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An X-ray absorption method for the identification of calcium phosphate species using peak height ratios

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Abstract

X-ray absorption near edge structure (XANES) studies on calcium phosphate species (Ca-P) deal with marginal differences among subtle spectral features despite a hitherto missing systematic breakdown of these differences. Related fingerprinting approaches depend therefore on spectral libraries that are not validated against each other, incomplete and scattered among publications. This study compiled a comprehensive spectral library from published reference compound libraries in order to establish more clear-cut criteria for Ca-P determination by distinctive phosphorus K-edge XANES features. A specifically developed normalization method identified diagnostic spectral features within the compiled library, e.g. by uniform calculation of ratios between white-line and secondary peak heights. Post-processing of the spectra ($n = 81$) verified distinguishability among most but not all phases, which included hydroxylapatite (HAP), poorly crystalline HAP, amorphous HAP, fluorapatite, carbonate fluorapatite (CFAP), carbonate hydroxylapatite, β -tricalcium phosphate, octacalcium phosphate (OCP), brushite, monetite, monocalcium phosphate, amorphous calcium phosphate (ACP), anapaite, herderite, scholzite, messelite, whiteite and P on CaCO_3 . Particularly, peak height ratios significantly improved analyte specificity, e.g. by supplementary breakdown into OCP and ACP. The spectral analysis also revealed Ca-P standards that were rarely investigated or inappropriately synthesized, and thus provides a basis for standard selection and synthesis. The developed method and resulting breakdown by species were subsequently tested on Ca-P spectra from studies on bone and sediment. The test indicated that bone material likely comprises only poorly crystalline apatite, which implies direct nucleation of apatite in bone. This biological apatite formation is likely opposed to that of sedimentary apatite, which apparently forms by successive crystallization. Application of the method to μXANES spectra of sediment particles indicated authigenic apatite formation by an OCP precursor.

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1 Introduction

Calcium phosphates (Ca-P) are a group of several mineral species that are present in various biological and environmental matrices, such as soil, sediment, ores, fertilizers, agroindustrial by-products, milk, bone and tooth enamel. For this reason these species play a crucial role in various scientific fields. However, speciation techniques are not yet fully capable to unequivocally identify certain key species due to sensitivity and specificity issues, and the complexity of biological or environmental samples. Moreover, some Ca-P species form naturally occurring polymorphs, which further increases the number of target phases. An important example for this polymorphism is apatite that may occur in amorphous (e.g. bone apatite), cryptocrystalline (e.g. collophane) or crystalline (e.g. igneous apatite) forms.

A central research focus of studies on Ca-P relates to natural formation processes of corresponding species, in particular of their end-members. The most common Ca-P end-members in biological or environmental systems are species of the apatite group. For instance, authigenically formed carbonate fluorapatite (CFAP) is suggested to be a primary sink of reactive phosphorus (P) in the ocean (Ruttenberg, 1992; Ruttenberg and Berner, 1993) and thus a key compound during the ultimate stage of a complex P removal mechanism within the global P cycle. It is thus distinguished from detrital fluorapatite (FAP), which is delivered to marine environments by continental weathering. Due to their occurrence in P-bearing rocks, apatites mark also the initial stage of P transformations within the global P cycle. Weathering of these rocks by soil genesis generates soluble P, which then becomes available for biotic and abiotic P transformation mechanisms (Giguet-Covex et al., 2013).

