

Errors in Quantitative Backscattered Electron Analysis of Bone Standardized by Energy-Dispersive X-Ray Spectrometry

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Summary: Backscattered electron (BSE) imaging has proven to be a useful method for analyzing the mineral distribution in microscopic regions of bone. However, an accepted method of standardization has not been developed, limiting the utility of BSE imaging for truly quantitative analysis. Previous work has suggested that BSE images can be standardized by energy-dispersive x-ray spectrometry (EDX). Unfortunately, EDX-standardized BSE images tend to underestimate the mineral content of bone when compared with traditional ash measurements. The goal of this study is to investigate the nature of the deficit between EDX-standardized BSE images and ash measurements. A series of analytical standards, ashed bone specimens, and unembedded bone specimens were investigated to determine the source of the deficit previously reported. The primary source of error was found to be inaccurate ZAF corrections to account for the organic phase of the bone matrix. Conductive coatings, methylmethacrylate embedding media, and minor elemental constituents in bone mineral introduced negligible errors. It is suggested that the errors would remain constant and an empirical correction could be used to account for the deficit. However, extensive preliminary testing of the analysis equipment is essential.

Key words: bone, backscattered electron imaging, standardization, energy-dispersive x-ray spectrometry, mineral content

Introduction

The scanning electron microscope (SEM) can be used to generate numerous signals that may be used to collect a wide variety of data from biological specimens. Of particular interest to bone biologists have been the elastically scattered, or backscattered electrons (BSE). Early studies

of BSE in the material sciences revealed that the backscatter signal is a function of the specimen's mean atomic number (Bishop 1966, Heinrich 1966). Consequently, grayscale BSE images could be obtained from the scanning electron microscope, where the pixel intensity (graylevel) was a function of atomic number. Because of this atomic number dependence, BSE imaging was applied to mineralized tissues (Boyde and Jones 1983, Reid and Boyde 1987). Experimental studies in bone have demonstrated that the mean graylevel of BSE images is linearly related to the bone's mineral content (Bloebaum *et al.* 1997; Roschger *et al.* 1995; Skedros *et al.* 1993a, b; Vajda *et al.* 1996). Therefore, BSE images obtained from bone are grayscale images where the relative graylevel can be interpreted as mineral content (weight mineral/weight dry bone), with bright regions signifying high mineral content bone and darker regions indicating low mineral content bone (Fig. 1). Microstructural features can also be clearly observed, allowing histomorphometry and mineralization to be analyzed on the same microscopic region. Both qualitative (Boyce and Bloebaum 1993; Boyde and Jones 1983, 1996; Boyde *et al.* 1986; Fratzl *et al.* 1996; Grynepas and Holmyard 1988) and quantitative (Bloebaum *et al.* 1997; Boyde *et al.* 1993, 1995b; Reid and Boyde 1987; Torontali *et al.* 1994) studies have shown BSE imaging to be a useful method for analyzing microscopic mineral variations in bone.

Backscattered electron (BSE) imaging in bone has been limited, however, by the difficulty obtaining quantitative measurements of mineral content. Experimental studies have demonstrated that the BSE signal can be calibrated and quantified using pure elemental standards, allowing comparison of images obtained within (Boyce *et al.* 1990) and between (Vajda *et al.* 1995) imaging sessions. This ensures that an individual instrument is properly calibrated and allows for a comparison of *relative*, but not *absolute* mineral content. Also, it makes comparisons between laboratories difficult because universally accepted calibration standards have not been developed. For many investigators, measurement of relative mineral differences may be sufficient, and quantitative BSE imaging can be applied to the study of microscopic bone mineralization with the technology and methods previously described (Boyce *et al.* 1990, Vajda *et al.* 1995). However, estimating the influence of bone mineralization with respect to mechanical

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properties (Currey 1988, 1990) would require *absolute* measurements of mineral content. Furthermore, obtaining *absolute* measurements of mineral content allows not only for instrument calibration, but also provides a method for standardization, making it possible to compare BSE images captured in different laboratories and to compare them with previous, accepted techniques for the measurement of mineral content.

Boyd *et al.* (1995a) developed halogenated dimethacrylate esters as reference standards, which would allow for the accurate standardization of BSE images. Micro-computed x-ray tomography has also been suggested as a method for standardizing BSE images (Boyd *et al.* 1993, Elliott *et al.* 1992, Mechanic *et al.* 1990). Both methods show promise, but at the present time quantitative data are lacking. It has not yet been demonstrated that dimethacrylate esters are suitable standards that can be consistently and uniformly manufactured and provide reliable results under electron bombardment. Resolution is an issue with x-ray microtomography, and relatively little published data address this aspect.

