Accurate determination of the Ca:P ratio in rough hydroxyapatite samples by SEM-EDS, PIXE and RBS – a comparative study

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The Ca:P ratio in a certified standard of hydroxyapatite was determined by X-ray spectrometry (XRS), with the X-rays excited by both electrons and ions using energy dispersive spectroscopy on the scanning electron microscope (SEM-EDS) and particle induced X-ray emission (PIXE). The certified value of Ca:P was accurately verified by 3 MeV ⁴He⁺ Rutherford Backscattering Spectrometry (RBS). We show that the demonstrably rough surface of this sample does not cause perturbation of the Ca:P ratio within the uncertainties of each of the XRS measurements.

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68.35.Ct Interface structure and roughness

68.55.Ln Defects and impurities: doping, distribution, concentration, etc

81.70.Jb Chemical composition analysis, chemical depth and dopant profiling

82.80.Ej X-ray, Mössbauer, and other γ -ray spectroscopic analysis methods

82.80.Yc Rutherford backscattering (RBS), and other methods of chemical analysis

83.80.Fg Granular solids

83.80.Lz Physiological materials (e.g. blood, collagen, etc.)

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Introduction

Determination of the Ca:P ratio in samples such as bone and teeth^{1,2,3} is of interest in biomedicine, and small variations in the Ca:P ratio are frequently studied in order to understand conditions such as osteoporosis^{4,5}, dental caries^{6,7,8} and the ontogenesis of calcified tissue^{9,10}. For this reason, hydroxyapatite (the most stable calcium phosphate found in the body) is one of the most researched biomaterials to date. For bulk analysis of such tissues, *in-vivo* methods involving absorptiometry of electromagnetic radiation have recently been developed^{11,12}, but these methods provide limited spatial information. Methods based on X-ray spectrometry (XRS) are particularly suitable for such studies because their mapping capabilities permit the rapid location of spatial variations in the Ca:P ratio.

In this paper, we investigate the accuracy of two XRS techniques which are frequently used to study calcified tissue; particle induced X-ray emission (PIXE) and energy dispersive X-ray analysis on the scanning electron microscope (SEM-EDS) for the determination of the Ca:P ratio in a certified standard of hydroxyapatite (HA). The certified value was verified by a traceable Rutherford Backscattering Spectrometry (RBS) analysis.

SEM-EDS is the technique of choice for many labs because it is widely available, relatively inexpensive, and because the sample can be imaged whilst simultaneously extracting compositional information. PIXE, despite the higher set-up cost compared with SEM-EDS, has distinct complementary value since both the cross-sections for X-ray production and also the information depth are much higher than for electrons. The latter is important to adequately average sufficiently large volumes of material in these samples whose structure may be very inhomogeneous. A further advantage of the PIXE technique is that the higher sensitivity (due to the almost complete absence of primary bremsstrahlung: $\mu\text{g/g}$ for PIXE compared with mg/g for SEM-EDS) allows trace elements to be studied.

Calcium phosphates present particular challenges for X-ray analysis due to the high absorption of these low energy X-rays in the HA matrix. This emphasises the importance of an accurate absorption correction, and, equivalently, the potential sensitivity of the spectra to roughness effects. Therefore a pitfall in determining the Ca:P ratio in the calcium phosphates may be the fact that these samples usually

exhibit surface roughness. In many cases, samples are embedded in resin and polished so as to present a flat surface to the beam, but of course the sample itself is still rough at the interface with the resin. Surface or interface roughness can seriously distort the interpretation of the X-ray data, and it is this that is considered in this paper.

Roughness effects are well documented to disturb SEM-EDS results (see for example Goldstein *et al*¹³) and can also be pronounced in RBS and PIXE data. At present, this data cannot be effectively corrected for roughness, although this problem was approached in the early days of PIXE¹⁴. The RBS data can be artificially fitted, as in a recent example¹⁵: and in this particular case the (uncorrected) PIXE results may be distorted by up to a factor of two. A new general algorithm for treating severe roughness effects correctly has recently been proposed¹⁶; this is applicable to RBS, PIXE (and also, in principle, SEM-EDS) data. The peak-to-background method used for quantifying data from rough or irregular samples in SEM-EDS¹⁷ cannot easily be used for PIXE data which has almost no primary bremsstrahlung background.

