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Limitations of ZAF Correction Factors in the Determination of Calcium/Phosphorus Ratios: Important Forensic Science Considerations Relevant to the Analysis of Bone Fragments Using Scanning Electron Microscopy and Energy-Dispersive X-Ray Microanalysis

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ABSTRACT: A series of calcium phosphate standards having calcium/phosphorus (Ca/P) molar ratios of 0.50, 1.00, 1.34, 1.50, and 1.67, respectively, was prepared for bulk specimen analysis using scanning electron microscopy (SEM) and energy-dispersive X-ray microanalysis (EDXA). The standards were mounted on carbon planchettes as either pure crystals or crystals embedded in epoxy resin. Ten different samples of each embedded and non-embedded standard were analyzed in a JEOL 100 CX electron microscope interfaced with a Kevex 8000 EDXA system using a lithium-drifted silicon detector and a multichannel analyzer. The Ca/P ratios were determined by calculating both net peak intensities without matrix corrections and atomic *k*-ratios using the MAGIC V computer program with ZAF correction factors for quantitative analysis. There was such extensive absorption of phosphorus X-rays in standards embedded in an epoxy matrix that the observed Ca/P ratios were statistically compatible with four different standards ranging in theoretical Ca/P ratios from 1.0 to 1.67. Although the non-embedded crystals showed a greater separation in the Ca/P ratios, both methods of preparation produced serious flaws in analysis. Direct application of the discovery of this caveat to the identification of suspected bone fragments for forensic science purposes is discussed.

KEYWORDS: forensic science, X-ray analysis, microscopy

Electron probe X-ray microanalysis [1,2] is a powerful tool that has been widely used in calcified-tissue research [3-17] and that recently has been added to the medical examiner's armamentarium [18]. The preparation and analysis of standards [19-21] is crucial to the proper interpretation of calcium/phosphorus ratios calculated from the emitted X-rays of probed biological specimens. Landis and Glimcher [10] and Landis [9] have been most thorough in the preparation of calcium phosphate standards (Ca/P molar ratios

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ranging from 0.50 to 1.62) for use with tissues analyzed by transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM). Unfortunately, other investigators using X-ray microanalytic methods on specimens probed in the scanning electron microscopy (SEM) mode have usually used standards with one theoretical Ca/P ratio [3,5,11–13,16,17]. Edie and Glick [22,23] state that irradiation effects can influence the absolute quantitation of calcium and phosphorus in mineralized tissue, but atomic number-absorption-fluorescence (ZAF) correction factors give a reliable estimate of Ca/P ratios. Boekstein et al. [24] state, however, that few systematic studies have been performed to investigate the reliability of quantitative electron probe X-ray microanalysis on biological bulk specimens.

We have recently analyzed, using energy-dispersive X-ray microanalysis (EDXA), a series of analytical grade calcium phosphate crystals probed in the SEM mode, for use as standards in the identification of bone fragments for forensic science purposes. Our results contradicted the findings of others [22,23] and raise serious questions regarding the reliability of determining Ca/P ratios in bulk specimens. There was such extensive loss of phosphorus X-rays in standards embedded in an epoxy matrix, that the experimental Ca/P ratios were statistically compatible with four different standards ranging in theoretical Ca/P ratios from 1.0 to 1.67. The discovery of this selective X-ray absorption in the analysis of these embedded standards has direct application to the identification of bone fragments [18] and to mineralized tissue research, in general. Many investigators embed these tissues in either epoxy resins [5,13,15,17], methacrylate [3], or Lucite [12] as a routine procedure for X-ray microanalysis in the SEM mode and may not be aware of this problem in semiquantitative analysis. Since bone is composed of an organic matrix in which an inorganic crystalline component is laid down in the course of development [3,25], and this organic matrix has the same background shape as any embedding medium [14], serious flaws in the analysis of suspected bone fragments for forensic science purposes are possible.