Roschger *et al.* (1995) proposed an alternative method to standardize the BSE signal using energy-dispersive x-ray spectrometry (EDX). They obtained BSE images and EDX spectra from the same microscopic region of bone and found a high positive correlation between weight percent calcium (wt.% Ca) and BSE weighted mean graylevel (WMGL). The authors suggested that BSE graylevels could be converted to wt.% Ca, allowing standardization between laboratories. Yet, Roschger *et al.* (1995) did not

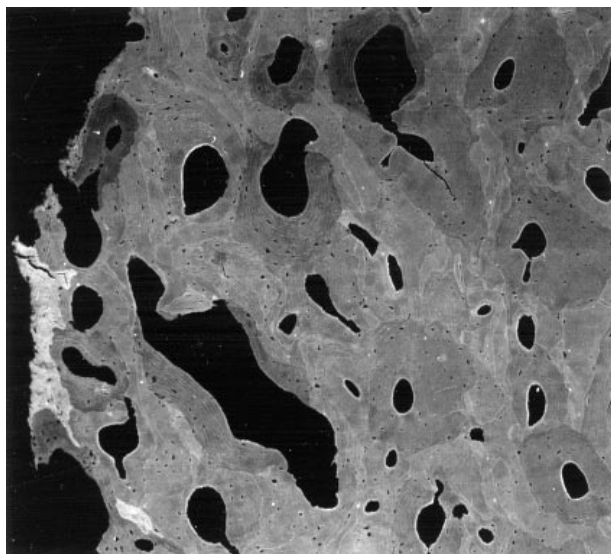


FIG. 1 Backscattered electron image from the posterior quadrant of a human femoral mid-diaphysis obtained from a 66-year-old woman. Dark regions signify low mineral content and bright regions indicate high mineral content. Edge effects (Reimer *et al.* 1986) and topography (Howell and Boyd 1994) may be partly responsible for bright margins seen near cracks and at the periphery of Haversian canals and lacunae. Field width = 1.6 mm.

compare their results with other accepted techniques for measuring mineral content. Without any a priori knowledge of the "true" mineral content of the bone tissue examined, they had no way of estimating the accuracy of their proposed technique.

We previously (Vajda *et al.* 1996) attempted to duplicate the findings published by Roschger *et al.* (1995) and tried to correlate their findings with traditional bulk measurements of ash content. Results confirmed the findings of Roschger *et al.* (1995) that wt.% Ca and WMGL are highly correlated. Furthermore, the method demonstrated good precision between multiple imaging sessions, but demonstrated poor accuracy and consistently underestimated the mineral content values obtained by traditional ash measurements by an average of 14.5 percentage points. Due to the consistency of the deficit, it was suggested that the EDX standardization technique might be used with an empirical correction for the accurate conversion of BSE graylevels to absolute mineral content measurements (Vajda *et al.* 1996).

The use of an empirical correction necessitates a clear understanding of the source of the deficit between EDX-standardized BSE images and ash measurements. It cannot be assumed that an empirical correction would be consistent between embedding techniques, BSE imaging equipment, or EDX x-ray analyzers. Although hypotheses were provided to explain the EDX standardization deficit (Vajda *et al.* 1996), experimental data were not available. The objective of this study was to investigate the reported deficit between EDX-standardized BSE images and traditional ash measurements. This study seeks to investigate the accuracy of the EDX analyzer in known standards, the influence of minor elemental constituents in bone mineral, and the effects of the organic component of bone. To accomplish these goals, several studies were performed: (1) Known reference standards were examined to determine the reliability of EDX measurements, (2) known reference standards were examined with a conductive coating and uncoated to determine the influence of conductive coatings on EDX measurements, (3) ashed-unembedded bone specimens were analyzed to determine the accuracy of EDX measurements in bone mineral, and (4) unembedded bone tissue was examined to determine the influence of the organic fraction of bone on the EDX signal.

Materials and Methods

Instrumental Error

To investigate the influence of errors in the EDX data collection process, pure fluorapatite (FA), hydroxyapatite (HA), and magnesium alloy reference standards were obtained for EDX analysis. The fluorapatite $[\text{Ca}_5(\text{PO}_4)_3\text{F}]$ standard was diamond milled by the manufacturer (Tousimis Research Inc., Rockville, Md.) and prepared for EDX analysis. Hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ powder

was compressed into pellet form at high pressure (Norian Corp., Cupertino, Calif.). Magnesium alloy (Johnson/Matthey Inc., Seabrook, N.H.) was ground and polished to an optically smooth finish. No conductive coating was applied to the standards.