Rutherford Backscattering Spectrometry

Ion beam analysis (IBA), which is a suite of techniques including PIXE and RBS, is complementary to microanalysis on the scanning electron microscope (SEM). The same energy dispersive spectrometry (EDS) detectors are used to detect the particle induced X-rays, whether the particles are electrons (SEM-EDS) or ions (PIXE).

In both techniques the backscattered particles are also useful, whether they are the backscattered electrons (BE) which give Z contrast on the SEM, or the backscattered ions which were first recognised by Lord Rutherford in 1911. Rutherford Backscattering Spectrometry (RBS) involves detection of the energy of particles that are backscattered from the sample, which depends both on the mass of the target nucleus and the depth at which the collision took place. The depth depends on the energy loss of energetic ions in material, which is now well known¹⁸. Therefore, RBS spectra can easily be quantified to determine the concentration and depth profiles of major elements in a sample, since the scattering cross-sections are determined analytically from the Coulomb potential¹⁹, and since almost all the detected particles have suffered only one scattering event. For faster moving incident particles, the Coulomb barrier is exceeded and the elastic scattering cross-sections become non-

Rutherford²⁰, due to the presence of short-range nuclear forces. This is called elastic backscattering (EBS). Great progress has recently been made to accurately determine the EBS scattering cross-sections, using nuclear data together with measured cross-section data to determine the parameters in Schrödinger's equation²¹. In contrast, although the BE signal on the SEM gives very useful qualitative information, it is almost impossible to quantify.

The great value of RBS in this context is that it provides direct information on the compositional depth profile of the sample (including the roughness). In this work, both RBS and EBS were used to verify the certified Ca:P ratio.

Experimental

The sample was a green (that is, not sintered) pressed pellet of hydroxyapatite powder HA P120 (from Plasma Biotol, UK), with a analysis (molar parts per million) of:

O:Ca:P = 608527:243661:143618.

The remaining 0.4 mol% had a composition mostly:

Mg:Si:Al:Na:Fe:Cu:Zn:Sr:Zr = 1661:1403:438:240:155:16:15:96:20.

(and there was also K:Ti:Mn:Sn:Ba:Hf:Pb = 53:31:48:8:8:6:6). The analysis was certified by CERAM Research in 1992 according to ISO12677 with an estimated expanded uncertainty in the Ca:P ratio of 0.5% (2k coverage). The pellet was pressed using a uniaxial steel die of 22 mm diameter and pressed at 250 MPa with no lubricant, and was carbon coated to a thickness of 17 $\mu\text{g}/\text{cm}^2$ (~75 nm) to enable SEM analysis and to reduce the secondary bremsstrahlung background in PIXE. An SEM image of the pellet surface (Figure 1) shows that the surface roughness has a structure with a scale of microns.

Stylus profilometry was not suitable for this sample due to the very large aspect ratio of the pits in the green pressed pellet. A Mitutoyo SurfTest SV-600 profilometer equipped with Surfpak SZ v1.002 software was used to measure surface roughness of samples. Calibration was carried out prior to every session using a Mitutoyo Precision reference specimen with an R_a value of 2.95 μm . The stylus had a 5 μm radius tip. A scan length of 15 mm with a scan speed of 0.2 mm s^{-1} and a range of 600 μm was

used giving an Ra of. 0.2 μm , which is much smaller than is credible on this sample, revealing only the relative flatness of the surface, from grain to grain.