Although SEM-EDXA remains a valuable tool to the forensic scientist [18,26–29], the discovery of this caveat in the X-ray microanalysis of calcified bulk specimens prompts this report.

Materials and Methods

Standards

The following three analytical grade calcium phosphate standards were obtained from Sigma Chemical Co. (St. Louis, Missouri): $\text{Ca}(\text{H}_2\text{PO}_4)_2$, Ca/P = 0.5; CaHPO_4 , Ca/P = 1.0; $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$, Ca/P = 1.67. Octacalcium phosphate $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$, Ca/P = 1.34, and tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$, Ca/P = 1.50 were a generous gift from Dr. William J. Landis,³ who prepared these standards *in vitro* and characterized them by powder X-ray diffraction and wet chemical analysis from which molar Ca/P ratios were calculated.

Mounting of Standards for Bulk Analysis

Two different methods of mounting the calcium phosphate crystals on planchettes were used (Fig. 1). The first method involved coating the bottom of a carbon planchette with a thin layer of unpolymerized Spurr's epoxy resin, applying the pure crystals to the wet resin, compacting the crystals with a spatula, and allowing the resin to harden at room

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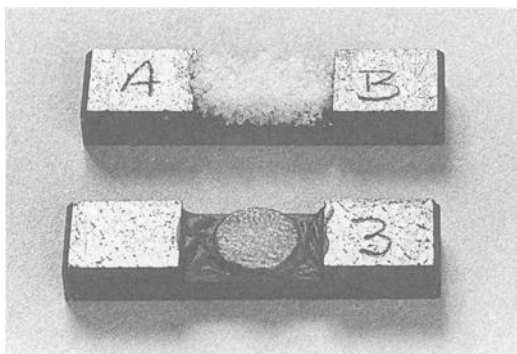


FIG. 1—Methods of mounting Ca/P standards for EDXA in the SEM mode. Analytical grade crystals are placed directly on a carbon planchette that has been previously coated with a thin film of epoxy resin (top). A disk of polymerized epoxy resin containing Ca/P standard is mounted on a carbon planchette using carbon paste (bottom).

temperature for several days. The loose crystals on top were then removed with compressed air.

The second method involved filling size 0 Beem capsules three-quarters full with the crystals, compacting the crystals by tapping the bottom of the Beem capsule on the table top, and then filling the capsule to the top with epoxy resin. The epoxy resin was allowed to polymerize in the oven overnight. Disks were then cut from these embedded standards using a jeweler's saw and mounted on carbon planchettes using carbon paste. The disks were purposely left unpolished to determine the standard deviation after microanalysis of these microscopically rough surfaces. The specially prepared octacalcium phosphate and tricalcium phosphate standards received as a gift from Dr. Landis were only mounted using the second method, since Method 1 required more sample than was available.

X-Ray Microanalytic Methods

Ten different samples of each embedded and nonembedded standard were analyzed in the SEM mode of a JEOL 100 CX electron microscope interfaced with a Kevex 8000 EDXA system using a lithium-drifted silicon detector and a multichannel analyzer. The standards were analyzed using 40-KeV accelerating voltage $\times 200$ magnification (0.067-mm² surface area probed) using a fast raster speed. Each spectrum was acquired for a total of 400 s. The specimen was angled toward the detector using a tilt angle of 35°. A Faraday cup was not used to monitor beam current since our study utilizes relative *k*-ratios which do not change with fluctuations in beam current [30].

Background Removal and Determination of Ca/P Ratios using Linear Regression Analysis

The Kevex automatic background removal program allows one to shape the background continuum by preferentially removing more background from the lower or higher end of the 10-KeV energy scale using a running average technique (window thickness from 1 to 99). The relative counts in the calcium and phosphorus peaks were then determined by Gaussian integration methods and the Ca/P atomic ratios determined using ZAF matrix correction factors and the ASAP routine (MAGIC V computer program). The phosphorus X-rays were integrated over a window thickness from 1.76 to 2.38 KeV and the calcium X-rays from 3.40 to 4.30 KeV. [ZAF correction factors provide actual intensity

ratios after adjusting for atomic number (Z), absorption (A) within the sample, and X-ray-induced fluorescence (F) within the sample, based on the average atomic number of the sample. The quantify modifier, ASAP, executes a standardless analysis for bulk samples using matrix corrections, calculates theoretical k -ratios and normalizes the sum of the k -ratios to 1.]