Species of the apatite group may directly nucleate or form by transformation of previously precipitated metastable precursors (successive crystallization), such as octacalcium phosphate (OCP), amorphous calcium phosphate (ACP), brushite (DCPD) and β -tricalcium phosphate (β -TCP). Natural transformations among these P compounds are generally driven by both thermodynamic and kinetic factors. In simplified terms,

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thermodynamically less stable but rapidly precipitating Ca-P species may allow for successive crystallization pathways until the thermodynamically favoured but kinetically slow end-member formation occurs. Direct nucleation of apatite or transformation of precursors to apatite can also be inhibited or slowed, for example by Mg^{2+} ions in biological matrices and marine environments (Ban et al., 1993; Golubev et al., 1999; Gunnars et al., 2004; Martens and Harriss, 1970). The initial phase during apatite formation including possible effects of precursors has been primarily investigated with regard to bone apatite and marine CFAP-bearing phosphorite deposits but this subject still remains controversial (e.g. Atlas and Pytkowicz, 1977; Baturin, 1981; Bentor, 1980; Rey et al., 2009; Sheldon, 1981). This has been due largely to the above-mentioned difficulties in identifying Ca-P within corresponding sample matrices down to species level. A brief description of the two major techniques for Ca-P determination in such matrices follows.

The conventional way to quantify the complete fraction of matrix-enclosed Ca-P is to perform sequential chemical extraction methods. Initial procedures were developed for soil, then adapted for sediment and more recently also employed for other matrices, such as agroindustrial by-products (e.g. Kruse et al., 2010). Advancements also facilitated separate quantification of CFAP (Ruttenberg, 1992), OCP (Oxmann et al., 2008) and biogenic apatite (Schenau and De Lange, 2000). A major advantage of these procedures is their high sensitivity that allows quantifications even in extremely P-poor environmental samples. However, the analyte specificity is limited and requires thorough verification because chemical extracts just provide operationally defined quantities.

In recent years, these extraction methods were applied together with X-ray absorption near edge structure spectroscopy (XANES) at the P K-edge (Beauchemin et al., 2003; Kruse and Leinweber, 2008; Kruse et al., 2010; Seiter et al., 2008; Shoher et al., 2006; Toor et al., 2005). The relatively novel P K-edge XANES technique makes use of X-ray absorption by P atoms of a sample (for an introduction to the fundamental theory see e.g. Newville, 2004). In brief, core shell electrons of these P atoms can be ejected from their quantum level thereby leaving core holes behind if their binding

energies are lower than energies of incident X-rays. If this occurs, energies in excess of the binding energies are released by the emission of photo-electrons and the corresponding P atoms are in a so-called excited state. Next, the excited state may decay by the drop of a higher shell electron into the core hole, which is accompanied by measurable emission of a fluorescence photon or measurable emission of another electron (termed Auger electron) into the continuum. Spectra obtained by these two emissions indicate the fluorescence yield (FY) or total electron yield (TEY) as a function of energy. In summary, the measured fluorescence intensity or electron yield is proportional to the absorption probability and a drop from an L-shell into a deeper K-shell by a prior absorption event causes measurable emissions at the absorption edge of the K-shell (Newville, 2004). This specific absorption edge is termed K-edge and corresponding XANES measurements provide spectra of the energy region near the K-edge. Equivalent analyses of environmental samples at the P L-edge or at extended regions of the K-edge (extended x-ray absorption fine-structure spectroscopy) have currently a limited application range due to their comparatively low sensitivity (Toor et al., 2006).

As the X-ray absorption probability depends on the chemical and physical state of an atom and its surroundings, XANES generally provides the capability to distinguish among crystalline Ca-P species. Moreover, XANES requires minimal sample preparation and is apparently not or only minimally affected by common sample matrices. Unlike most other structural probes it is also capable to provide analysable signals of amorphous compounds (Newville, 2004). However, quantification of matrix-enclosed Ca-P species by XANES is currently restricted because quantitative XANES probes all P atoms within a sample. Hence, different species contribute to the measured spectrum, which is problematic because their spectral features overlap (Doolette and Smernik, 2011). Despite approaches to tackle this issue by least-squares linear combination fitting or principal component analysis with target transformation (Ajiboye et al., 2007b; Beauchemin et al., 2003 and references therein), it has been therefore recommended to use XANES together with sequential extraction methods (Ajiboye et al., 2007a, 2008; Kruse and Leinweber, 2008). Fortunately, inevitable merging of spectra

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by bulk XANES analysis can be overcome by resolving particular species within individual matrix particles using a focused beam at sub-micron spatial resolution (μ XANES; e.g. Brandes et al., 2007). Although μ XANES is not quantitative, it provides a more straightforward approach to ensure that apparent compositions of bulk XANES analyses represent actual compositions.