The IBA was carried out on a 2 MV Tandetron²². An RBS/EBS spectrum with a total collected charge of 336 μC (collected by a charge integrator on the back of the suppressed sample plate) is shown in Figure 2. The collection area was over about 7 mm^2 with a beam flux of about 11 nA/mm^2) using $3060 \pm 3 \text{ keV } ^4\text{He}^+$ at normal incidence and a surface barrier detector of 1.22 msr solid angle at a scattering angle of 172° . The Tandetron generating voltmeter was calibrated against the 992 keV $^{27}\text{Al}(\text{p},\gamma)^{28}\text{Si}$ reaction. The C coating is clearly seen and easily quantified.

The He^+ beam energy was chosen to get extra sensitivity to the oxygen content. For O with a ^4He beam energy near the 3044 keV resonance, the $^{16}\text{O}(\alpha,\alpha)^{16}\text{O}$ cross-section reaches 26 times Rutherford at this scattering angle. The IBANDL website²³ shows that $\text{Ca}(\alpha,\alpha)\text{Ca}$ elastic scattering cross-sections are Rutherford to approximately 4.6 MeV. No $\text{P}(\alpha,\alpha)\text{P}$ cross-sections are available, but we assume that they are Rutherford at this energy since $\text{Si}(\alpha,\alpha)\text{Si}$ is Rutherford to 3.7 MeV and $\text{Al}(\alpha,\alpha)\text{Al}$ is Rutherford to 4.2 MeV.

The SEM-EDS was carried out using an Oxford Instruments EDS system with a 60 eV Si(Li) spectrometer crystal and an ultra-thin entrance window. The take-off angle of the detector was 35° to the sample. Analysis was performed using ZAF (atomic number, X-ray absorption and X-ray fluorescence) corrections to the order of two iterations on the Oxford Instruments Link ISIS v3.35 program. The electron beam (LaB₆ filament) voltage was run at 20 keV with a working distance of 10 mm and a collecting time of 50 seconds. Ten randomly selected spots were analysed. Approximately 90,000 total spectrum counts were taken per sample area, of which approximately 10,000 were characteristic of Ca or P. Instrumental sensitivity factors (ISF) were derived from standards of GaP and a Wollastonite (CaSiO_3) prism. The relative uncertainty of a Ca/P ratio measurement depends on the uncertainty of transferring ISFs between two separate measurements with no common element, which comes down to the electron fluence uncertainty which we estimate at 5%.

PIXE spectra were collected simultaneously with RBS/EBS spectra using both He and H beams at various energies using 148 eV Si(Li) detectors 3 mm thick with 21 μm Kapton and 122 μm Be filters to exclude the backscattered particles. The filter

thicknesses were determined (see later) using the lead glass certified reference material BCR-126A (from the Institute of Reference Materials and Measurement, Geel, Belgium)²⁴.

Data Analysis

The fitted RBS spectrum is shown in Figure 2. The spectra were fitted using the method of Jeynes *et al*²⁵ and using DataFurnace²⁶ (NDF, a validated code²⁷). The fit of the backscattering data (with NDFv9.2d²⁸) uses SRIM2003 stopping powers^{29,30,18} and evaluated EBS cross-sections from SigmaCalc^{31,32,33} for $^{16}\text{O}(\alpha,\alpha)^{16}\text{O}$ and $^{12}\text{C}(\alpha,\alpha)^{12}\text{C}$. The EBS was calculated with the best available convolutions in depth³⁴ to reproduce the spectrum accurately in the vicinity of the 3044 keV $^{16}\text{O}(\alpha,\alpha)^{16}\text{O}$ resonance. Chu's correction to Bohr's (1915) theory of energy straggling^{35,36} was used, and no correction was made for double scattering.

The lower yield at the high energy edges in the RBS spectrum is evidence of sample roughness due to the beam re-entering the sample through the asperities causing extra energy loss. To simulate this, the extra energy loss was artificially introduced by pretending that the near-surface volume is full of "He". Helium is invisible in He-RBS, and so this calculational artifice has the effect of introducing extra energy loss without otherwise affecting the spectrum. We denote it as "He" to emphasise that it is not real. Figure 3 shows the fitted sample structure. The sample is fitted by modelling the rough surface as 95% hydroxyapatite, and 5% invisible "He", with the invisible "He" content decreasing as a function of depth. Notice that this artifice affects both the Ca and the P signals in the same way, so that it is not biasing the results. Roughness was artificially simulated in the same way by Beck *et al*¹⁵, whose spectra were subsequently interpreted correctly by Molodtsov *et al*¹⁶ with an algorithm not yet available in standard IBA codes.