Five EDX spectra were captured from each sample using a lithium-doped silicon detector equipped with a beryllium window (Link Pentafet system, Oxford Instruments, Cambridge, U.K.) and interfaced with a JEOL 6100 SEM (JEOL, USA, Inc., Peabody, Mass.). Working distance was set at 15 mm, accelerating voltage at 20 kV, and probe current at 2.50 nA with a live capture time of 130 s. The take-off angle of the EDX detector was set at 35°. Commercially available software (Link ZAF-4, Oxford Instruments) was used to calculate atomic number (Z), absorbance (A), and fluorescence (F) correction factors. Pure CaCO₃ and InP (Tousimis Research Inc.) were used as reference standards for the major constituents of bone—Ca and P. In addition, Al, Au, NaF, KCl, and MgO (Tousimis Research) were used as reference standards for the metals, conductive coatings, and other minor elemental constituents present in bone (LeGeros 1981). The software for ZAF corrections used in this investigation allows for one element to be unanalyzed, and the weight fraction of the unanalyzed element is calculated by a difference method. ZAF corrections were performed with oxygen as the unanalyzed element for the apatites. The use of an unanalyzed element for the Mg alloy was not necessary because all three elements of the alloy (Mg, Al, Zn) can be readily detected by EDX analyzers. A pure Cu reference standard (Tousimis Research) was used for gain calibration of the EDX spectra.

Error Due to Conductive Coatings

To investigate the potential influence of a gold conductive coating, an additional sample of hydroxyapatite and Mg alloy were obtained and prepared as described above. After polishing, the specimens were sputtercoated with a thin layer of gold for 75 s at 70 μm Hg and 10 mA, yielding a gold coating approximately 250 Å thick (Hummer Model VI-A Sputtering System, Anatech, Ltd., Alexandria, Va.). Five EDX spectra were obtained from each sample as described above. Wt.% Ca and wt.% Mg were determined for each sample, respectively, after eliminating the fractional weight contributed by the gold coating. For example, a sample with EDX data indicating 47.5% Al, 47.5% Mg, and 5% Au would be adjusted to 50% Mg and 50% Al after correcting for the gold present.

Errors in Bone Mineral Measurements

To investigate the potential influence of minor elemental inclusions in bone (LeGeros 1981, Lowenstam and Weiner 1989), bone mineral obtained by ashing was examined. Four bone specimens were obtained from three different species (Table I). Specimens were stored in 70% ethyl alcohol for a minimum of 2 weeks. Each bone segment was manually cleaned of soft tissue and periosteum, where applicable, and defatted in a large volume of reagent-grade chloroform (Omnisolv, EM Industries, Inc., Gibbstown, N.J.) under vacuum for 20 days. Bone segments were placed in an oven at 80° C for 4 days to remove residual chloroform. Bone segments were subsequently placed in a furnace at 550°C for 24 h (Gong *et al.* 1964). Ashing at higher temperatures would substantially remove inorganic carbonates (Gong *et al.* 1964), which are present and detectable in the BSE mode. This method of ashing has previously demonstrated a strong linear correlation with BSE graylevels (Bloebaum *et al.* 1997).

The ashed bone segments were not embedded in methylmethacrylate as is common in SEM imaging. The ashed bone specimens were glued into drill holes in a Plexiglas® block with a quick-drying epoxy resin. The specimen block was milled, ground, and polished to an optically smooth finish. The polished surface was lightly sputtercoated with a thin layer of gold. The specimen was placed in the SEM, and EDX standardization of BSE graylevels was performed as has been previously described in detail (Roschger *et al.* 1995, Vajda *et al.* 1996). Briefly, the procedure involved capturing BSE images at high magnification in a region that appeared relatively homogeneous. Immediately after BSE image capture, an EDX spectrum was captured from the same region (Fig. 2). The BSE and EDX capture was repeated nine times, yielding a total of ten BSE/EDX data pairs. All BSE images were captured with a solid state annular backscatter detector (Tetra, Oxford Instruments) configured around the electron beam. Digital BSE images with a resolution of 512 × 512 pixels and 8 bits/pixel (256 distinct graylevels) were stored on magnetic media using a computer-controlled image capture and retrieval system (eXL, Oxford Instruments). All BSE images were captured using nine scans and a Kalman frame averaging technique (Oxford Instruments). Instrument calibration was performed at 20 min intervals by imaging a magnesium alloy (99.8% pure; 93% Mg, 6% Al, 1% Zn; Johnson/Matthey, Inc.) and aluminum (99.9999% pure, Johnson/Matthey, Inc.) refer-

