not employing standards are based on the principle that the parameters that determine the theoretical amount of specific X-rays emitted by the element per incident electron can be calculated and compared with the experimental values (Russ, 1974a; Nasir, 1976). Standardless analysis with conventional energy-dispersive detectors has, so far, not been considered to be possible for biological specimens if the absolute concentrations have to be determined, since some elements remain undetectable (H, C, N, O). This situation may be overcome if it is sufficient to know the ratio in which the elements are present in the sample (relative concentrations); but, if an absolute quantitative determination of concentration of elements is necessary, reference standards should be used (see, however, the contribution of Nicholson (1994) to this conference).

The principles of standardization and the use of various standards for quantitative X-ray microanalysis of biological specimens have been reviewed by Spurr (1975), Morgan *et al.* (1975), Chandler (1977), Roomans (1979, 1988b), Russ (1980), Condron and Marshall (1986), Warley (1990, 1993) and Patak *et al.* (1993).

The basic characteristics required for a compound to be used as a standard were defined by Anderson (1967): the material must have a well defined chemical composition, must be homogeneous at the spatial resolution used during the analysis and it must resemble the specimen to assure similar interactions with the electron beam. The standards for quantitative EPMA should be homogeneous at the level of resolution used: obtaining reproducible results when different areas with equivalent volumes are analyzed is a sign of the homogeneity of the standard. A definite advantage is chemical resemblance between the standard and the specimen. This will reduce the error during the correction procedure necessary in quantitative analysis (Roomans, 1988a). In addition, the preparation technique (e.g., cryosections versus sections of embedded tissue) and the thickness of the specimen will determine the choice of standard chosen for the calibration.

Standards may be classified into two types depending on the resemblance to the specimen in chemical composition. Ideal standards must have the same matrix and composition as the specimen. The choice of ideal standards implies that the composition of the specimen has already been established by other analytical techniques. Non-ideal standards are commonly used in routine microanalytical determinations, but mathematical corrections accounting for differences in absorption between specimen and standard must be applied (Condron and Marshall, 1986).

Since mineralizing tissues are composed of an organic matrix in which an inorganic crystalline component is laid down in the course of development

Table 1. Types of calcium inorganic compounds found in biological systems.

Mineral	Formula	Organism	Function
Calcium carbonate			
Calcite	$CaCO_3$	Vertebrates Algae Trilobites	Gravity device Exoskeletons Eye lens
Aragonite	$CaCO_3$	Fish Molluscs	Gravity device Exoskeleton
Vaterite	$CaCO_3$	Ascidians	Spicules
Amorphous	$CaCO_3$ n H_2O	Plants	Ca store
Calcium phosphate			
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	Vertebrates	Endoskeletons Teeth, Ca store
Octa-calcium phosphate	$Ca_8H_2(PO_4)_6$	Vertebrates	Bone formation
Amorphous	$Ca_9(PO_4)_6$	Vertebrates Mussels	Bone formation Ca store
Calcium oxalate			
Whewellite	CaC_2O_4 H_2O	Plants	Ca store
Weddellite	CaC_2O_4 2 H_2O	Plants	Ca store
Gypsum	$CaSO_4$	Jellyfish larvae	Gravity device

(Boyde *et al.*, 1961), and this matrix has the same background shape as any embedding medium (Nicholson and Dempster, 1980), standards used for soft biological tissues may be used in the initial stages of mineralization, but high density standards should be used in fully mineralized structures.

Applications of EPMA to Biomineralization

Electron probe X-ray microanalysis has been applied to calcification studies in the vertebrate tissues of bone, cartilage, dentin, enamel and otoconia. The organic matrices of bone, dentin and early enamel are known to constitute approximately 25-30 % of their respective dry weights and are largely acellular (Landis, 1979). These calcified tissues contain a solid inorganic mineral phase of a calcium phosphate salt, of which hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is the final form and contributes 70-75 % of the dry weight (Nicholson *et al.*, 1977). Since the otoconia, the calcified structures of the vestibular system, consist of a $CaCO_3$ mineral phase with a high density, they will be discussed separately later.

The first determinations of Ca/P ratios were made by Mellors (1964) in human bone and by Tousimis (1963) in mineralized epiphyseal cartilage from guinea pig tibia and gave results consistent with the stoichiometry of hydroxyapatite. Wergedal and Baylink (1974) determined bone mineralization rates of the tibial diaphyses from growing rats from Ca/P molar ratios. Mineral deposition in bone consisted of two phases: a first, rapid deposition of an amorphous calcium phosphate (Ca/P = 1.35), followed by a second phase with deposition of hydroxyapatite in an exponentially decreasing rate, with a maximum Ca/P = 1.60, which

was lower than the theoretical value predicted for hydroxyapatite alone (1.67). Analysis of calcified tissues have been carried out in dentin using characteristic intensities and ZAF correction factors (Sánchez-Quevedo *et al.*, 1989), and in the otoconia by measuring the P/B ratios without standards (Anniko *et al.*, 1984, 1987). Landis and Glimcher (1978) produced a set of calcium phosphate crystals characterized by X-ray powder diffraction from which molar Ca/P ratios were calculated, but only relative values for Ca and P concentrations were obtained, and inaccuracies in determination of X-ray intensities of P may appear (Payne and Cromey, 1990). De Bruijn (1981) suggested the use of crystalline-like standards when the elements in the specimen are present in a crystalline phase. Although this approach will not create major problems if the material is analyzed in a scanning electron microscope (SEM), difficulties will arise when the standards have to be embedded and thin- sectioned (De Bruijn, 1981).

