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FTIRI Parameters describing Acid Phosphate Substitution in Biologic Hydroxyapatite

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Abstract

Acid phosphate substitution into mineralized tissue is an important determinant of their mechanical properties and their response to treatment. This study identifies and validates Fourier Transform Infrared Spectroscopic Imaging (FTIRI) spectral parameters that provide information on the acid phosphate (HPO_4) substitution into hydroxyapatite in developing mineralized tissues. Curve fitting and Fourier self-deconvolution were used to identify subband positions in model compounds (with and without HPO_4). The intensity of subbands at 1127 cm^{-1} and 1110 cm^{-1} correlated with the acid phosphate content in these models. Peak height ratios of these subbands to the ν_3 vibration at 1096 cm^{-1} found in stoichiometric apatite, were evaluated in the model compounds and mixtures thereof. FTIRI spectra of bones and teeth at different developmental ages were analyzed using these spectral parameters. Factor analysis (a chemometric technique) was also conducted on the tissue samples and resulted in factor loadings with spectral features corresponding to the HPO_4 vibrations described above. Images of both factor correlation coefficients and the peak height ratios $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$ and $1112\text{ cm}^{-1}/1096\text{ cm}^{-1}$ demonstrated higher acid phosphate content in younger vs. more mature regions in the same specimen. Maps of the distribution of acid phosphate content will be useful for characterizing the extent of new bone formation, areas of potential decreased strength, and the effects of therapies such as those used in metabolic bone diseases (osteoporosis, chronic kidney disease) on mineral composition. Because of the wider range of values obtained with the $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$ parameter compared to the $1110\text{ cm}^{-1}/1096\text{ cm}^{-1}$ parameter, and the smaller scatter in the slope, it is suggested that this ratio should be the parameter of choice.

Keywords

FTIR imaging; hydroxyapatite; bone mineral; dentin mineral; acid phosphate

Fourier transform infrared spectroscopic imaging (FTIRI) and microscopy (FTIRM) have been used to describe variations in mineralized tissue composition at a spatial resolution of 7–10 μm [1–4]. Knowledge of the spatial variation in mineral and matrix composition is important as it allows interpretation of alterations in mechanical properties [5,6], fracture risk [7], effects of therapies [8,9] and the effects of genetic and metabolic abnormalities on each of these properties [10–12]. In the cited examples, FTIR spectroscopy combined with a focal plane array (FPA) detector provided IR images showing the distribution of specific

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compounds within the mineralized tissue, without the need for staining or use of antibodies. With FTIRI there are simultaneous measurements of multiple spectra within a single tissue section. Each individual spectrum contains absorption bands related to the chemical components within that specific region of the specimen, producing a “chemical image” or “chemical photograph” [13]. Spectra can be processed to provide maps of specific parameters [14] or analyzed with multivariate statistics to provide information about the components contributing to the complex spectrum at each pixel in the image [15].

Three mineral-based spectral parameters routinely used for specimen characterization, namely mineral/matrix peak area ratio [16], carbonate/phosphate peak area ratio [17] and mineral crystallinity [18] have been independently validated. The crystallinity parameter, based on the relative intensities of peaks at 1030cm^{-1} and 1020cm^{-1} , respectively, reflects the relative amount of crystal perfection (or amount of non-stoichiometric apatite in the lattice) and is related to the line broadening of the c-axis (002) wide angle x-ray diffraction peak [18].

FTIRM and FTIRI studies have used several different parameters to assess acid phosphate substitution in hydroxyapatite crystals in mineralized tissues. Since the major acid phosphate vibration ($537\text{--}539\text{cm}^{-1}$) is not detectable with current FTIRI imaging detectors, a variety of different parameters have been used to provide this information. Only with synchrotron FTIR could maps [19] or images [20] of relative acid phosphate substitution be obtained using the ratio of acidic phosphate intensity (538cm^{-1}) over the peak area of the ν_4 phosphate band. Previously, we had used peak intensities of the 1143cm^{-1} band over the total area of the ν_1, ν_3 band [21]; the peak height ratios of $1120\text{cm}^{-1}/960\text{cm}^{-1}$ [22–23] and the peak height ratio of $1106\text{cm}^{-1}/960\text{cm}^{-1}$ [24] to evaluate acid phosphate substitution into the apatite lattice. Which of these ratios or other parameters should be used in current FTIRI protocols required further investigation.