TABLE I Bone specimens used and their anatomical provenance

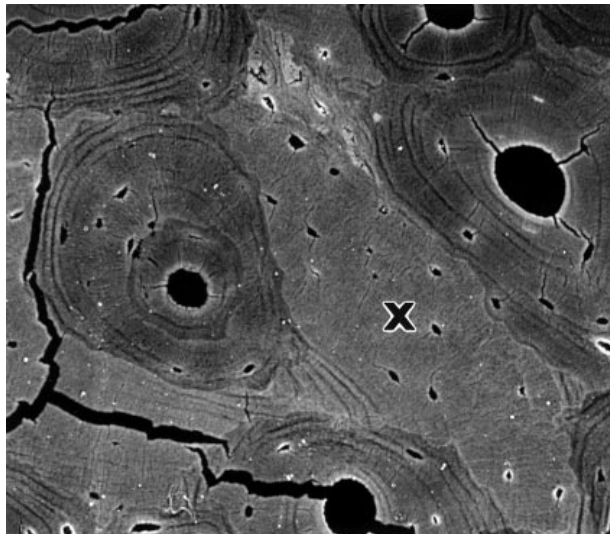
Species		Anatomical location
Human	<i>Homo sapiens</i>	Midshaft, anterior cortex, femur
Dog	<i>Canis familiaris</i>	Midshaft, anterior cortex, femur
Rocky Mountain mule deer	<i>Odocoileus hemionus hemionus</i>	Midshaft, cranial cortex, calcaneus
Rocky Mountain mule deer	<i>Odocoileus hemionus hemionus</i>	Antler

ence standards following published protocols (Vajda *et al.* 1995). Prior to every image capture, beam current was monitored with an SM-16100 probe current detector (Oxford Instruments) connected to an external picoammeter (Keithley Instruments, Cleveland, Ohio). Fine adjustments of the condenser lens strength were made as necessary to insure a stable beam.

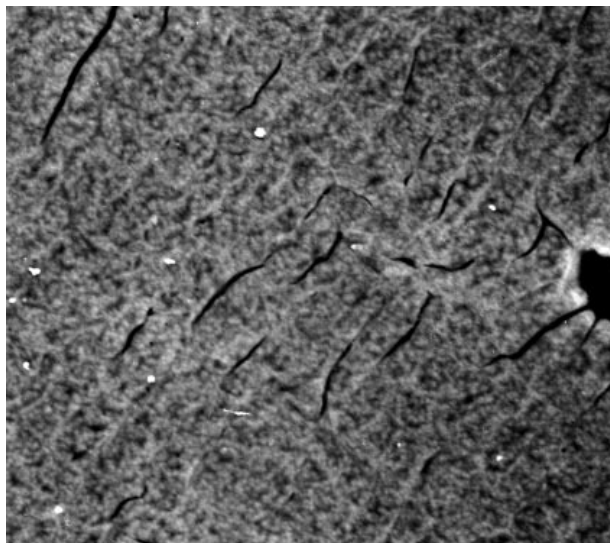
The weighted mean graylevel was calculated for each BSE image following previously published protocols (Bloebaum *et al.* 1997, Boyce *et al.* 1990, Vajda *et al.* 1996). Wt.% Ca was calculated for each EDX spectrum using ZAF corrections. Because all organic components

had been removed during the defatting and ashing protocol, oxygen was treated as the dominant unanalyzed element for ZAF corrections. Linear regression analysis was used to establish a standardization equation relating BSE WMGL and wt.% Ca. Using the standardization equations, all subsequent BSE images captured under the same operating conditions can be converted to wt.% Ca measurements.

Subsequently, five 200X BSE images were captured from each of the bone segments. A WMGL was calculated for each image. Using the EDX standardization equations, BSE WMGLs were converted to wt.% Ca. Analysis regions were selected to avoid the periphery of the bone in order to reduce the influence of any epoxy resin that may have infiltrated bone tissue before drying.