Applications of these two XANES techniques are at present mainly based on fingerprinting approaches because ab initio fitting of corresponding spectra is currently impaired by the complex theory behind XANES-typical multiple scattering regimes. As P K-edge XANES approaches therefore rely on a comparison between samples and reference materials, the analyte specificity depends on the degree of conformity between species of a sample, reference species and related measurements (e.g. Giguet-Covex et al., 2013). Due to variability among XANES measurements, for instance by differing energy calibrations, self-absorption and particle size effects, P XANES fingerprinting approaches generally collect own spectral libraries of apparently best suited reference species. Because of time-consuming acquisition and synthesis of reliable reference compounds and specifically due to the temporary use of synchrotron facilities, these libraries usually include project-oriented and, consequently, incomplete subsets of spectra. Further, verification of synthesized reference compounds is difficult because these libraries are not systematically validated against each other. Finally, spectra of different Ca-P species show just marginal differences among subtle spectral features and compatibility of two spectra is often deduced from apparent conformities among those subtle features. Due to the lack of a systematic spectral comparison, clear-cut criteria for the identification of Ca-P species are not available to date.

Hence, the study objectives were (i) to establish a spectral library that encompasses a compiled suite of P K-edge XANES spectra of various Ca-P species published in the literature; and (ii) to conduct a systematic comparison of spectra included in this library in order to identify diagnostic spectral features. An insightful comparison was achieved by a specifically developed normalization method that identified subtle but distinctive spectral disparities for the differentiation of calcium phosphate species.

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2 Methods

2.1 Collection of P K-edge XANES spectra

Phosphorus K-edge XANES spectra of Ca-P reference compounds were collected from literature resources that included corresponding spectral libraries (Table 1; 17 libraries). The majority of these libraries ($n = 13$) were drawn from a comprehensive compilation of XANES studies published in a review of spectroscopic approaches for P speciation in environmental matrices (Kizewski et al., 2011) and assumed to be representative of common measurement variations. Libraries of these studies were selected if they comprised more than one species for Ca-P and sufficient energy ranges from white-line peaks to oxygen oscillations. One exception was the study of Beauchemin et al. (2003), whose library did not include oxygen oscillations, yet contained a valuable set of reference compounds. As the developed method generally required spectral coverage of oxygen oscillations (see Sect. 2.2), spectra of Beauchemin et al. (2003) were consequently processed by an additional alignment with other spectra. Potential duplicates were excluded, such as spectra of Lombi et al. (2006). Their study comprised personally supplied spectra of researchers, who published studies already selected (Hesterberg et al., 1999; Peak et al., 2002). Additional libraries of four other studies were included, which were all but one from the period after the review until collection (see underlined reference numbers in Table 1). Plot data were retrieved by optical plot reading using a line-following routine (OPR; CurveSnap V1.0).

The compiled library (Table 1) comprised 76 spectra of reference compounds, which included spectra of hydroxylapatite (HAP), poorly crystalline HAP, amorphous HAP, FAP, CFAP, carbonate hydroxylapatite (CHAP), β -TCP, OCP, DCPD, monetite (DCPA), monocalcium phosphate (MCP), ACP and P on CaCO_3 . Five additional spectra of anapaite, herderite, scholzite, messelite and whiteite (Ingall et al., 2011) were used for a more general comparison but excluded from a detailed analysis due to their unusual and easily distinguishable spectral features. In addition to the above-mentioned reference compounds, the library also included Ca-P of two sediment particles (Brandes

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et al., 2007; not listed in Table 1) and six bone apatites (deer, sheep, chamois, chicken, pig, bovine; Rajendran, 2011; Rajendran et al., 2013; not listed in Table 1). Most original spectra were already normalized by subtracting a linear regression function for the pre-edge region and fitting the post-edge region to a quadratic polynomial function.