In contrast, we have used bulk crystalline standards and the P/B ratio method to determine absolute concentrations of Ca and K in the otoconia (Campos *et al.*, 1992; Lopez-Escamez *et al.*, 1992, 1993b).

Preparation of Standards

The assumption that standards matching the specimen should be prepared in the same way as the specimen and to expect that contamination or loss of elements will occur to the same extent in both standard and specimen is not necessarily true. In our view, manipulation of standards should be simplified, or better, avoided. There may be a greater degree of certainty in having stable and stoichiometrically well defined standards and estimating the extent of mass modification in the specimen, and subsequently correcting for this change, than in adopting the "matching standard approach" (Patak *et al.*, 1993). Any manipulation of these standards (e.g., fixation, freeze-drying, carbon coating, air-exposure) may cause addition or loss of elements to the standard which may result in chemical changes.

Although methods for obtaining reference standards for biological specimens have been described (see Warley, 1990 for review), few authors have developed standards specifically for quantitative analysis of calcified tissues with a high density matrix (Janossy and Neumann, 1976; Krefting *et al.*, 1981; Campos *et al.*, 1992; Lopez-Escamez *et al.*, 1992, 1993b).

Standards for thin specimens in the transmission electron microscope (TEM) may be prepared as cryosectioned matrix (Warley, 1990) or thin crystals of inorganic salts (Roinel, 1975; Hyatt and Marshall, 1985). When standards are prepared for SEM, films of protein or dextran containing salts (Sumner, 1990; Crespo *et al.*, 1993), bulk crystals of salts (Zs.Nagy *et al.*, 1977; Lopez-Escamez *et al.*, 1993b) or glass standards (Fiori and Blackburn, 1982) can be used.

Standards for Thin Specimens

Cryosectioned matrix containing salts

Roomans (1979) proposed criteria for standards consisting of salts dissolved in an organic matrix:

1. The matrix should have the same composition of C, H, O and N elements as the tissue.

2. The element added to the matrix should be homogeneously dispersed and the concentration of elements should be adjusted within a range close to the biological tissue.

3. The matrix must be loadable with a wide variety of elements, and the concentration of these elements must be assessable by analytical techniques other than EPMA.

4. It should be possible to prepare ultrathin sections, or, even better, cryosections, of the standard. 5. The matrix must be stable under the electron beam.

To prepare these standards, known concentrations of water-soluble salts are added to a matrix (i. e., gelatin) to obtain a standard similar in composition to the specimen. A droplet of this matrix is mounted on stubs and cryofixed. The frozen standards are cut in a cryoultramicrotome to obtain thin sections similar to the specimen (Roomans, 1979; Warley *et al.*, 1983). These sections can be analyzed after freeze-drying or in the frozen-hydrated state. In addition, cryoprotectants such as glycerol may be added to minimize ice crystal formation during the cryofixation (Roomans and Sevéus, 1977). This type of standard is suitable for the analysis of thin specimens in the STEM mode in the TEM (Hagler *et al.*, 1983; Warley *et al.*, 1983).

However, calcified tissues are characterized by a mineralized matrix with a high density, and the matrix in these type of standards does not resemble the specimen, because the difference X-ray absorption between the "hard" specimen and the "soft" standard. This difference in interaction with the electron beam will lead to errors in the estimation of the background. Thus, the amount of salt required to increase the density of the matrix can not be solubilized properly and will tend to form aggregates resulting in an inhomogeneous standard. This method is also unsuitable for the analysis of high Z elements, such as Pt, Pb or Cd which form precipitates in the protein solution (Warley, 1990).

Aminoplastic standards

Roos and Barnard (1984) used a matrix composed of glutaraldehyde, urea and different concentrations of inorganic salts to obtain a standard which it is similar in chemical composition to biological tissue. However, these authors noticed several problems associated with the preparation of aminoplastic thin sections including shrinkage of the blocks after polymerization, collection of the dry sections without flotation, and that the sections had to be flattened mechanically. Lupton and Saubermann (1986) simplified the method using conventional Cu grids which were dipped into the liquid standard solution after which polymerization occurred directly on the grids. Although these standards have not been carefully investigated, inhomogeneity problems have been found when high Z elements had to be solubilized (Morgan and Winters, 1988, 1989). This type of standard has a matrix with lower density compared to calcified material, and it will be difficult to use with mineralized tissues because of the different absorption between standard and specimen.