Observation of a broadened phosphate ν_1, ν_3 band in the $1100\text{--}1150\text{cm}^{-1}$ region of spectra of newly mineralizing tissues suggested these tissues underwent a change in composition with development in addition to the previously reported [6] increase in crystal size and perfection. Subbands in this region have previously been attributed to HPO_4 (acid phosphate) vibrations [25–27] which, as noted above, are usually identified by a band at $\sim 530\text{--}560\text{cm}^{-1}$, frequencies not accessible to the current generation of imaging spectrometer detectors. Based on analysis of model compounds with and without acid phosphate components, two acid phosphate bands centered at $1110\pm 2\text{cm}^{-1}$ and $1127\pm 2\text{cm}^{-1}$ were confirmed in the current work. To determine if using these bands would provide additional information about the composition of mineralized tissue, we calculated two peak height ratios ($(1127\text{cm}^{-1}/1096\text{cm}^{-1})$ and $(1112\text{cm}^{-1}/1096\text{cm}^{-1})$) and compared these to other FTIRI parameters, where 1096cm^{-1} is a ν_3 vibration found in stoichiometric apatite [25 and references therein]. Both the calculated ratios and the mineralized tissue images showed an inverse correlation with the FTIRI crystallinity parameter in most but not in all tissues, and thereby provided new insight into the importance of acid phosphate content in the mineralized tissues.

Materials and Methods

Model Compounds

Hydroxyapatite and Calcium Phosphate Salt Preparations and Characterization

Hydroxyapatite powders were prepared at pH7, pH7.4, pH8, and pH9 by the wet chemical process described elsewhere [28]. In brief, amorphous calcium phosphate (ACP) was synthesized from 400mM CaCl_2 (Sigma-Aldrich, St. Louis, MO) and 300mM dibasic

ammonium phosphate (Sigma-Aldrich) in 1 L of Tris-hydroxy methyl amino methane (Tris) (Sigma-Aldrich) buffer. The pH of the preparation was adjusted to pH 7.0, 7.4, 8.0, and 9.0 via the Tris buffer. The ACP thus formed was allowed to convert to hydroxyapatite and ripen at that same pH by 24 hr incubation in the mother liquor. Three other calcium phosphate compounds were also used; tribasic calcium phosphate (TCP, an HPO_4 -free hydroxyapatite), brushite (a structurally distinct calcium acid phosphate compound), and octacalcium phosphate (OCP, an apatite-like compound containing acid phosphate substitutions). The synthetic and commercially acquired calcium phosphate salts (Table 1) were identified based on powder x-ray diffraction of the materials ground to a fine mesh. Mixtures of different compounds were prepared on a weight/weight basis, and were characterized by x-ray diffraction and IR as detailed below.

X-Ray Diffraction

All model compound powders were sieved to 325 mesh and analyzed by wide-angle X-ray diffraction with Ni-filtered $\text{CuK}\alpha$ radiation (D8 Advance X-Ray diffractometer, Bruker Industries, Madison, WI). Composition was confirmed by comparison to ASTM standards [29]. The crystallinity, defined as the broadening of the 002 band of hydroxyapatite, was measured for all the synthetic hydroxyapatites and related mixtures.