A linear regression line was then calculated for each sample using different background shape factors (progressive background count removal from 1 to 10 KeV) and the x -intercept used to determine the experimentally derived Ca/P ratio for that standard. Background (bremsstrahlung) shape factors of 70, 80, 90, and 99 (window thicknesses in Kevex set-up parameters) were used to calculate linear regression lines with a 95% confidence level. The use of background shape factors below 70 (lower end of the 1 to 10 KeV energy scale) introduced errors in the calculation of Ca/P ratios because of the difficulty in integrating the phosphorus peaks. The inclusion of these lower background shape factors decreased the confidence in calculating a linear regression line below 90% and therefore were not used in the present analysis.

Net peak intensities were also calculated after background removal using shape factors 70 to 99 (higher end of the 1 to 10 KeV energy scale).

Linear Regression Analysis

A least-square fit was determined for each set of data points to determine the relationship between the variables. Regression lines that fit the equations derived from the method of least squares ($y = Ax + B$; $A = \text{slope}$, $B = \text{y-intercept}$) were then graphed. A correlation coefficient (r) was obtained for each set of variables to determine how well the data points fit the linear regression. The correlation coefficient is a value between -1 and $+1$. At $r = 0$ there is no fit, while at $r = \pm 1$ there is a perfect fit.

Statistics

The mean Ca/P ratio \pm S.D. (standard deviation) for each standard was then determined using ten separately acquired spectra. The coefficient of variation was expressed as

$$\frac{\text{S.D.}}{\text{mean}} \times 100$$

The means were statistically compared using Student's t -test.

Results

A comparison of the mean Ca/P atomic k -ratios between embedded and nonembedded standards indicated a greater standard deviation and coefficient of variation in two (Ca/P = 1.0 and 1.67) of the three standards prepared as pure crystals compared to the embedded standards (Table 1). A calibration curve of the unembedded standards (Fig. 2) shows a clear separation between all three standards using linear regression analysis. The difference between the Ca/P ratios of the 0.50 and 1.00 standards was statistically significant ($p < 0.001$), whereas the difference between the 1.00 and 1.67 standards was not ($p > 0.05$). Although the coefficient of variation of the mean Ca/P atomic k -ratios of the five embedded standards varied between 5 and 9%, the calibration curve (Fig. 3) revealed a distinct crowding of four different standards (Ca/P ratios from 1.00 to 1.67) using linear regression analysis. Indeed, there was no statistical difference between these four standards (p -value > 0.1 between the observed Ca/P ratios of the 1.00 and 1.67

TABLE 1—Comparison of Ca/P atomic ratios between embedded and nonembedded standards.

Analytical Grade Standards	Theoretical Ca/P Ratio	Observed Mean Ca/P Atomic k-Ratios \pm S.D. ^a	Coefficient of Variation
Ca(H ₂ PO ₄) ₂ embedded ^b	0.50	0.83 \pm 0.06	7.5
nonembedded ^c	0.50	0.72 \pm 0.04	5.0
CaHPO ₄ embedded	1.00	1.58 \pm 0.08	5.0
nonembedded	1.00	1.49 \pm 0.14	9.4
Ca ₈ H ₂ (PO ₄) ₆ · 5H ₂ O embedded	1.34	1.52 \pm 0.10	6.3
nonembedded	...	NP ^d	...
Ca ₃ (PO ₄) ₂ embedded	1.50	1.73 \pm 0.15	8.9
nonembedded	...	NP ^d	...
Ca ₁₀ (OH) ₂ (PO ₄) ₆ embedded	1.67	1.66 \pm 0.15	9.1
nonembedded	1.67	2.38 \pm 1.62	68.1

^aDetermined after linear regression analysis of each spectrum.