Embedding resins containing inorganic salts

Mixtures of inorganic salts and embedding resins were initially used by Spurr (1975), but difficulties in dissolving the salts were observed early. Embedding resin containing salts have been used to determine relative concentrations in thin sections of calcified tissues with the Cliff-Lorimer molar ratio method (Cliff and Lorimer, 1975; Ali *et al.*, 1977). The value of such a ratio method lies in its applicability to a wider range of thicknesses (Russ, 1974b).

The introduction of crown ethers as solvents of inorganic salts in embedding resins (Spurr, 1974; Chandler, 1976; Harvey *et al.*, 1980), was an improvement for this type of standard. Crown ethers are large organic molecules which may form complexes with alkali metals and which are soluble in epoxy resins (Condron and Marshall, 1986). These compounds have been mainly used for the preparation of standards for Na and K, although they also can be used for transition elements (Chandler, 1976). Instead of using carrier molecules to incorporate elements into the embedding resin, elements which form complexes with organic compounds that are directly soluble in the resin have also been employed (Roomans and Van Gaal, 1977; Weakley *et al.*, 1980).

Calculations using absorption corrections based on Phi-Ro-Z curves (Marshall, 1982) have led to the identification of irregularities in the distribution of Na and K crown ether complexes in epoxy resin standards, that were probably due to sedimentation during polymerization. Although this might be avoided by continuous stirring during polymerization, nominal concentrations in epoxy resin standards should not be accepted without an independent chemical analysis of the selected region of the block for EPMA (Condron and Marshall, 1986).

De Bruijn (1981) used Chelex^{100}- ion exchange beads with negatively charged iminodiacetate groups which could bind different cations in a controlled way as reference standards. These beads could be satisfactorily embedded in Epon resin and sectioned, but not in Spurr resin because of the shrinkage of the polymer. Ultrathin sections of these beads can be used to calibrate the X-ray signal against concentration, but again differences in the matrix make it difficult to use this standard for thin sections of calcified tissues.

Thin crystals of inorganic salts

Janossy and Neumann (1976) produced microcrystals of $CaHPO_4$ by pipetting solutions of the salt onto coated Cu grids, which were air-dried and carbon-coated to measure ultrathin sections of dentine. However, they found problems with the Hall method, since large uncertainties arose during determination of the background. The Ca/P ratios of mineralized tissues were calculated in the TEM with calcium phosphate standards (Ca/P molar ratios ranging from 0.50 to 1.62), but only relative concentrations were obtained (Landis, 1979). Krefting *et al.* (1981) used mixtures of inorganic salts dissolved in distilled water, pipetted in narrow lines and rapidly frozen in liquid nitrogen and freeze-dried, similar to the microdroplet technique described by Roinel (1975) and compared them to epoxy-resin standards. Using this mixture, Krefting *et al.* (1981) measured the concentrations of elements in the mineralized and unmineralized epiphyseal growth plate. They recommended the recrystallized salt standards because of the easy preparation, the known composition and the stability under the electron beam. However, the recrystallized standards are not particularly homogeneous at a resolution level of a micrometer or less, and they are probably too thick for quantitative EPMA of thin specimens in the TEM.

Thin crystals of binary salts can be produced by spraying an aqueous solution of the appropriate salt onto Pioloform coated grids (Morgan, 1983; Hyatt and Marshall, 1985), and heating them in a hot plate to obtain small crystals. Salts which are insoluble in water can be resuspended in water and sprayed in the same way (Patak *et al.*, 1993). Addition of polyvinylpyrrolidone (PVP) facilitates production of thin droplets and reduces crystal size (Rippon *et al.*, 1993). These standards are also easy to produce for a wide range of elements, they are chemically well defined, and

the composition is known exactly. Although the vast difference in matrix composition of these standards from biological specimens could be inconvenient, this may actually be an advantage for the analysis of calcified thin specimens.

Standards for Bulk Specimens

Protein or dextran films

Shuman *et al.*, (1976) used several proteins with known concentrations of covalently bound elements (P or S) as standards. They prepared thin films of these proteins on film-covered grids, heating the grids at 60° C during 1 h. Several salts can be added to the protein solution to obtain standards for different elements. Sumner (1978) made up 1- 1.5 μm thick protein standards by dipping grids into a gelatin solution containing inorganic salts. These grids can either be air-dried (Sumner, 1978; 1990) or plunge frozen and freeze-dried. Wyness *et al.* (1987) prepared slam-frozen gelatin standards with different salts for bulk specimens and analyzed them in the frozen-hydrated state with the aid of a cryochamber attached to the SEM.