(a)



(b)

FIG. 2 (a) Low magnification backscattered electron (BSE) image. A homogeneous area (X) is identified and a high magnification image is captured. Field width = 400 μm . (b) High magnification BSE image from region in (a). The weighted mean graylevel of the entire image is calculated and an EDX spectrum is collected, allowing direct correlation of BSE graylevels with wt.% Ca. Field width = 55 μm .

Errors Due to the Organic Phase of Bone

To investigate the effects of bone proteins on EDX measurements, four bone specimens were obtained from three different species (Table I). Each bone was then sectioned into two adjacent segments. One bone specimen was too small to be sectioned and was used only for the EDX standardization procedure (human). One segment from each of the other three bones was used for BSE analysis and the other segment from each bone was used for ash percent measurements. Ash percent was calculated as: $100 \times (\text{weight of ashed bone} / \text{weight of dry bone})$.

Bone segments used for BSE analysis were ground, polished, and goldcoated as described above. The specimen block was placed in the SEM and EDX standardization of BSE graylevels was performed. Due to the inclusion of bone proteins, carbon was treated as the dominant matrix element and was unanalyzed in ZAF corrections.

To facilitate comparison with the other portions of this study, ash values have been converted to wt.% Ca values based upon stoichiometry. Neglecting minor constituents and phase differences, bone mineral is typically described as a form of apatite, specifically HA $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$. Calcium occupies 39.9% of stoichiometric HA by weight. Ash fraction can therefore be converted to wt.% Ca by the multiplicative factor 0.399.

Results

Instrumental Error

All five measurements of FA showed very consistent and accurate results (Table II). Wt.% Ca was determined to be $40.11 \pm 0.14\%$, which closely matches the expected value of 39.7% Ca by weight. Similarly, all five measurements of Mg alloy showed results consistent with the expected value (93% Mg); measured wt.% Mg was $93.46 \pm 0.06\%$. The small standard deviations suggest repeatability of measurements with minimal error. Wt.% Ca measurements in the HA standard, however, were $37.33 \pm$

1.24%, which consistently underestimated the expected value of 39.9%.

Error Due to Conductive Coatings

Wt.% Mg was determined to be $93.56 \pm 0.28\%$ in the Mg alloy. Wt.% Ca in the HA standard was $37.06 \pm 0.73\%$. These values matched those determined above for the uncoated samples (Table II). Minor variations between coated and uncoated specimens were observed, but they were not statistically significant ($p = 0.79$) using an analysis of variance (ANOVA). However, the statistical power was low because of the small sample size, and it cannot be conclusively stated that the conductive coating had no influence on EDX measurements.

Errors in Bone Mineral Measurements

The mean wt.% Ca of the EDX-standardized BSE images of ashed bones was $36.66 \pm 0.38\%$. The values were significantly lower ($p < 0.0001$) than the wt.% Ca of stoichiometric HA (39.9% Ca) using a one-sample Student's *t*-test (Table III). Comparisons of wt.% Ca among the four ashed bone specimens revealed small but statistically significant ($p < 0.01$) variations using a one-way ANOVA (Table III).

Errors Due to the Organic Phase of Bone

In unembedded bone, the wt.% Ca values determined by EDX-standardized BSE imaging were significantly lower ($p < 0.05$) than the wt.% Ca values determined by bulk ashing using a paired *t*-test (Table IV). The mean wt.% Ca (ashing) was $26.99 \pm 2.23\%$ and the mean wt.% Ca (BSE/EDX) was $21.74 \pm 2.54\%$, representing an absolute difference of 5.25 percentage points.

Discussion

Roschger *et al.* (1995) originally proposed standardizing the BSE signal in bone with calcium $K\alpha$ -line x-rays, but the technique was criticized for several reasons. It was argued that x-rays and BSEs have different emission volumes and a direct correlation would not be expected. Furthermore, EDX data are inherently noisy, whereas BSE data are much more stable. In spite of these limitations, Roschger *et al.* (1995) demonstrated a highly linear correlation between wt.% Ca and BSE WMGL in human bone. In an independent investigation, we (Vajda *et al.* 1996) achieved similar results in a series of bones from varied vertebrate species. Thus, as Roschger *et al.* (1995) argued, the errors inherent in EDX data collection are small enough that it is adequate to standardize the BSE signal.

Although the results of Roschger *et al.* (1995) were promising, they never corroborated their findings with other methods of measuring mineralization. We previously com-

pared this EDX standardization method with traditional ash measurements and found that it underestimated ash fraction by an average of 14.5 percentage points (Vajda *et al.* 1996). This value was based on a comparison of ash fraction data with wt.% HA data from EDX-standardized BSE images. To facilitate comparison with the current study, the wt.% HA values previously reported can be converted to wt.% Ca measurements as described above, resulting in a deficit of 5.79 percentage points by weight. The current study has helped to determine the source of these errors.