FTIR of Model Compounds and Mixtures

Two-component mixtures of different ground and lyophilized model compound powders (ranging from 0.0–100% weight percent) were prepared by weighing the individual powders, and then mixing them during grinding with 1 wt % KBr. The homogenized powders were pressed in a pellet press to optical transparency (10 tons, 30 seconds). Mixtures were prepared by combining a compound with no HPO_4 (TCP or pH 9 HA) with a compound with structures similar to HA known to contain HPO_4 (OCP or pH 7.4 HA). From each model compound or mixture, 3–4 separate KBr pellets were prepared, scanned, and analyzed. FTIR spectra were acquired on a Nicolet 4700 IR spectrometer (Thermo Electron North America LLC). Scanning was performed in transmission mode in the 4000–400 cm^{-1} range at 2 cm^{-1} resolution, accumulating 64 scans. The spectra were truncated to the phosphate absorbance region (1200–900 cm^{-1}), baselined and analyzed. The analysis included Fourier self-deconvolution (FSD) and second derivative spectroscopy with Grams 32 software version 6 (Galactic Industries, Salem, NH) and Win-IR PRO software (Bio-Rad Laboratories, Cambridge, MA), respectively.

FSD is a method for decomposing complex spectra based on the assumption that an absorption band lineshape is the convolution of a Dirac delta function with a broadening function. The goal of FSD is to reduce the width of the broadening function without altering the peak position or band area. FSD is a special high pass fast Fourier transform (FFT) filter which synthetically narrows the effective trace bandwidth features. The application in Grams software is based on the method described by Griffiths and de Haseth [30]. Two filters are employed in this method. First, an exponential filter is used to “sharpen” spectral features; a constant (γ') is varied to change the filter shape. This imposed filter is the Fourier transform of a Lorentzian line shape. This function is multiplied by the Fourier transformed trace, and the data is then reverse Fourier transformed to give the result. In the current study, we used the well resolved band at 962 cm^{-1} to evaluate γ' so our resultant FSD spectrum did not contain side lobes. The constant γ' was chosen to be 6 cm^{-1} which, for the half-widths of the bands studied, falls within the acceptable limits for band area preservation [30]. Second, as noted by Griffiths and de Haseth, the functions used to change the decay characteristics in FSD increase the spectral noise level. The smoothing function used for this study was a Bessel smoothing function of the form:

$$J(x)=[1-(x/\Delta)^2]^2$$

where Δ is the maximum retardation. As noted [30], the FWHH is narrowed more by the exponential function than it is broadened by the smoothing.

The second technique used to assign peak positions was second derivative spectroscopy (FD). FD is a computationally-efficient, widely applied technique used to provide accurate frequency measurements. Operationally, the Fourier transform of absorbance spectrum is multiplied by a function of the form $F(x) = cx^n$ where “c” is a constant and “n” a positive integer. Computation of the product of this function and the Fourier transform of an absorbance spectrum produces the “n”th derivative of the spectrum. We have found that every order of derivation reduces the S/N ratio by a factor of 3–5. Thus computing the second derivative reduces the S/N by ~9–25. The second derivative peaks which appear as minima, may be inverted for convenience.

The acid phosphate content was assessed utilizing the subband at $\sim 530\text{ cm}^{-1}$ in the ν_4 phosphate wavenumber region ($500\text{--}650\text{ cm}^{-1}$) [27,31]. In addition, broadening in the ν_1, ν_3 phosphate absorbance region ($900\text{--}1200\text{ cm}^{-1}$) was evaluated. The mixtures containing combinations of acid phosphate-free and acid phosphate-containing calcium phosphates were analyzed. The intensity ratios were calculated following truncation and baseline correction ($900\text{--}1200\text{ cm}^{-1}$) using the peak positions identified in the FSD spectra and second derivative spectra of the pure compounds. Correlations between the weight percent acid phosphate containing component in the mixtures and the measured parameters were calculated in SlideWrite 5.0 (Advanced Graphics Software, Rancho Santa Fe, CA) based on a linear fit of the equation $y=a_0 + a_1 x$. The coefficient of determination (R^2) and the goodness of fit (fit standard error) are reported for each parameter examined in each mixture.