We prefer to use dextran solutions containing inorganic salts instead of gelatin, because S is already present in gelatin (Crespo *et al.*, 1993; Lopez-Escamez *et al.*, 1993a). Small droplets of a dextran solution are deposited on Ni grids glued with Leit C carbon cement to SEM stubs. The mounted standards are rapidly plunge-frozen in liquid nitrogen-cooled freon 22 and freeze-dried (Crespo *et al.*, 1993). These standards are much simpler to prepare than standards made from gelatin cryosections (Roomans, 1979; Warley *et al.*, 1983). The coefficient of variation of measurements from the same standard was in the range of 5% (Sumner, 1990), which is similar to those obtained from cryosectioned standards (Roomans and Seveus, 1977; Warley *et al.*, 1983; Saubermann *et al.*, 1981). We have found that the coefficient of variation of the P/B ratio increases in dextran standards containing KH_2PO_4 when the concentration of the salt was higher than 0.5 M (Lopez-Escamez *et al.*, 1993a), which indicates that solubilization problems could appear at higher concentrations. This detracts from the application of these type of standards for the analysis of calcified structures, because the difference in the density of the mineral matrix as compared to either gelatin or dextran.

Bulk crystals of salts

Zs.Nagy *et al.* (1977) developed a freeze-fracture, freeze-drying technique for quantitative analysis of bulk specimens in SEM, using the continuum normalization method and bulk crystals of salts as standards. Zs.Nagy and Casoli (1990) also demonstrated that the calibration curves obtained were similar, regardless of whether the continuum radiation used for the calculation of the P/B ratio was selected under the respective elemental peaks or in a peak-free region of the spectrum. These findings confirmed the validity of the Hall continuum normalization method even for bulk specimens. However, Zs.Nagy *et al.*, (1977) noted that not all crystalline salts were suitable for the calibration, because some of them were unstable under the electron beam (i.e., potassium oxalate) or showed a deviation from the expected values due to secondary fluorescence (KCl). In addition, the crystals containing crystalline water tended to lose it during the local warming up of the crystals under the electron beam. The limitation of this approach was the difference in matrix composition between the crystals and the soft biological tissue. This led to the use of crystals composed of low atomic number elements ($Z < 11$) plus the element to be analyzed (e.g., Na, K).

Payne and Cromey (1990) used calcium phosphate crystals in the SEM as standards for quantification of bone fragments, and found a selective absorption of P when calcium phosphate standards were embedded in epoxy resin. Crystalline standards were produced by applying the pure crystals to a thin layer of unpolymerized Spurr resin and allowing the resin to harden at room temperature. According to Roomans (1979), P X-rays are absorbed six times more than Ca X-rays in a 5 μm-thick carbon film. This ratio is probably much higher in a bulk specimen, which probably reflects the fact that epoxy resin has a higher mass absorption coefficient for P X-rays (lower energy) compared with Ca X-rays. This problem limits the use of bulk crystals for quantitative analysis of P in calcified structures and raises serious doubts about the values of relative Ca/P ratios in the SEM with energy dispersive detection systems (Payne and Cromey, 1990).

We have used bulk crystals of salts to calibrate the P/B ratio against concentration to measure absolute Ca and K concentrations in the otoconia (Table 2). Bulk crystals were mounted on a pre-glued carbon disc on a SEM stub with a nickel grid used to avoid overpenetration of the crystal (Lopez-Escamez *et al.*, 1992). These standards fulfill Anderson's criteria for the analysis of calcified structures (Anderson, 1967). Their composition is stoichiometrically defined, and their crystalline structure guarantees a constant relationship between the atoms resembling the otoconia in the analyzed microvolume. We tested the reproducibility of the standards using different bulk microcrystals of the same salt mounted at the same time (Fig. 1): they showed similar values for the P/B ratios under a given instrumental configuration. Stability and homogeneity

Table 2. Bulk crystalline salts of Ca and K used to calibrate the P/B ratio against concentration in the X-ray microprobe in an SEM.

Crystalline salt	Formula	MW	WF	Z^2/A	P/B ratio
Calcium salts					
Calcium bis(dihydrogen-phosphate) monohydrate	$Ca(H_2PO_4)_2H_2O$	252.08	15.89	5.68	20.08
Calcium bis(dihydrogen-phosphate)	$Ca(H_2PO_4)_2$	234.10	17.12	5.83	19.40
Calcium hydrogen-phosphate dihydrate	$CaHPO_4\ 2H_2O$	172.09	23.29	5.89	30.61
Calcium acetate	$(CH_3\text{-}COO)_2Ca$	158.20	25.33	5.09	34.12
Calcium hydrogenphosphate	$CaHPO_4$	136.10	29.45	6.48	30.61
Dicalcium pyrophosphate	$Ca_2P_2O_7$	254.10	31.54	6.68	32.86
Tricalcium phosphate	$Ca_3P_2O_8$	310.18	38.76	6.97	38.67
Calcium carbonate	$CaCO_3$	100.09	40.04	6.27	44.02
Calcium hydroxide	$Ca(OH)_2$	74.09	54.09	7.15	47.28
Potassium salts					
Potassium lactobionate	$KB[C_6H_3(CF_3)_2]_4$	902.32	4.30	3.79	9.11
Potassium chromium sulfate	$KCr(SO_4)_212H_2O$	499.41	7.83	3.93	11.16
Potassium aluminium sulfate	$AlKSO_4\ 12H_2O$	474.40	8.24	3.46	12.59
Potassium dichromate	$Cr_2O_7K_2$	294.21	13.29	7.89	12.53
Potassium dihydrogen-phosphate	KH_2PO_4	136.09	28.73	6.02	25.68
Potassium hydrogen-phosphate trihydrate	$KHPO_4\ 3H_2O$	228.23	34.26	5.58	47.53
Potassium ferrocyanide	$K_3Fe(CN)_6$	329.26	35.62	6.89	40.67
Potassium hydrogen phosphate	K_2HPO_4	174.18	44.89	6.91	47.27
Potassium carbonate	K_2CO_3	138.20	56.59	6.87	56.50