TABLE II Energy-dispersive x-ray data for gold coated and uncoated specimens (mean \pm SD)

	Uncoated	Conductive coating	Expected value
Fluorapatite ^a	40.11 ± 0.14	NA	39.74
HA ^a	37.33 ± 1.24	37.06 ± 0.73	39.89
Mg Alloy ^b	93.46 ± 0.06	93.56 ± 0.28	93.00

^aWt.% Ca measurement.

^bWt.% Mg measurement.

Abbreviations: NA = not available, SD = standard deviation, wt.% Ca = weight percent calcium.

TABLE III Wt.% Ca measurements in ashed bone specimens (mean \pm SD)

Specimen	Wt.% Ca
Stoichiometric HA ^a	39.89
Deer antler	36.77 ± 0.08
Deer calcaneus	36.34 ± 0.09
Canine femur	36.32 ± 0.06
Human femur	37.21 ± 0.12
Mean of ashed bones	36.66 ± 0.38
Bovine femur ^b	36.60

^aNot a measured value, calculated based on stoichiometry.

^bData from Elliot (1994).

Abbreviations: wt.% Ca = weight percent calcium, SD = standard deviation, HA = hydroxyapatite.

TABLE IV Wt.% Ca measurements determined by energy-dispersive x-ray/backscattered electron compared with wt.% Ca measurements determined by ashing in unembedded bone tissue (mean \pm SD)

Specimen	Ash ^a	EDX	Absolute difference
Deer antler	24.44	18.41 ± 0.25	6.04
Deer calcaneus	28.64	24.11 ± 0.55	4.53
Canine femur	27.87	22.69 ± 0.29	5.18
Mean	26.99	21.74	5.25

^aAsh % is converted to wt.% Ca based upon stoichiometry (see text).

Abbreviations: EDX = energy-dispersive x-ray, BSE = backscattered electron. Other abbreviations as in Table III.

Instrumental Error

The EDX data obtained from the FA standard and Mg alloy indicate that the EDX measurement methods employed in this study are accurate in inorganic compounds. This is anticipated, as EDX technology in the material sciences has been well established (Heinrich 1995). The results of the EDX measurements in pure HA were not as reliable. Because the results were within acceptable error in the FA and Mg alloy standards, the poor results in pure HA cannot be attributed to errors in the x-ray collection process. The errors in HA may be the result of inaccuracies in the application of ZAF corrections. The HA standard used in this study may not fulfill the requirements for the ZAF program used. This explanation is questionable, however, because it would be anticipated that ZAF errors would have led to similarly poor results with the FA standard. Variations in surface structure as a result of different specimen preparations may also explain some of the error in HA. No visible differences in surface roughness could be observed using secondary electron imaging, but smaller more subtle variations may explain some of the error. The most likely source of error was the presence of contaminants or inhomogeneities in the HA that may have led to deviations from the ideal stoichiometry. Such inhomogeneities are known to occur in industrial HA preparations like the one used in the present study. We were unable to determine the composition of the HA standard using alternate analysis methods to confirm this hypothesis, but this explanation is supported by the relatively larger standard deviation in HA in comparison to the Mg Alloy and FA.

Error Due to Conductive Coatings

Previous reviews (Vajda *et al.* 1996) had suggested that the gold coating may be a source of error and carbon coating would be preferable. Gold, as a result of its high atomic number, would absorb incident electrons and could account for the deficit previously observed (Vajda *et al.* 1996). An effort to account for the gold coating was attempted in this and the previous study (Vajda *et al.* 1996) by measuring the wt.% Au and eliminating it from the wt.% Ca and wt.% Mg values. This simple adjustment is a crude attempt to account for the gold coating and undoubtedly suffers from errors, because the method of ZAF corrections assumes that the gold is evenly distributed within the sampled region, which is untrue in the current investigation. Nonetheless, it appears to be sufficient because a comparison of gold-coated and uncoated specimens in the present study revealed no statistically significant differences between measured EDX values. Conductive coatings may play a role in the accuracy of the EDX technique, but they do not explain the large errors previously reported. This is true even with a gold coating, which some investigators would argue is a worst-case situation, as a result of the high atomic number of gold which could reduce Z-contrast.