5 However, all data were post-processed by a uniform procedure in order to standardize retrieved spectra.

2.2 Post-processing of P K-edge XANES spectra

A specific absorbance and energy normalization processed all spectra for a preliminary spectral comparison and a following determination of peak height ratios and relative peak positions (Fig. 1a). Absorbance was scaled relative to a unit edge jump at 2180 eV and energy normalized by adjusting main (white-line) peaks to 2150 eV and tertiary peaks (oxygen oscillations) to ca. 2167 eV. Energy normalization standardized differing spectra with respect to their white-lines and oxygen oscillations and therefore accounted for species-specific secondary peak positions relative to white-lines and oxygen oscillations. This procedure also allowed for an averaging of multiple spectra of equal species and setting peak baselines for all spectra at equal energies. For a more general comparison of species-specific spectral features (Sect. 3.2; Fig. 2) absorbance was normalized to a step of one at the energy of the maximum first-derivative (threshold energy). The normalized spectra thus obtained were subsequently averaged among equal species.

In order to determine main and secondary peak heights and relative peak positions, uniform baselines were operationally defined across all energy normalized spectra by linear fits between energies of 2147.05, 2156.4, 2158.25 and 2162.2 eV (Fig. 1a) and subsequently subtracted (Fig. 1b). The baseline-subtracted spectra were again scaled relative to maximum main peak intensities of one (or 100 %) and then averaged among equal species. This final normalization was employed to visualize differences among peak intensity ratios (secondary peak heights relative to white-line peak heights) for the reference compounds analysed (Figs. 3 and 4). It is important to note that absorbance

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normalizations did not affect determined peak height ratios. Thus, only the initial energy normalization influenced species-specific peak height ratios due to adjustment of the spectra relative to fixed baseline positions. Additional post-edge background subtraction was omitted due to the limited energy range of most available spectra. Peak height ratios between main and secondary peaks (see A and B in Fig. 1a and b) were expressed as relative secondary peak heights (arbitrary units relative to maximum main peak intensities of one or percentages of main peak heights). All curves were slightly smoothed by a cubic B-spline connection. B-splines, baseline subtractions, peak positions, peak heights and averages of multiple curves were calculated using the data analysis program OriginPro 7.5 (OriginLab Corp., USA). Quality of OPR was verified by performing the above-mentioned analytical procedure for both available primary data and corresponding digitized plot data (see Fig. S1). The correlation between results of these two input data types was highly significant ($r = 0.99$; $p < 0.0001$).

3 Results and discussion

3.1 Spectral library

Among the 76 spectra of the compiled library (Table 1), HAP had the highest number of available spectra ($n = 17$) followed by DCPA ($n = 13$), DCPD ($n = 9$), FAP ($n = 8$), OCP ($n = 8$), carbonated apatites ($n = 6$), β -TCP ($n = 4$), ACP ($n = 4$), MCP ($n = 3$), P on CaCO₃ ($n = 2$), amorphous HAP ($n = 1$) and poorly crystalline HAP ($n = 1$). As most of the different spectra for certain particular species were similar, representative model spectra were obtained by averaging multiple curves. Additionally, five easily distinguishable standard spectra of non-apatitic Ca-P compounds (anapaite, herderite, scholzite, messelite, whiteite; Ingall et al., 2011) are shown for a more general comparison in Fig. 2.