The He^+ beam energy was determined very precisely to about 1 keV by the O signal, since the FWHM (full-width half-maximum) of the 3044 keV resonance is about 8 keV. The beam energy determined from the spectrum (3063 ± 1 keV) was not significantly different from the calibrated beam energy. Note that the distortion of the resonant O signal by roughness effects is not modelled correctly by our artificial approach which cannot correctly calculate the energy straggling in the sample, but that we have minimised this distortion by using a high backscattering angle. When

the algorithm of Molodtsov is implemented in DataFurnace it will be possible to explicitly determine the roughness parameters and simulate the spectral shape from them, but here we need only to precisely fit the data in the near surface region to extract the Ca:P ratio, assuming that this ratio is constant to the surface.

Because the roughness is not modelled correctly, the shape of the O resonance signal and the leading Ca edge are both poorly fitted in the RBS spectrum. Because the fits are rather poor and almost the whole of the Ca signal at a higher energy than the P edge is affected by the roughness, we have only demonstrated that the spectrum is consistent with the certified stoichiometry, with the misfit in both the P and Ca signals being <1%. At the precision of the fitting, the O content is stoichiometric to the surface, using the very high sensitivity given by the resonant scattering cross-section at 3044 keV. Therefore the high fluence used for this data did not noticeably damage the sample. The SEM-EDS was undertaken prior to the RBS analysis, and the PIXE data was taken simultaneously with the RBS. Therefore the composition of the sample did not degrade during the analysis.

The systematic uncertainty for the RBS for this type of analysis, which is not sensitive to the details of the calibration, is in principle <1%²⁵. Where unmodelled roughness affects the signal shapes, as here, the true value of the expected uncertainty cannot be determined. A free fit with the artificial modelling of the roughness described gave a Ca/P ratio of 1.77, a 4% increase. But it is clear that the artificial model must overestimate the Ca since the artificial "roughness" depresses the Ca signal (falsely) more than the P. So we assign a value of 2% to this uncertainty.

The SEM-EDS spectra were fitted using the Oxford Instruments Link code^{37,38,39} which is based on a semi-empirical general determination of the depth distribution of the X-ray excitation function due to the collision cascade of the electron beam, utilising both Monte Carlo calculations and an extensive series of measurements of tracer layers of one material in another together with self-supporting thin layers. The accuracy with which this function is estimated depends on the uncertainty in the tracer layer thicknesses (3%) and the demonstrable accuracy of the MC (also about 3%). The absorption correction (more difficult than the mass or fluorescence corrections) is obtained by integration of the excitation function, and was critically compared to an extensive measurement database and found to have an uncertainty of about 5% for

"light" elements (Ca and P are "light" in this context). Thus the total systematic uncertainty is estimated as 7%.

The PIXE spectra were fitted using both DataFurnace⁴⁰ and OMDAQ⁴¹ (which implements GUPIX⁴², a validated code⁴³). The PIXE data analysis follows a similar method as for SEM-EDS, but for ion beam induced X-rays the absorption correction is far easier to calculate since the bulk of the excitation volume is defined by the beam and not by the collision cascade. Ion beams are much stiffer than electron beams, so that the collision cascades are largely restricted to the end of range, which is usually far too deep for these low energy X-rays to escape. Therefore the PIXE absolute quantification should be more reliable than for electrons, although the same methods using relative standards are used to make use of the high precision available. In particular, the uncertainty of 4% for SEM-EDS in the estimation of the excitation volume is entirely avoided for PIXE; and similarly, the 5% uncertainty in the absorption correction for SEM-EDS is largely due to the uncertainty of X-ray exit pathlength because of the complicated shape of the excitation volume, and this is also almost entirely avoided for PIXE.