(MW= molecular weight, WF= weight fraction). Analytical conditions: accelerating voltage 15 kV, tilt angle 30°, take-off angle 45°, working distance 45 mm, spot size 100-200 nm, count rate 1000 counts per second, live time 50 seconds.

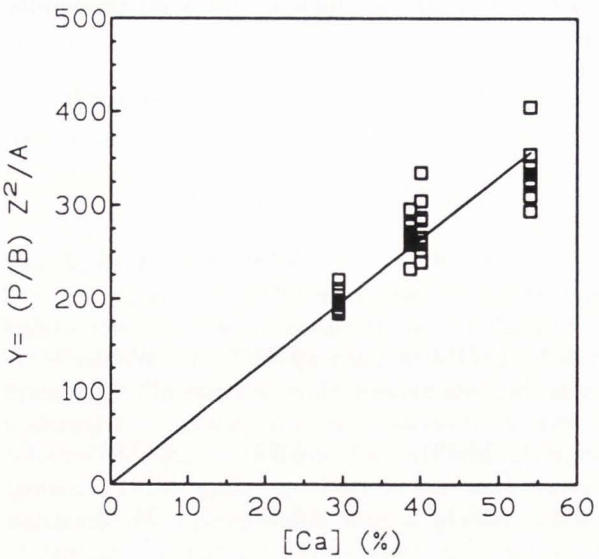


Fig. 1. Calibration curve for $Y = (P/B) \bullet Z^2/A$ against Ca concentration for $CaHPO_4$, $Ca_3P_2O_8$, $CaCO_3$, $Ca(OH)_2$ crystalline bulk standards. Regression line through origin was $y = 6.60\ x$ ($N = 41$, $r = 0.8741$, $P < 0.0001$). Spectra were obtained during 50 s live time at 10,000 X at the following analytical conditions: voltage 15 kV, tilt angle 35°, take off angle 50°, count rate 1000 counts per second.

are some of the advantages of crystalline standards for mineralizing tissue (Krefting *et al.*, 1981; Lopez-Escamez *et al.*, 1992). The accuracy of the calibration was in agreement with the results obtained by other authors (Zs.Nagy and Casoli, 1990; Reid *et al.*, 1992). The coefficients of correlation were close to 1.00 for the wide range of concentrations studied (Lopez-Escamez *et al.*, 1993b). We also found that the accuracy of the calibration was not dependent on the accelerating voltage of the SEM in the range of 10-25 kV when the P/B ratio was corrected for Z^2/A (Lopez-Escamez *et al.*, 1993b). We recommend the application of these salt standards in the SEM to obtain calibration curves for other elements.

Glass standards

Fiori and Blackburn (1982) used lithium borate glasses as microprobe standards. They have many properties which make them very attractive as analytical standards for microbeam analysis. The numerous oxide glass-forming systems allow selection of the glass matrix in which the elements of analytical interest are to be incorporated. However, one of the most interesting features of glass is that it can be fabricated in a variety of shapes and sizes all from the same parent glass melt of well-characterized composition, resembling the shape

Fig. 2. Characteristic, continuum and P/B ratio obtained for Kα emission line of calcium in $Ca_3P_2O_8$ crystalline standard as a function of spectra acquisition time. The slope of the regression lines were not statistically significant from zero ($P > 0.05$) and none of the signals measured depend on the time of irradiation of the standard.

Fig. 3. Characteristic, continuum and P/B ratio obtained for Kα emission line of calcium in $CaCO_3$ crystalline standard as a function of spectra acquisition time. As observed for $Ca_3P_2O_8$, the slope of the regression lines were not statistically significant from zero ($P > 0.05$). The volume irradiated in both standards, measured as the continuum signal, is approximately the same.