The majority of reference materials were either self-synthesized minerals or certified synthetic minerals of chemical supply companies. Fluorapatites and carbonated

apatites were natural specimens acquired from mineral dealers. For HAP, the library included several spectra of natural and synthetic specimens thereby facilitating a spectral comparison of both sources. Additional Ca-P spectra of sediment particles (Brandes et al., 2007) and bone apatites (Rajendran, 2011; Rajendran et al., 2013) demonstrated the capability of the developed normalization method for determining particular Ca-P minerals in sample matrices (see Sect. 3.4).

Despite certain advantages, such as surface-sensitivity and prevention of self-absorption, collected spectra were rarely obtained by measuring the intensity of emitted electrons (TEY; Table 1). Most spectra were collected in fluorescence mode and most procedures describe measures taken to reduce self-absorption (e.g. using diluted or finely grounded and thinly mounted samples) or to correct data by appropriate equations. One advantage of FY spectra is their occasionally richer structure compared to TEY spectra (see Toor et al., 2006). However, spectra for certain species that were collected by both techniques yielded comparable results (see Sect. 3.5).

3.2 General species-specific spectral features

Despite several remaining technical issues of the relatively novel P speciation by XANES, spectra were in good agreement. Virtually all publications indicated comparable spectral features of mineral Ca-P at the P K-edge, which provided the opportunity to detect the abundance of this P fraction in complex matrices (see e.g. Ca-P content in soil by XANES vs. chemical fractionation; Beauchemin et al., 2003; Kruse et al., 2010; Shober et al., 2006). These spectral features were distinctive and characterized by (i) a post-edge shoulder at the high-energy side of the main (white-line) peak, which is located at about 2150 eV; (ii) an oxygen oscillation centred at about 17 eV above the main peak; and (iii) a secondary peak between the main peak and the oxygen oscillation (Fig. 2a). Averaged spectra of apatite ($n = 30$), β -TCP ($n = 4$), OCP ($n = 7$) and ACP ($n = 2$) exhibited all three features and positions of secondary peaks between main peaks and oxygen oscillations were almost identical. The distance between main peaks and secondary peaks was larger (ca. 10 eV) than the distance between sec-

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ondary peaks and oxygen oscillations (ca. 7 eV). For brushite ($n = 7$), all three features were also present but secondary peaks exhibited a slight shift towards main peaks. The spectra of monetite and MCP were comparable and displayed a lack of the post-edge shoulder (secondary peak and oxygen oscillation omitted in Fig. 2a for visual clarity).

5 A few spectra of the reference library were excluded from averaging in Fig. 2a due to a limited energy range (spectra of Beauchemin et al., 2003) or inconsistency with common species-specific spectra (see next section).

Cryptocrystalline apatites and P adsorbed on CaCO_3 were generally characterized by broadened peaks, particularly in terms of their oxygen oscillations (Fig. 2b). Co-precipitation is frequently an issue for the synthesis of P adsorbed on CaCO_3 . Thus, the line shape of spectra of adsorbed P, which was comparable to those of poorly crystalline apatite or ACP, may be attributable to mineral Ca-P (Peak et al., 2002). Co-precipitation probably also affected the spectrum of amorphous apatite because it showed the brushite-typical secondary peak shift. Spectra of different additional non-apatitic Ca-P minerals (Ingall et al., 2011, Fig. 2c) were generally easily distinguishable from those of apatite, β -TCP, OCP, ACP, brushite and monetite due to unusual features. These features included very broad secondary peaks and/or very broad oxygen oscillations for whiteite, herderite, anapaite and messelite and a comparatively high intensity of the oxygen oscillation for scholzite.

20 Differences among spectra of apatite, β -TCP, OCP and ACP were most difficult to unravel due to very similar centre positions and energy ranges of peaks (Fig. 2). In order to distinguish these spectra, it proved necessary to consider peak intensities. However, these intensities are frequently influenced by self-absorption and generally given as arbitrary units. To solve this problem, peak height ratios between main and secondary peaks were analysed. Corresponding results are described in the following section.