For the PIXE we use a pure "fundamental parameters" method, where the data are analysed with a first-principles calculation which critically depends on the databases used. In particular, the X-ray detector filter thicknesses are chosen to give the certified values for the lead glass standard, and it is the uncertainty in the filter thickness which controls the total PIXE uncertainty. For GUPIX, both the Si and the Ca concentrations in the standard are reproduced, and therefore the P in the HA will also have the correct relative ISF. For DataFurnace the relative Si yield was adjusted with 400nm Al as an extra "filter". However, the relative transmission of P is twice that of Si, so for NDF there is potentially quite a large uncertainty here. And in neither case is the P excitation cross-section database experimentally checked. However, using two different codes we access these (different) databases in two different ways, and find (in this case) about 2.5% difference. Thus, we assign a 2.5% uncertainty in Table 1, although this may be an underestimate

Unlike the e-beam methods, the IBA methods allow quantitative elemental concentration depth profiles to be determined directly from the backscattered particle energy spectrum, and hence the correct X-ray absorption to be determined. This is currently implemented in the DataFurnace code using the algorithm of Reis⁴⁴.

However, DataFurnace does not presently calculate the secondary fluorescence correction, but for hydroxyapatite the secondary fluorescence yield is negligible, being less than 0.5%. For the present analysis the sample is homogeneous in depth, so that GUPIX and DataFurnace should be exactly equivalent for H^+ PIXE. At present DataFurnace cannot treat PIXE data using a He^+ beam.

A potential source of error in all X-ray spectroscopic methods of analysis of P in a Ca matrix using Si(Li) detectors is the interference between the P K_α line (2.10 keV) and the silicon K escape peaks due to the Ca K_α and K_β lines (1.95 keV and 2.27 keV). The tails of the escape peaks can introduce uncertainty into the calculated P K peak area if they are not correctly modelled. Escape peaks are treated in GUPIX using a semi-empirical approach⁴⁵ which yields an escape peak intensity close to 1% of the primary lines for the Ca K lines. Thus an error of 20% in the escape peak model would result in a maximum error of 0.2% of the Ca peak area in the P peak determination. This can be a serious problem for measuring trace levels of P in Ca, but in this case, where the peak areas of P and Ca are comparable, the effect is negligible.

Discussion

The Ca:P mole ratios obtained are presented in Table 1 together with the estimated uncertainties. The sample uncertainty is the variation in Ca:P ratio from repeated measurements at different places on the sample. It is interesting to note that the sample variation is lowest (1%) for He PIXE, where a broad beam ($\sim 1\text{ mm}^2$) was used. This beam spot is sufficient to average over the observed fluctuations in the Ca:P ratio that the other (microbeam) techniques detect. The relative uncertainty is the uncertainty due to either counting statistics (RBS) or the uncertainty in the filter thickness (PIXE).

The electron and ion induced X-ray data are not strictly comparable since the instrumental sensitivity factors (ISF) were determined differently in both cases. In both cases the fundamental parameters method was being used: for SEM-EDS because the ISFs were determined in dissimilar materials and the shape of the excitation volume was therefore *calculated*, and not normalised; and for PIXE

because the ISF for P was inferred from the Si signal of the lead glass standard using database cross-sections. The systematic uncertainties presented in Table 1 therefore reflect differences in the databases used and the computational differences of the codes. For H-PIXE, NDF and GUPIX give answers differing by 2.5%. A systematic uncertainty of 2.5% has therefore also been assigned to He PIXE, for which no direct comparison can be made.

What effect of roughness might we expect to see? The Ca:P line intensity ratio is quite a strong function of absorption, and effects recognisably due to roughness extend to a depth of around 300nm in the particle spectrum. But even if we assumed that there was distortion of the Ca:P ratio due to roughness effects equivalent to 1 μm of material, we would only see a 2% effect.