^bPrepared as a disk in epoxy resin (see Materials and Methods).

^cPure crystals on a carbon planchette (see Materials and Methods).

^dNot prepared according to Method 1 (see Materials and Methods).

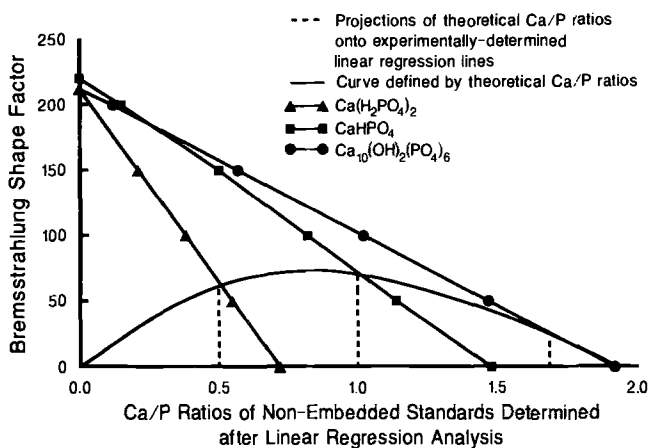


FIG. 2—Calibration curves of nonembedded Ca/P standards after linear regression analysis. Bremsstrahlung shape factors used to determine points for the y-axis included window thicknesses (KeveX setup menu parameter) of 70, 80, 90, and 99. (Low shape factor values model and remove more background counts from the lower end of the 10-keV energy scale, resulting in lower phosphorus counts. High shape factor values model and remove more background counts from the higher end of the 10-keV energy scale, resulting in lower calcium counts.) Note clear separation along the x-axis between 1.00:1.67 Ca/P standards. The nonlinearity of this set of standards is underscored by a curvature obtained after projecting theoretical Ca/P ratios of the standards onto experimentally determined linear regression lines, and by the necessity to expand the values of the y-axis beyond experimental limits to extend the linear regression line back to zero. A Ca/P ratio of unknown crystals would be determined by using the x-intercept of a linear regression line obtained after computer processing of actual spectral acquisitions.

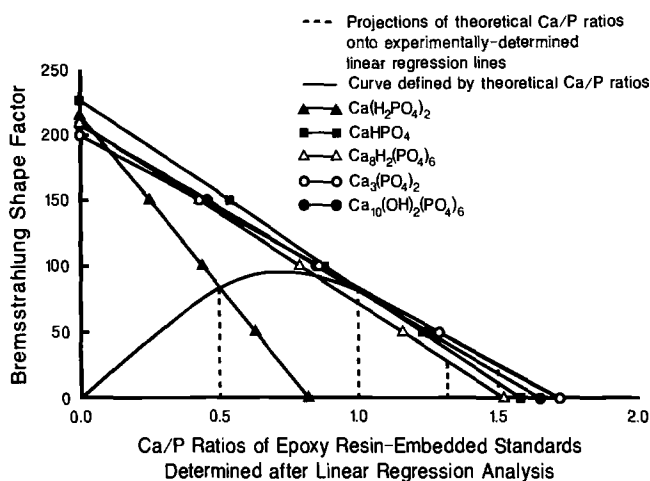


FIG. 3—Calibration curves of epoxy resin-embedded calcium phosphate standards after linear regression analysis. Bremsstrahlung shape factors used to determine points for the y-axis included window thicknesses (KeveX setup menu parameter) of 70, 80, 90, and 99. See Fig. 2 caption for explanation of the meaning of background shape factor values. Note "crowding" along the x-axis of observed Ca/P ratios derived from Ca/P standards having theoretical ratios of 1.00:1.67. The lack of a first-order relationship between known and observed Ca/P ratios over the entire range is also evident using embedded standards.

standards). If these standards were actual specimens of unknown Ca/P ratios to be analyzed, our results indicate that the same acquired spectra from one test sample would be consistent with *four* distinct theoretical ratios ranging from 1.00 to 1.67. There was, however, a statistical difference between the mean Ca/P ratio of the 0.50 and 1.00 standards ($p < 0.001$).