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3.3 Species-specific peak height ratios

Ratios between main and secondary peaks indicated relatively high method specificity for the discrimination among certain species (Fig. 3). For all spectra analysed, the relative secondary peak heights of FAP, HAP, CFAP, CHAP and β -TCP were higher than those of OCP and ACP. Reference materials for DCPD generally showed OCP-typical secondary peak heights but their secondary peak positions were at lower energies, as described above. Two atypical DCPD spectra had secondary peaks at higher energies (dotted curves in DCPD plot; excluded from averaging in Fig. 2). These spectra were, however, still distinguishable from spectra of OCP because they lacked OCP-typical post-edge shoulders. Due to consistently absent post-edge shoulders, the spectra of DCPA and MCP were also different from OCP despite many OCP-typical secondary peaks of DCPA and MCP. Furthermore, spectra of reference materials for P adsorbed on CaCO_3 and for amorphous and poorly crystalline HAP partly exhibited OCP-typical secondary peak heights but they showed other distinctive features, as described above. Therefore, spectra of the eight analysed OCP specimens were unique among the 81 analysed spectra of various Ca-P species, albeit with one probably insignificant restriction: two of the four ACP reference materials showed secondary peak heights that were comparable to those of OCP (dotted curves in ACP plot of Fig. 3; excluded from averaging in Fig. 2). However, Evedorn et al. (2009) noticed by ATR-FTIR and XRPD analyses that the method suggested by Christoffersen et al. (1990) for synthesis of two ACP polymorphs (frequently referred to as ACP1 and ACP2) produced significant amounts of OCP. Yet the method of Christoffersen et al. (1990) was employed to synthesize samples for the two ACP spectra, which were comparable to those of OCP (Table 1, italics). Evedorn et al. (2009) subsequently developed their own procedure and the produced OCP-free sample generated a spectrum, which was comparable to that of an ACP reference material produced by a chemical supply company (HiMed Bioactive Materials Resource Center; Table 1; GÜNGÖR et al., 2007). These two reliable ACP spectra exhibited significantly lower secondary peak heights than all eight OCP

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spectra and were therefore easily distinguishable from OCP. Finally, both of these reliable ACP spectra also showed a less-pronounced shoulder, whereas spectra of the putatively OCP-contaminated ACP exhibited a larger OCP-like shoulder width (Fig. 3; see also discussion below). These results therefore strongly suggest that the reference materials for the two OCP-like ACP spectra were indeed OCP-contaminated and that particular care must be taken to avoid this contamination. Similar relative secondary peak heights of the eight OCP spectra indicated that OCP can be reliably synthesized using the described procedures (Table 1). Furthermore, these similar peak heights resulted in a relatively narrow secondary peak intensity range that did not overlap with that of apatite. All in all, then, OCP, ACP, DCPD and DCPA/MCP are likely distinguishable by P K-edge XANES using the proposed method that implements an analysis of peak height ratios. Moreover, this method can also be applied to differentiate between these thermodynamically less stable Ca-P minerals and apatite or β -TCP.

As secondary peak intensity ranges of different spectra for FAP, HAP, CFAP, CHAP and β -TCP generally overlap, these compounds could not be separately identified by their relative secondary peak heights. However, results of a one-way ANOVA revealed significant differences between those reference compounds in terms of relative secondary peak heights, $F(2, 34) = 6.10$, $p = 0.05$. Post-hoc analyses indicated that FAP or the group of FAP, CFAP and CHAP had significantly higher normalized secondary peaks than HAP or β -TCP. Averaged secondary peak heights were obtained from model spectra, which were received by averaging multiple normalized and baseline-subtracted spectra among each species (Fig. 4). These average heights (% of white line peak height) decreased in the following order: FAP (8.5%) > carbonated apatites (8%) > HAP (7%) > β -TCP (5.9%) > OCP (4%) > DCPD (3.7%) > ACP (1.6%). The averaged post-edge shoulder height (Fig. 4; % of main peak height) decreased in precisely the same sequence, and hence these two parameters were generally correlated (also among sets of equal species; not shown), except for ACP. However, ACP showed a less-pronounced shoulder if compared to the other species shown in Fig. 4.