The relative lack of coating influence is most likely because the conductive coating layers are extremely thin and do not substantially hinder high-energy incident electrons. Gold coatings might be more of an issue in low-voltage applications, where the relative influence of conductive coatings may increase due to a reduced electron emission volume (Kanaya and Okayama 1972). Also, different coating techniques (e.g., evaporative plasma sputtering) may produce more or less uniform coatings and hence influence specimen topography. For these reasons, carbon may be the preferred coating material. Carbon does have the disadvantage, however, of producing the same x-ray spectral peak as the material being investigated, making it impossible to eliminate the contribution of the carbon coating to the EDX signal. Quantitative data addressing the effects of various coating materials and methods on bone mineral analysis are lacking in the literature and are under investigation in our laboratory.

Errors in Bone Mineral Measurements

Results for the ashed bone specimens revealed wt.% Ca to be significantly lower than the wt.% Ca in stoichiometric HA. The slightly lower wt.% Ca values in ashed bones is consistent with the literature regarding bone mineral. Bone mineral is a calcium-deficient form of HA, with the inclusion of 4–6% CaCO₃ by weight as well other minor elemental constituents (LeGeros 1981, Legros *et al.* 1987). Elliott (1994) reported the wt.% Ca as a fraction of bone mineral in bovine bone to be 36.6%, matching the measurements in the present study (Table III). Furthermore, the small variation among the species is anticipated based on previous data demonstrating species-specific differences in a series of 16 vertebrate species (Biltz and Pellegrino 1969). Results of the present study suggest that minor elemental constituents and phase differences in bone are accurately detected by EDX and cannot explain the large errors previously described (Vajda *et al.* 1996).

Errors Due to the Organic Phase of Bone

In contrast to the other specimens examined, the wt.% Ca data for the unembedded bone tissue in this study underestimated ash fraction by an average of 5.25 percentage points (a 19.5% difference). This suggests that EDX measurements become inaccurate when the organic fraction of bone is present. Alternatively, it could be argued that the errors are the result of comparing a bulk measurement (ashing) to an average value obtained from several microscopic measurements (BSE). This explanation is, however, unlikely as we have previously demonstrated a high linear correlation ($r^2 > 0.97$) between bulk ash content and average BSE data collected from five microscopic regions of bone (Bloebaum *et al.* 1997).

It is interesting that the 5.25 percentage point deficit closely matches the 5.79 percentage point deficit previously reported (Vajda *et al.* 1996). The tissues used in the

present study, however, were not embedded in polymethylmethacrylate (PMMA), while those used by Vajda *et al.* (1996) were embedded in PMMA. This implies that the embedding medium is not the source of the observed deficit. This is encouraging, because a large error in the standardization technique as a result of specimen preparation would hamper the universal applicability of the BSE technology and graylevel-based analysis.

The results of the present study indicate that the light elements (primarily C, N, O), which compose the organic fraction of bone and a portion of the mineral phase, are the major source of the inaccuracies in the EDX measurements made by Vajda *et al.* (1996) and Roschger *et al.* (1995). The x-ray detector employed in the present study was equipped with a Beryllium window, which blocks low-energy x-rays. In this system, proper accounting for light elements is performed entirely by ZAF corrections. The available ZAF-4 software only allows for one element to be selected as the dominant unanalyzed matrix element. It has been reported that selecting one element as the matrix element for ZAF corrections in biological analysis can lead to errors as large as 15% (Boekestein *et al.* 1980). To illustrate this point, the ZAF corrections for the data from one of the three trials in Vajda *et al.* (1996) were repeated with oxygen as the unanalyzed element. The choice of oxygen is not arbitrary. Oxygen comprises a large portion of the mineral fraction of bone and could arguably be the dominant matrix element, rather than carbon, especially for highly mineralized bone tissue. The results of these calculations are displayed in Figure 3. A highly linear correlation between standardized BSE image data and ash data was observed with a slope approximately equal to one. This is consistent with results when carbon was the unanalyzed element. There is no statistically significant difference ($p > 0.75$) between the slopes of the two trials using a test for equality of slope (Sokal and Rohlf 1995). However, the y-intercepts are significantly different when compared with an analysis of covariance ($p < 0.05$). When carbon is used for ZAF corrections, the method underestimates ash data by an average of 5.68 percentage points. Using the same EDX spectra, but oxygen as the unanalyzed element, ash data are underestimated by an average of 4.34 percentage points. The selection of the unanalyzed element significantly alters the data. Improvements in software to account for the correct ratios of the light elements may reduce the errors observed. Until recently, however, the absorption coefficients for light elements were not well defined, making this a difficult task (Bastin and Heijligers 1990, 1991; Goldstein *et al.* 1992).