FTIR Spectroscopy and Imaging of Developing Mineralizing Tissues

FTIRI spectra from several different mineralized tissues were examined using factor analysis [32] to identify components contributing to their complex phosphate spectra. Earlier published FTIR imaging studies provided the spectra for these analyses. They included developing bovine dentin [33]; baboon osteons over a wide range of ages [6], and cortical and trabecular bone from wild type mice at different developmental ages [34,35,36]. These spectra had been obtained from $2\mu\text{m}$ thick polymethylmethacrylate (PMMA) embedded mineralized tissue specimens using a Perkin Elmer Spectrum-Spotlight-100 system (Waltham, MA, USA) at 4 cm^{-1} spectral resolution and $6.25\mu\text{m}$ pixel size in the transmittance mode. IR images were analyzed using ISYS Chemical Imaging software (Spectral Dimensions (presently Malvern) Inc., Olney, MD). Spectra extracted from these images were truncated to the ν_1, ν_3 phosphate absorbance region ($1200\text{--}900\text{ cm}^{-1}$). To detect simple patterns (components) in the observed absorbance spectra throughout the image, factor analysis was performed using the ISYS score segregation routine. Score segregation begins by normalizing the principal component analysis (PCA) scores by channel over the range 0–1 and sharpening them to the power specified by the score segregation acceleration parameter. The default value of 10 was used. If the data did not converge, the number of selected factor loadings was decreased by one and the cycle began again with renormalization of the scores. Usually 4–6 factor loadings were generated. Because score segregation enforces normalization by channel, it tends to produce good spatial segregation of an image, with factor loadings that are not necessarily pure spectra. Statistical correlation mapping produced a set of images (one image per factor loading)

whose pixel values are the correlation coefficients between the reference spectrum (or factor) and the spectrum of a corresponding single pixel. The highest correlation coefficient would be one where a single pixel spectrum is identical to the factor whereas two orthogonal spectra yield a correlation coefficient of zero. Images were also prepared using the parameters identified from the model compounds correlating to acid phosphate distribution.

Results

We and others had previously used several different ratios [21–24] based on subbands in the 1100–1150 cm^{-1} region to assess acid phosphate substitution in mineralized tissues using FTIRI. Herein, a series of model compounds and mixtures thereof containing different amounts of acid phosphate (Table 1) are examined. The FTIR spectra of acid phosphate-containing and related compounds demonstrated the presence of acid phosphate vibrations in the 520–560 cm^{-1} region (Figure 1A, arrow). There was an evident relationship between the subband intensity at $\sim 530 \text{ cm}^{-1}$ and the pH of HA preparations. The model compounds studied contained variable amounts of acid phosphate based on the 530 cm^{-1} absorbance with brushite having the greatest relative amount of HPO_4 , followed by octacalcium phosphate, and then HA prepared at pH 7. The pH 7 HA was very unstable, and converted to a more crystalline form within a few hours, thus for other model compound studies, we used HA prepared at pH 7.4. HA prepared at pH 8 and pH 9 showed very weak subbands at 530 cm^{-1} , and tricalcium phosphate, which does not contain substituted HPO_4 , displayed no subband in this region. Fourier self deconvolution was applied to the several of the spectra (Figure 1B). Only hydroxyapatite prepared at pH 7 and octacalcium phosphate showed peaks at 1127 ± 2 and $1110 \pm 2 \text{ cm}^{-1}$. In addition, second derivative spectra for octacalcium phosphate and pH 7 HA (Figure 1C, bottom) displayed negative peaks at ~ 1127 and 1109 cm^{-1} , whereas comparable spectra of TCP and HA prepared at pH 8 and 9 (Figure 1C, top) do not display these features. The ratio of the acid phosphate subband intensities (in the original spectra) to a subband at 1096 cm^{-1} and the previously used 1030 cm^{-1} stoichiometric apatite phosphate band were then calculated from spectra of the mixtures of these compounds.