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Several authors commented that the post-edge shoulder appeared more well-defined with decreasing thermodynamic stability of the investigated Ca-P minerals (Ajiboye et al., 2008; Eveborn et al., 2009; GÜngör et al., 2007; Hesterberg et al., 1999; Sato et al., 2005). This thermodynamic stability decreases as follows: DCPD > DCPA > ACP > TCP > OCP > HAP (Sato et al., 2005). Indeed, all shoulders for averaged spectra of crystalline HAP, FAP, CFAP, CHAP, β -TCP and OCP were relatively well-defined. Apart from the missing post-edge shoulder for DCPA and MCP, ACP (Fig. 4) and most but not all DCPD specimens (Fig. 3) showed subtle shoulders. Therefore, more soluble species actually tend to exhibit less pronounced shoulders although a more consistent trend was mainly impaired by three spectra for DCPD, which contained well-defined shoulders. Besides these spectra, white-line peaks of few DCPA samples also had clearly visible post-edge shoulders. However, these were near the base of white-line peaks and therefore significantly lower than for all other spectra (Fig. 3). As both poorly crystalline apatite (Fig. 2b) and ACP (Fig. 4) exhibited less pronounced shoulders, this spectral feature might be partly affected by the degree of crystallinity. As the shoulder width of HAP and OCP was larger in comparison to DCPD and DCPA, Brandes et al. (2007) hypothesized that the width may depend on the coordination number. However, a comparison of the averaged spectra clearly showed that, on average, all apatite species exhibited both higher secondary peak heights and higher post-edge shoulder positions than potential apatite precursor phases (Fig. 4).

In summary, these results show that the developed normalization method allows for a high degree of differentiation among Ca-P species although this had previously been thought not to be achievable by P K-edge XANES due to striking spectral similarities (see e.g. the problematic differentiation between HAP and OCP mentioned by Eveborn et al., 2009). This degree of differentiation is achieved despite measurement deviations among different spectral libraries. In general, within-study differences between spectral features of two species were even more pronounced than could be assumed from the peak intensity ranges shown in Figs. 3 and 4. For example, each OCP spectrum was collected together with an HAP spectrum (see reference numbers in Fig. 3) and

corresponding spectral discrepancies between these two species of each individual library were consistently large.

3.4 Speciation examples

To exemplify the applicability of this novel method for identification of individual Ca-P species in complex matrices, spectra of bone apatites (Rajendran, 2011; Rajendran et al., 2013) and sediment particles (Brandes et al., 2007) were also examined. Rajendran et al. (2011, 2013) investigated dried and calcined bones by XANES and observed that the spectra changed by heating dried bones to 600 or 800 °C. Spectra of those calcined bone apatites were normalized, baseline-subtracted and averaged according to the reference compounds (Fig. 4, CB, $n = 6$). The spectrum thus obtained was compared with averaged spectra for eight synthetic (Fig. 4, S) and five natural HAP (Fig. 4, N) specimens. This comparison showed that increased temperatures obviously generated relatively pure hydroxylapatites because the secondary peak of the averaged calcined bone apatite spectrum matched exactly that of synthetic HAP. The five natural HAP specimens of the compiled library exhibited higher secondary peaks than synthetic HAP or calcined bones, which could be a consequence of various impurities, such as fluoride substitutions. The HAP sample from Snarum (Norway, Brandes et al., 2007) was the only natural HAP that well matched to the secondary peak of synthetic HAP (cf. synthetic HAP and HAP-1 in Fig. S2). Hence, the selection of natural vs. synthetic HAP reference materials appears to be important for commonly used quantitative XANES approaches, such as least-squares linear combination fitting.