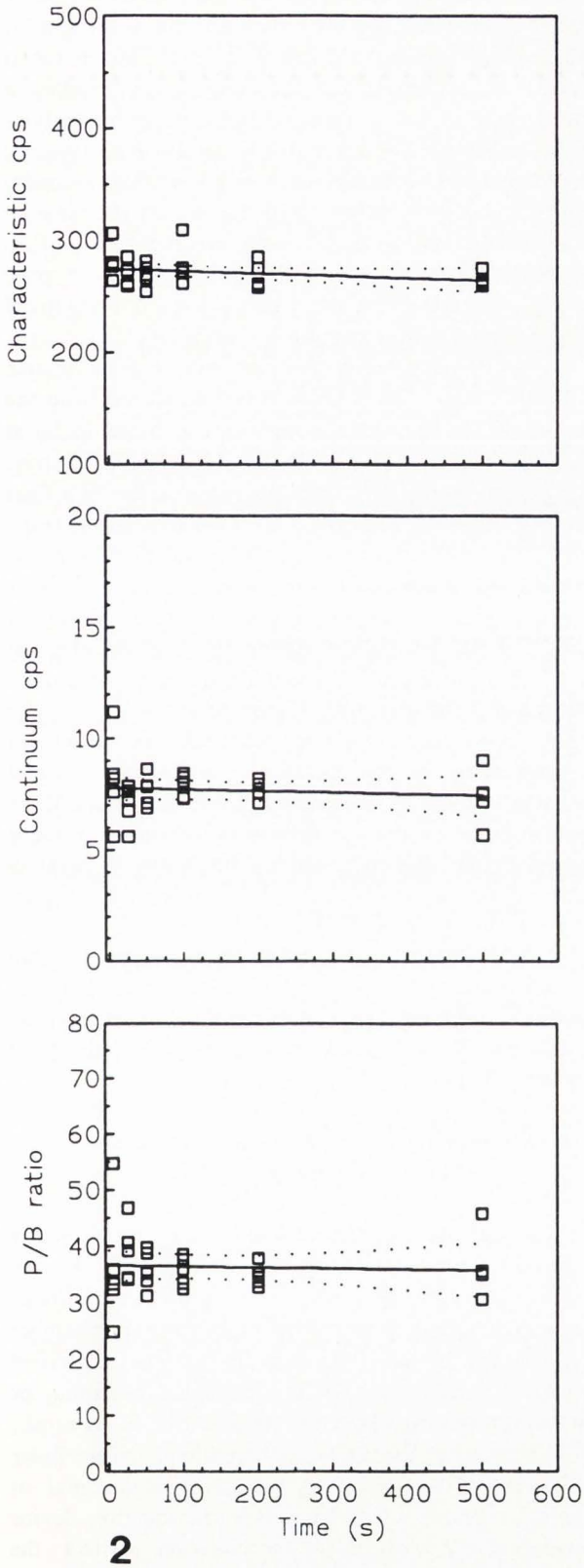
and size of the specimen (Fiori and Blackburn, 1982). Almost any element in the periodic table may be included in these glasses at a concentration up to several weight per cent. These standards have been used to calibrate the P/B ratio against Mg concentration and applied to individual lymphocytes (Hook *et al.*, 1986).

The proposed glasses are suitable as standards for the microanalysis of bulk specimens and they satisfy the requirement for a microanalytical standard: the glass structure is very homogeneous even with several elements present and they are resistant to electron beam induced irradiation damage, and could be very convenient for the quantitative EPMA of calcified tissues.

Analysis of Standards: Calibration of the X-ray Detector

The relative efficiency of the X-ray detection system for measuring all elements in the detectable range must be evaluated by choosing appropriate sets of standards (Chandler, 1977). Calibration curves of standards are characteristic for a given instrumental configuration and they are not valid under different analytical conditions (e.g., voltage, take-off angle, tilt angle). This is inconvenient because each instrument needs to be calibrated every time that any of these parameters are modified. A relatively easy way to assess the sensitivity and reproducibility of our microprobe for specific X-ray emission lines is to measure a standard containing the element of interest in a known concentration to check that the system is calibrated. The value obtained should not be statistically different from the one estimated by the calibration curve. This should be done every time that a specimen with an unknown concentration of the element is measured.