As shown in Figure 2A–C, both the $1112 \text{ cm}^{-1}/1096 \text{ cm}^{-1}$ and $1127 \text{ cm}^{-1}/1096 \text{ cm}^{-1}$ peak height ratios showed significant linear correlations with acidic phosphate salt content in these mechanical mixtures. OCP and HA7.4 are used as models of an acid phosphate containing apatite in these figures, whereas tribasic calcium phosphate (Figure 2A and C) or HA9 (Figure 2B) represented materials with little to no acid phosphate. The $1127 \text{ cm}^{-1}/1030 \text{ cm}^{-1}$ peak height ratio was also highly correlated with gravimetric weight percent HPO_4 content. Plots of $1112 \text{ cm}^{-1}/1030 \text{ cm}^{-1}$ and $1020 \text{ cm}^{-1}/1030 \text{ cm}^{-1}$ (the inverse of the $1030 \text{ cm}^{-1}/1020 \text{ cm}^{-1}$ intensity ratio used as a measure of crystallinity) also showed linear correlations. For the model compound mixtures there was a similar correlation with the acid phosphate content. The R^2 calculated for the linear fits, the goodness of fit, and the intercept (ao) and slope (a1) are summarized in Table 2. The slopes were small, but statistically significant, for the three mixtures presented here, even for the mixture of pH 7.4 HA and TCP, which are the most similar in composition. The goodness of fit was comparable among all acid phosphate parameters measured (mean value 0.034 ± 0.016) indicating any of these ratios could be used to assess acid phosphate content. We plotted $1127 \text{ cm}^{-1}/1096 \text{ cm}^{-1}$ for the mineralized tissues examples (below) in this paper as the relative SD of the slope of $1127 \text{ cm}^{-1}/1096 \text{ cm}^{-1}$ was the smallest for all model compound mixtures examined. A plot of $1020 \text{ cm}^{-1}/1030 \text{ cm}^{-1}$ vs. $1127 \text{ cm}^{-1}/1096 \text{ cm}^{-1}$ was also linear (Figure 2D) indicating the $1127 \text{ cm}^{-1}/1096 \text{ cm}^{-1}$ ratio increased parallel to the loss of crystallinity in the model compounds.

Applications to mineralized tissues

Factor analyses (see Material and Methods for details) of the ν_1, ν_3 phosphate peak region ($900\text{--}1200\text{cm}^{-1}$) in IR spectra extracted from images at different stages of development in (A) baboon osteonal bone and (B) bovine dentin generated 4–6 factors (Figure 3). The spatial distribution of the correlation coefficients associated with each factor loading is shown in color images of the osteon (Figure 4B) and the dentin (Figure 5A) along with images of several of the peak height ratio parameters discussed earlier. The spatial distribution of the highest correlation coefficients and the increase in the 1127 and 1112 cm^{-1} subbands relative to the 1096 cm^{-1} band were always noted in the newly deposited mineral. For this reason, and based on the FSD and second-derivative results we selected two ratios ($1112\text{cm}^{-1}/1096\text{cm}^{-1}$) and ($1127\text{cm}^{-1}/1096\text{cm}^{-1}$) to display the acid phosphate content in mineralizing tissues. For the osteon, images of the correlation coefficients for factors 1–3 were closest to the images of the $1112\text{cm}^{-1}/1096\text{cm}^{-1}$ and the $1127\text{cm}^{-1}/1096\text{cm}^{-1}$ peak height ratios (Figure 4B and C, respectively). The distribution of the ratios of $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$ and $1112\text{ cm}^{-1}/1096\text{cm}^{-1}$ for newly forming bovine dentin were most similar to the correlation images for factors 1 and 4 (Figure 5B and A, respectively). Furthermore, factors 1 and 4 for the bovine dentin (Figure 3B) clearly show enhanced relative intensity in the 1127 cm^{-1} region. Hence we recommend the use of the $1127\text{cm}^{-1}/1096\text{cm}^{-1}$ for future descriptions of acid phosphate substitution.

The section of the unerupted tooth showing newly formed mineral is indicated in the visible image in Figure 5A with the least mature mineral deposited on the right hand side of the zoomed in images. The figures of the osteon and of the tooth are accompanied by maps of mineral/matrix ratios (Figure 4C and 5B) and maps of crystallinity is shown for the osteon (Figure 4D) and dentin sample (Figure 5B). Note that the regions with the lowest mineral/matrix ratio have the highest $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$. Images of the $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$ peak height ratio for mouse tibias of different ages (Figure 4E,F) display a continuous decrease in the average values of these ratios as a function of mouse age consistent with the osteon and dentin images. The average values for $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$ ratio were consistently higher in trabecular struts than in cortical shell (Figure 4F), most likely reflecting the increased remodeling of the trabeculae. The values in each image were the highest on newly forming surfaces of both trabecular and cortical tissues (Figure 4E,F).