Discussion

Although quantitative methods for the X-ray microanalysis of thin specimens probed in the TEM or STEM mode have been well worked out [9,10,14,31–35], the reliability of quantitative bulk specimen analysis is not known [21,24]. It has become apparent from the present systematic study of a series of calcium phosphate standards using SEM and EDXA that serious flaws in the analysis of calcified tissue are possible. Although most authors use a standard to analyze along with their test specimens, they usually choose a specimen close to the presumed composition of their test specimen [3,5,11–13,16,17]. This is in keeping with the requirements of a good standard [19]. Unfortunately, we have experimentally determined that there was preferential loss of phosphorus X-rays when calcium phosphate standards were embedded in epoxy resin. Since many authors routinely embed their calcified tissues in epoxy resins [5,13,15,17], methacrylate [3], or lucite [12], and since the organic matrix of normal bone resembles embedding medium in having low-density properties [14], the observed Ca/P ratios acquired in the analysis of a bulk specimen cannot be very reliable. DiMaio et al. [18] used Ca/P ratios to help identify foreign material adherent to bullets as being "bone" in nature. It is obvious from the present study that the inaccuracies of Ca/P ratios obtained from the analysis of calcified bulk standards using SEM and EDXA makes such a conclusion untenable.

We have shown that an observed Ca/P ratio in the range of 1.52 to 1.73 would be consistent with *four* distinct embedded standards whose theoretical Ca/P ratios ranged from 1.0 to 1.67. Although matrix corrections were applied in these analyses, ZAF corrections were originally developed for metallurgical specimens [6], which are usually

devoid of organic material. The preferential loss of phosphorus X-rays relative to calcium in the present study is explained, in part, by the known absorption of low-energy X-rays (<3 KeV) in emerging from a target [36]. The physics of this situation would apply, however, to both types of bulk specimens analyzed in this study (pure crystals and embedded crystals). The presence of an element may be obscured if the matrix in which it occurs has a high mass absorption coefficient for the characteristic X-rays [2]. According to Roomans [20], phosphorus X-rays are absorbed six times more than calcium X-rays in a 5- μm -thick carbon film. This ratio is probably much higher in a bulk specimen, and from the present study, it is probable that epoxy resin has a higher mass absorption coefficient for phosphorus X-rays ($K_{\alpha} = 2.01$ KeV) compared with calcium X-rays ($K_{\alpha} = 3.69$ KeV). This would imply that, in the analysis of actual bone specimens using SEM and EDXA, the contribution of the organic matrix to the relative ratio of the emerging phosphorus and calcium X-rays from the target, could obscure real differences in Ca/P molar ratios present in different specimens.

Roomans [21] does not recommend crystal standards over gelatin- or resin-embedded standards because of the higher standard deviation of the probed crystals. We agree with this statement, but have discovered serious flaws inherent in both types of crystal preparation. The calibration curves of both the embedded and unembedded standards obtained in the present study illustrate an important point regarding semi-quantitative analysis of calcium phosphate-containing bulk specimens. The limits in theoretical Ca/P ratios that are actually calculatable is probably <2.0 (even using the more reliable unembedded standards). This raises serious doubts about the value of relative k -ratios, in general, using SEM and EDXA.

It is evident that quantitative techniques in the analysis of bulk specimens need to be investigated further. We hope that the results of the present systematic study, using a series of calcium phosphate standards prepared for bulk analysis using SEM and EDXA, will help other investigators avoid this pitfall. The possibility of a misdiagnosis of suspected bone fragments for forensic purposes with serious legal consequences has therefore been curtailed by the discovery of this caveat in semi-quantitative analysis.

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