Energy-dispersive x-ray technology has been previously used with good results in mineralized biological specimens. Typically, these studies have used thin specimens (Landis 1979, Nicholson and Dempster 1980). Other investigators have used the EDX technique in bulk specimens of mineralized tissue with varying results (Åkesson *et al.* 1994, Boekestein *et al.* 1980, Payne and Cromey 1990, Roomans 1981). Payne and Cromey (1990) report-

ed inaccurate Ca/P molar ratios in a series of analytical standards. Unlike the present study, Åkesson *et al.* (1994) reported EDX results that slightly overestimated wt.% Ca measurements performed by neutron activation analysis and chemical analysis. Payne and Cromey (1990) and Åkesson *et al.* (1994) used relative k-ratios and ZAF peak-to-background methods, respectively. To our knowledge, a detailed comparison of the various correction methods in mineralized tissue has not yet been performed. The limitations of ZAF corrections could be reduced with thin or windowless technology, allowing direct measurement of the light elements. Windowless technology has undergone great progress in the past decade (Goldstein *et al.* 1992, Lund 1995), but detailed studies of its accuracy in mineralized tissue have not been performed. As an alternative, Phi-Rho-Z correction techniques (Goldstein *et al.* 1992) may yield results superior to ZAF correction techniques in bone. Further research into this area is warranted.

The results presented here as well as in previously published studies (Roschger *et al.* 1995, Vajda *et al.* 1996) indicate that BSE standardization with EDX can be performed with good precision and, with the use of an empirical correction, acceptable accuracy. The major source of error appears to be in the application of ZAF corrections to account for the light elements. This error is, therefore, a problem inherent in the bone tissue and cannot be eliminated without improvements in ZAF corrections. However, an empirical correction to account for errors in ZAF corrections should be consistent, provided the ZAF correction methods and data acquisition protocols are not altered as has been previously demonstrated (Vajda *et al.* 1996). Unfortunately, the use of different x-ray analyzers or ZAF

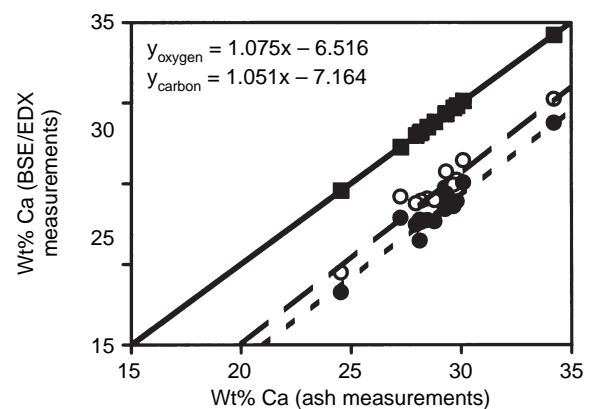


FIG. 3 Scatterplot between weight percent calcium (wt.% Ca) measurements using energy-dispersive x-ray (EDX)-standardized backscattered electron (BSE) images and wt.% Ca measurements from ash fraction data. Solid circles are for EDX measurements with carbon as the unanalyzed matrix element. Open circles are for EDX measurements with oxygen as the unanalyzed matrix element. Squares represent a theoretically perfect linear correlation. ○ = oxygen, ● = carbon, ■ = theoretical.

software would require performing a study similar to that of Vajda *et al.* (1996) to obtain the necessary empirical correction between various laboratories. Similarly, sample preparation should be standardized to eliminate any possible contribution to the EDX analysis. Therefore, every laboratory may initially need extensive testing of their equipment before the method can be utilized. Corroboration with techniques such as ashing, which is relatively simple and has a long history in the literature, is essential to obtain reliable results. Furthermore, once a correction factor has been established, no adjustments in equipment or analysis can be made without reidentifying the empirical correction. Given these limitations, the method does allow for the standardization of the backscatter signal and quantitative measurement of microscopic variations in mineral content. Large-scale studies, imaging numerous samples and requiring multiple days can be performed (Vajda *et al.* 1995). It is an advance in the application of BSE imaging to bone, but investigators must be aware of the calibration and standardization required. Alternative techniques which do not suffer from these limitations, such as x-ray microtomography or composite reference standards, may need to be investigated.

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