Discussion

This study based on uni- and multi-variate analyses of FTIR data from model compounds and images from mineralized tissues, defines two peak height ratios in the ν_1, ν_3 phosphate region that provide insight into acid phosphate substitution within these samples. Although the precise lattice location at which acid phosphate substitutes in the hydroxyapatite lattice is not known [37], acid phosphate substitution is known to be associated with new mineral deposition [24–26]. The ν_1, ν_3 phosphate band has a complex spectra arising from phosphate species' vibrations in stoichiometric and non-stoichiometric hydroxyapatites [38,39]. This band has been analyzed to provide information on hydroxyapatite crystal size and perfection [1,6], the maturity (relative age) of the crystals [40], and their chemical composition [6,17,26,39]. In this paper we focused on the composition as reflected by acid phosphate substitution that can be determined by FTIR.

The acid phosphate subbands at 1112 cm^{-1} and 1127 cm^{-1} [27,36,41,42] had previously been associated with HPO_4^{2-} containing HA, was confirmed here by both second derivative and Fourier self deconvolution analyses of model compounds. There are relative advantages and disadvantages for both these data processing methods. Second derivatives of spectra do not preserve comparative intensity information between spectral features, especially if they have different initial line-shapes. However, the frequency information is accurately retained.

The process of derivation also induces side lobes in the derivative spectra, as noted here. On the positive side, the process of derivation requires no input of operator-defined parameters but smoothing functions, often included in the analysis, require minimal operator intervention. In contrast, FSD requires operator selection of the extent of the narrowing process and the limitations have been well discussed elsewhere [30]. The interference from side lobes is easily controlled. We demonstrate the process in Figure 2B. The approach we used was to observe the effect of FSD on a relatively isolated spectral mode, in this case the phosphate ν_1 mode near 960 cm^{-1} . The parameters we have chosen and the resulting deconvoluted spectra show no side lobes in the 960 cm^{-1} feature, with some line narrowing of the band, suggesting that our parameter is sufficiently conservative to minimize spectral distortions in the more overlapped spectral regions.

The rationale for using the 1127 cm^{-1} and 1112 cm^{-1} band intensities in analyzing FTIR images is based on the inability of the current generation of IR imaging detectors to detect the $530\text{--}560\text{ cm}^{-1}$ band characteristic of acid phosphate. The $1112\text{ cm}^{-1}/1096\text{ cm}^{-1}$ and $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$ ratios in model compounds correlated with the “crystallinity parameter” which we previously validated [16,41] by x-ray diffraction line-broadening analysis of similar model compounds. Their use was also supported by FSD and second derivative spectroscopy in the model compounds. Recently, Farley et al. [40], using FTIR microscopic data defined a “crystallinity” parameter based on ν_4 subbands in the $595\text{--}650\text{ cm}^{-1}$ region, and indicated that the previously validated [16,41] $1030\text{ cm}^{-1}/1020\text{ cm}^{-1}$ peak height ratio for imaging data and their crystallinity parameter for FTIR microspectroscopy have different structural origins. They defined a new mineralized tissue parameter called “mineral maturity” as the intensity ratio of $1030\text{ cm}^{-1}/1110\text{ cm}^{-1}$ (reflecting the stoichiometric fraction of apatite present), and used the $595\text{ cm}^{-1}/650\text{ cm}^{-1}$ peak height ratio as an index of crystallinity. When FTIR imaging and point-by point microscopy samples are compared, with data generated possibly on different IR instruments with different spectral resolutions and levels of zero filling, small spectral differences (i.e. 1110 cm^{-1} vs. 1112 cm^{-1}) may sometimes be observed. Thus we point out that Farley et al.’s “mineral maturity” factor is the inverse of our $1112\text{ cm}^{-1}/1096\text{ cm}^{-1}$ parameter which parallels the $1112\text{ cm}^{-1}/1030\text{ cm}^{-1}$ parameter, reflecting the proportion of acid phosphate vs. stoichiometric apatite, and does in fact measure “mineral maturity” or the amount of acid phosphate substitution. Again, for describing acid phosphate substitution, we recommend and plan to use the $1127\text{ cm}^{-1}/1096\text{ cm}^{-1}$ ratio to avoid any confusion.