The monitoring of the background signal in the


Characteristic cps
500
400
300
200
100
Continuum cps
20
15
10
5
0
P/B ratio
80
70
60
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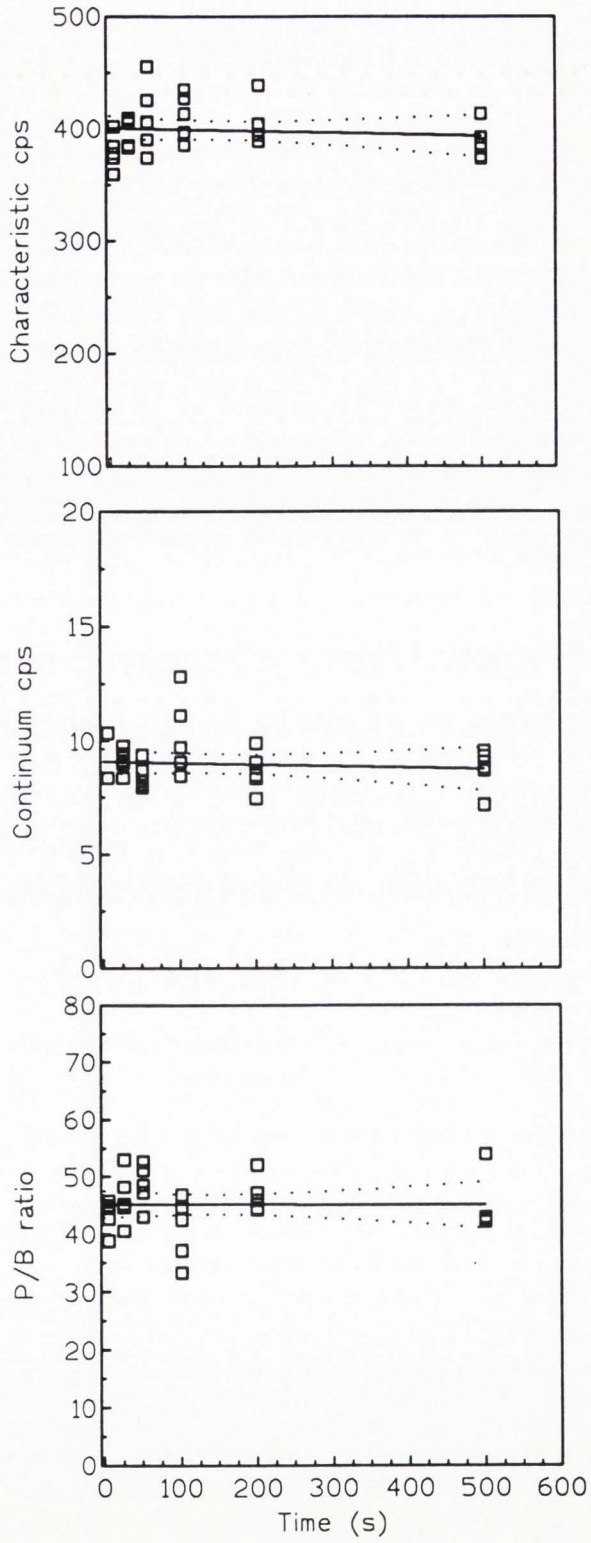
Time (s)

2


Characteristic cps
500
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Continuum cps
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P/B ratio
80
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Time (s)

3

different standards is a useful parameter to avoid differences in the excited mass between the standards and the specimens (Lopez-Escamez *et al.*, 1993b). Regardless of the method of quantification used, the measured signal (i.e., P/B ratio) for the element of interest must be plotted as a function of the concentration of the element (Fig. 1). The usual procedure is to analyze a number of standards which contain this element at different concentrations and to demonstrate a linear relationship between the measured signal and the concentration of the element. The calibration constant k, calculated for each element, depends on the instrumental configuration used. However, the demonstration of a linear relationship itself between the P/B ratio (corrected for Z^2/A if necessary) and the concentration, does not necessarily indicate the accuracy of the calibration by itself (Warley, 1990). Accuracy can be evaluated, if the results found in the microanalytical study are in agreement with those obtained by other independent analytical techniques. The accuracy of the calibration also depends on the correct estimation of the background (Hall and Gupta, 1984; Roomans, 1988c; Zierold, 1988). This is important in thin specimens, where the extraneous contribution (film, grid, the surroundings in the microscope) to the total background can introduce inaccuracies in the estimate of the specimen contribution (Warley, 1990).

Sensitivity to the Electron Beam: Irradiation Effects

The physics of the electron beam-specimen interactions impose limitations upon the accuracy of the calibration, and the choice of an unstable standard can results in large errors in the quantitative EPMA (Edie and Glick, 1980). Electron beam sensitivity has been defined as alterations affecting the structural and chemical integrity of the irradiated specimen (Hobbs, 1987).

Edie and Glick (1979) observed that irradiation effects can influence the absolute quantification of Ca and P in mineralized tissue and correction factors are necessary for a reliable estimate of Ca/P ratios. About 10^{11} electrons per area analyzed are necessary to obtain a significant spectrum (Hall and Gupta, 1984). This necessary electron dose also depends on the thickness of the specimen, but radiation damage is unavoidable in most EPMA determinations (Roos and Morgan, 1990; Von Zglinicki, 1993). Therefore, standards matching the radiation sensitivity of the biological specimen should be analyzed with the same range of radiation doses.

In this study, we present new data regarding the problem of radiation damage in bulk standards. We have checked the stability of crystalline standards under the electron beam, monitoring characteristic and continuum signals at different spectra acquisition times for $CaCO_3$ and $Ca_3O_8P_2$ standards (Figs. 2 and 3). This makes it possible to determine whether there is any irradiation effect (mass loss or contamination) during the analysis of the standards. Figs. 2 and 3 show that both types of signals remained constant even for a long (500 seconds) live time and that they do not depend on the time of acquisition of the spectra. These observations confirm the findings of Morgan and Davies (1982) in pure mineral microdroplets standards: Ca is not volatized from uncooled specimens during the analysis.