Conclusions

We have carried out a thorough preliminary analysis of a hydroxyapatite green pressed pellet made of certified material using particle backscattering (RBS & EBS), together with SEM-EDS and both He- and H-PIXE. The particle backscattering is shown to be consistent with the certified composition with a 2% uncertainty, demonstrating that the certified composition of the pressed pellet samples extends right up to the surface.

We have also shown that although the pressed-pellet roughness could be readily seen in the backscattering data, and is of course clear in the SEM, it does not have a measurable effect on the observed Ca:P ratio; although the instrumental sensitivity factors were not determined to better than 3.5% (7% for SEM-EDS) which is not nearly enough to see roughness effects, expected in this case to be less than 2%.

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Table and Figure Captions

Table 1: Summary of Results and Uncertainties

Figure 1: SEM of a green pressed pellet of hydroxyapatite powder. The image is 60 μm across

Figure 2a: RBS/EBS spectrum of HA (certified composition)

Symbols = data, line = fit. Fitted energy of 3063 keV $^4\text{He}^+$, assuming the certified stoichiometry to the surface. 172° scattering angle, 1.22 msr, normal beam incidence, 336 μC collected charge. The partial spectra are shown for C, O, P, Ca (increasing maximum energy). The C and O signals are non-Rutherford.

Figure 2b: RBS/EBS spectrum of HA

Log_e ordinate scale to show minor and trace elements. Partial spectra are shown for Na, Mg, Al, Si, Fe, Cu+Zn, Zr+Sr, Sn, Ba, Hf, Pb (increasing maximum energy). Pb and Hf are depleted at the surface, and the unfitted roughness accounts for the high energy tail of the HA into the C surface layer.

Figure 3: The fitted structure simulated in Figure 2

Extra energy loss was artificially introduced by invisible "He", to reduce yield in the near-surface region. The carbon layer was the 75nm anti-charging coating.

Table 1: Summary of Results and Uncertainties						
Technique	Ca:P ratio	Uncertainty				Analysis depth in HA (3.16g/cc)
		Sample ^a	Measurement			Projected range and straggle ^d
			Relative ^b	Systematic ^c	Total	(microns)
3.06 MeV He RBS	(1.70) ^e	----	0.5%	2%	2.1%	0.54 ^f
20 keV SEM-EDS	1.63 ± 0.14	5%	----	7%	7%	1.1 ± 0.9
3.06 MeV He PIXE	1.73 ± 0.06	1%	2.4%	(2.5%) ^g	3.5%	8.6 ± 0.5
2.58 MeV H PIXE (DataFurnace)	1.60 ± 0.08	4%	2.3%	2.5%	3.4%	50.4 ± 3.1
2.58 MeV H PIXE (GUPIX)	1.56 ± 0.08	4%	2.3%	2.5%	3.4%	50.4 ± 3.1
<p>a: Sample variation from repeated measurements (SEM) or identifiable areas (PIXE). 10 randomly chosen spots were measured for SEM and He PIXE. 5 randomly chosen spots were measured for H PIXE.</p> <p>b: Relative uncertainty from filter thickness uncertainty (PIXE) or counting statistics (RBS)</p> <p>c: Systematic uncertainty – see text</p> <p>d: Except for RBS (see note f), calculated by SUSPRE⁴⁶ which uses the projected ranges algorithm (PRAL) of Biersack⁴⁷. The information depth is 100% of this for electrons and around 50% for particles</p> <p>e: Model-free fit was not made for RBS data. Consistency at <1% for both Ca and P signals</p> <p>f: Thickness of HA for which there is no P background on the Ca signal</p> <p>g: Inferred from the comparison of GUPIX and NDF for H-PIXE</p>

Figure 1: SEM of a green pressed pellet of hydroxyapatite powder. The image is 60μm across

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Figure 3: The fitted structure simulated in Figure 2

Extra energy loss was artificially introduced by invisible "He", to reduce yield in the near-surface region. The carbon layer was the 17 $\mu\text{g}/\text{cm}^2$ ($\sim 75\text{nm}$) anti-charging coating. Roughness effects persist to a depth of 80 $\mu\text{g}/\text{cm}^2$ (250 nm full density HA)

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