Knowledge of the location and amount of acid phosphate substitution is important for studying normal mineralized tissue development, alterations in disease, and tissue regeneration. The location of the highest amounts of acid phosphate substitution indicates areas of new bone formation [19,31,44]. It thus might be possible to use images of acid phosphate substitution in place of tetracycline labeling [45] to locate the most recent site of bone deposition without the need for other interventions. Altered acid phosphate content has also been associated with modifications in bone strength. For example, comparing the strength of three strains of male mice (B6, C3H, A/J) at 16 weeks, the C3H mice, which had the highest mechanical strength, albeit not significantly higher based on tensile testing, had the lowest acid phosphate content based on imaging of the $1106\text{ cm}^{-1}/960\text{ cm}^{-1}$ peak height ratio [24]. In the ovariectomized adult monkey, an animal model for human osteoporosis, the HPO_4 content, measured from synchrotron data was increased in the fracture prone animals [46]. Similarly, in micro-damaged bone, based on synchrotron FT-IR, there was an increase in HPO_4 content at the sites of damage [20]. Based on FT-IRM measurements of biopsies from humans with both low and high turnover osteoporosis, the absorbance peak height ratios of $1120\text{ cm}^{-1}/960\text{ cm}^{-1}$ reveal a difference in acid phosphate content of tissue on the trabecular surface relative to the center of the trabeculae [22]. This suggests that the fractured bones contained older mineral.

In conclusion we recommend that the term “acid phosphate content” be applied to the 1112 cm^{-1} /1096 cm^{-1} ; 1127 cm^{-1} /1096 cm^{-1} or 1112 cm^{-1} /1030 cm^{-1} ratios to make parameters comparable and that in future studies one of these parameters (preferably 1127 cm^{-1} /1096 cm^{-1}) be included with FTIR analysis of bone and dentin mineral where knowledge of acid phosphate content is important. Knowledge of the distribution of acid phosphate can be significant in determining bone age, in assessing changes in bone or dentin quality with disease and treatments, and in assessing whether secondary mineralization is proceeding appropriately in conditions such as osteoporosis [7], chronic kidney disease [47], osteomalacia [16], and osteogenesis imperfecta [14] where mineral crystals are known to differ in composition from those in age-matched controls.

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Figure 1.

Spectra of Model Compounds (see Table 1 for abbreviations). (A) Spectra top to bottom: brushite, OCP, HA7, HA8, HA9, TCP. (B) spectra (dashed), FSD (solid). Top to bottom: OCP, HA7, HA8, HA9, TCP. (C) 2nd derivative spectra. Top: HA8-solid; HA9-dashed; TCP-dash-dot. Bottom: OCP-solid; HA7-dash.

Figure 2.

Calculated correlations relating FT-IR ratios for mixtures of Model Compounds to Composition (see Table 1 for abbreviations). The weight percent acid phosphate was based on the percent of the HPO₄ free component included in the mechanical mixture.

A. Peak height ratio vs. % OCP in OCP/TCP mixtures

B. Peak height ratio vs. % OCP in OCP/HA9 mixtures

C. Peak height ratio vs. % HA7.4 in HA7.4/TCP mixture

For the above: (■) 1127/1030, (□) 1112/1030, (○) 1020/1030, (▽) 1112/1096, (◆) 1127/1096

D. Correlation plot of 1020/1030 vs. 1127/1096 ratios

Figure 3.
Factor loadings for a baboon osteon (A) and newly forming bovine dentin close to the root (B).

Figure 4.

Spectral changes in the baboon osteon; for each set of figures the arrow indicates the size of the image, the color scale bar the range of values in each image

(A) Light micrograph showing the morphology of an individual osteon. The arrow indicates the Haversian canal (blood vessel) with the most recently formed mineral closest to the canal. The age of the mineral increases with distance from the Haversian canal.