Mass loss depends on the amount of organic matrix is the specimen. The concentrations estimated from the analysis of the mid-dentine region of a human molar at 12 kV, which possess 25-30 % organic matrix, increased exponentially from the initial value to a final stable value after prolonged electron irradiation (Edie and Glick, 1980). In contrast, there was no irradiation effect in the analysis of mature enamel since there is minimal organic content in this structure (Edie and Glick, 1979). Irradiation effects for a given electron dose were reproducible in mineralized tissues: little mass loss occurred in the 10^{-10} C/μm^2 analysis, but as the dosage increased, the apparent Ca and P increased and approached to the concentrations obtained from direct chemical analysis of the mineral phase. The effect of the electron beam on the specimen was to remove a major portion of the specimen leaving the heavy mineral (a form of hydroxyapatite) in apparently higher concentration (Edie and Glick, 1980).

The choice of crystalline standards, either for thin or bulk specimens, for the quantitative analysis of calcified structures will reduce the electron beam irradiation effects (e.g., mass loss or contamination) and produce reliable calibration curves.

The Otoconia: Calcified Structures in the Vestibular System

The saccule and the utricle are the two sensory organs of the vestibular system characterized by the presence of calcified structures lying upon the ciliated sensory epithelium located in the endolymphatic chamber (Hillman and Lewis, 1971; Lim, 1973). These calcified structures, made of $CaCO_3$ crystals (otoconia or statoconia) which contain a protein matrix (Ross *et al.*, 1985; Pote *et al.*, 1993), are tethered to an extracellular matrix called the otolithic, otoconial, statoconial or gelatinous membrane and act as a transduction device where gravity and linear acceleration deflect the stereocilia of the hair cells in the vestibular sensory epithelium.

EPMA studies have centered on the determination of the elemental composition of developing (Anniko *et al.*, 1987; Campos *et al.*, 1984) and mature otoconia (Anniko *et al.*, 1984, 1985; Campos *et al.*, 1990), and the effect of aminoglycoside-induced toxicity in the otoconia (Cañizares *et al.*, 1990; Campos *et al.*, 1994). However, it has been difficult to compare the results from different authors because of differences in preparation of inner ear samples, the redistribution of diffusible ions and the preservation of the morphological features (Anniko *et al.*, 1981, 1984). In addition, the relatively brief experience in quantitative EPMA of biological bulk specimens and the lack of suitable standards for a fully quantitative analysis of the otoconia, has been overcome only recently (Campos *et al.*, 1992, 1993; Lopez-Escamez *et al.*, 1992, 1993a), when absolute Ca and K concentrations in otoconia were measured by using a set of bulk microcrystalline standards. We have also shown that mass loss, measured as the number of background counts per second as a function of live time, does not occur in otoconia (Lopez-Escamez *et al.*, 1993b).

The otoconia and the gelatinous membrane form a dynamic system where Ca and other ions are exchanged during the process of otoconial biomineralization, and calcite is assumed to be constantly removed and redeposited at the otoconial surface throughout life (Ross and Pote, 1984). Recently, a linear relationship has been found between Ca and K concentrations in the otoconia of adult mice in the saccule and the utricle (Campos *et al.*, 1994). However, this relationship was not observed after chronic gentamicin treatment, indicating a dissociation between Ca and K in the otoconia. These results are consistent with the hypothesis that K is present in the mineral phase of otoconia of both organs.

A detailed knowledge of the biochemical environment of the otoconia (gelatinous membrane structure, local endolymphatic composition) is essential if we want to understand the complex interactions between organically controlled and purely physical processes that result in a specific calcified structure. This information can be provided by quantitative EPMA, by using different types of standards resembling the otoconia, the gelatinous membrane and the endolymph. We expect that this quantitative approach will lead to a better understanding of the physiological phenomena that occur in the stereociliary apical surface of vestibular sensory epithelia.

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Discussion with Reviewers

A.J. Morgan: You state that "...glass could be very convenient for the quantitative EPMA of calcified tissues". You also describe how this material possesses a number of advantages, including availability as thin shards and as a bulk material containing homogeneously distributed elements. The case that you, and the original authors of the papers that you refer to, make for the use of glass is compelling. How do you, therefore, justify the use of crystalline standards?

Authors: We do not have any experience with glass as standard for SEM. A comparative study between microcrystalline and glass standards will be very useful to determine the best approach. However, the homogeneity of crystalline standards is a definite advantage.

A. Warley: You use dextran as a matrix for the standards that you recommend for soft (not mineralized) tissue. The use of dextran worries me a little because those who use this compound regularly do so using a cold stage which will minimize mass loss. Since dextran is a carbohydrate polymer and Shuman *et al.* (1976) has shown that mass loss from carbohydrates differs in extent to that from proteins, it is possible that dextran may have different mass loss properties compared to the usual protein standards. Do you have any comment to make about this?

Authors: It is possible that dextran and protein standards have a different mass loss rate. Shuman *et al.* (1976) (text reference) compared mass loss, as measured by X-ray continuum counts, in sucrose and serum albumin thin specimens in TEM. Mass loss was about a 30% higher in sucrose than in albumin standards. However, these results are not comparable to ours since we have analyzed thicker films of dextran (molecular weight 300,000), not thin films of sucrose, in an SEM configuration.