(B) Images of correlation coefficients (factor scores) for the same osteon (see Figure 3A for loadings).

(C). Images of Mineral/Matrix (Min/Mat) ratio, 1112 $\text{cm}^{-1}/1096 \text{ cm}^{-1}$, and 1127 $\text{cm}^{-1}/1096 \text{ cm}^{-1}$ for the same osteon.

D. Image of the crystallinity parameter (1030 $\text{cm}^{-1}/1020 \text{ cm}^{-1}$) for the same osteon.

E. Images of 1127 $\text{cm}^{-1}/1096 \text{ cm}^{-1}$ and (F) average values of this parameter for cortical (cort.) and trabecular (trab.) mouse bone at different stages of development.

Figure 5.

Spectral changes in developing bovine dentin

A. Visible image of the bovine tooth indicating area shown in the adjacent images of correlation coefficients for dentin factor loadings (see Figure 3B for loadings)

B. Images of: $1126\text{ cm}^{-1}/1096\text{ cm}^{-1}$, $1112\text{ cm}^{-1}/1096\text{ cm}^{-1}$, $1030/1020\text{ cm}^{-1}$ and mineral/matrix ratios corresponding to the region indicated in 5A..

Table 1

Calcium Phosphates Tested as Model Compounds

Chemical Name	Formula	Source	Abbreviation
Calcium Phosphate Tribasic (Hydroxyapatite)	$\text{Ca}_5(\text{OH})(\text{PO}_4)_3$	Sigma-Aldrich	TCP
pH 7 HA	$\text{Ca}_5(\text{OH})(\text{HPO}_4)_x(\text{PO}_4)_{3-x}$	Synthesized *	HA 7
pH 7.4 HA	$\text{Ca}_5(\text{OH})(\text{HPO}_4)_x(\text{PO}_4)_{3-x}$	Synthesized	HA 7.4
pH 8 HA	$\text{Ca}_5(\text{OH})(\text{HPO}_4)_x(\text{PO}_4)_{3-x}$	Synthesized	HA 8
pH 9 HA	$\text{Ca}_5(\text{OH})(\text{HPO}_4)_x(\text{PO}_4)_{3-x}$	Synthesized	HA 9
Calcium Phosphate Dibasic (Brushite)	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	Sigma-Aldrich	DCPD
Octacalcium Phosphate	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	(Dr. LeGeros) NYU Dental	OCP

* See Materials and Methods for Synthetic Methods

Table 2

Linear Regression Coefficients and Goodness of Fit for data in Figure 2

	OCP/TCP			
Parameter	R²	GoF	a₀	a₁
1127/1030	0.91	0.048	0.21±0.03	0.0033±0.0014
1112/1030	0.63	0.045	0.44±0.03	0.0013±0.003
1020/1030	0.89	0.048	0.66±0.04	0.0030±0.0006
1112/1096	0.95	0.071	0.32±0.05	0.0075±0.0009
1127/1096	0.95	0.041	0.72±0.03	0.0041±0.0005
	OCP/HA 9			
1127/1030	0.97	0.013	0.33±0.02	0.00191±0.0001
1112/1030	0.081	0.014	0.498±0.0001	0.0001±0.0017
1020/1030	0.94	0.022	0.75±0.02	0.0020±0.0003
1112/1096	0.95	0.027	0.87±0.02	0.00276±0.00002
1127/1096	0.97	0.038	0.61±0.002	0.0052±0.004
	HA7.4/TCP			
1127/1030	0.79	0.027	0.21±0.02	0.0010 ±0.00001
1112/1030	0.96	0.037	0.43±0.003	0.00050±0.0005
1020/1030	0.34	0.043	0.705±0.035	0.00072±0.0005
1112/1096	0.57	0.037	0.76±0.001	0.0010±0.0003
1127/1096	0.92	0.031	0.38±0.019	0.0020±0.0003

GoF- goodness of fit is the fit standard error, or the root mean standard error, where the best fit's value would be zero.

The linear regression fit the equation $y = a_0 + a_1 * x_1$