Further, Rajendran et al. (2013) speculated in respect of their spectral analysis that dried bone probably contained HAP and β -TCP. Indeed dried bone apatite (Fig. S2) showed a much lower secondary peak than synthetic HAP, which could theoretically indicate the presence of more soluble precursor phases. For instance, all secondary peaks of dried bone apatite were even smaller than those of OCP. However, the secondary peak height of dried bone was also comparable to that of poorly crystalline HAP. By a closer observation of the entire spectra it may be deduced that dried bone actually

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consisted of poorly crystalline apatite because both of these had relatively broad oxygen oscillations (Fig. 2b). This assumption would be in line with the conclusion of Rey et al. (2009), who inferred from a thorough review of several studies that no substantial evidence has been established for the presence of any Ca-P phase in bone except poorly crystalline apatite. Thus, apatite may form in bone by direct nucleation and not by prior formation of more soluble precursor phases.

Interestingly, this direct nucleation in bone is opposed to the well-documented apatite formation by precursor phases in sediment (Gunnars et al., 2004; Jahnke et al., 1983; Krajewski et al., 1994; Schenau et al., 2000; Van Cappellen and Berner, 1988; see also Morse and Casey (1988) and Nancollas et al. (1989) for the Ostwald step rule of successive crystallization; note also the significant apparent OCP levels in high pH substrates e.g. determined by Beauchemin et al. (2003) using linear combination fitting of XANES spectra). This precursor pathway for the formation of authigenic CFAP, which appears likely, is also substantiated by the second example for the applicability of the proposed method. Post-processing of μ XANES spectra from two sedimentary Ca-P particles (Brandes et al., 2007) revealed that the secondary peak height of one particle was comparable to that of FAP (Fig. 4, Sed1), whereas the secondary peak of the other particle very closely matched the averaged secondary peak of OCP (Fig. 4, Sed2 and Sed2*). That the latter particle most likely comprised OCP was also confirmed by all other important spectral features, such as a missing shift of the secondary peak for exclusion of DCPD (Fig. 4, D), an OCP-typical shoulder height and a broader shoulder feature. Finally, the spectrum exhibited a well-separated oxygen oscillation that rules out P adsorbed on CaCO_3 and poorly crystalline apatite. In view of the two different μ XANES spectra, Brandes et al. (2007) suggested that X-ray fluorescence spectroscopy may offer a good opportunity to approach the subject of the very early formation stages of authigenic CFAP by a novel technique. The formation of CFAP-bearing phosphorite deposits, for instance, still remains controversial after several decades of intense research (e.g. Bentor, 1980; Sheldon, 1981; Tribble et al., 1995). Brandes et al. (2007) further concluded that a suite of appropriate reference

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materials will be required to tackle these issues by XANES. By considering 81 Ca-P reference materials of 17 spectral libraries, this study provides evidence that P K-edge XANES is indeed excellently suited for investigations on apatite formation. This study also provides evidence that authigenic apatite formation proceeds most likely via an OCP precursor pathway.

3.5 Limitations, possible sources of error and recommendations

Based on the relatively large number of naturally occurring Ca-P species numerous identifying features are required to obtain adequate analyte specificity. The presented spectral breakdown considered a representative set of the most abundant Ca-P species in environmental and biological matrices and should therefore be well-suited for studies in different fields. Constraints due to the presence of P species other than those of Ca-P appear to be insignificant because features of Ca-P at the P K-edge were found to be distinctive. For instance, spectra of Ca-P are easily distinguishable from those of potassium phosphates, aluminium phosphates, iron phosphates and organic P compounds (e.g. Brandes et al., 2007; Giguët-Covex et al., 2013; Sato et al., 2005).

Further, self-absorption effects impair the identification of species by XANES. However, using diluted or finely grounded and thinly mounted samples during FY measurements appears to efficiently mitigate these effects because species-specific features were comparable for TEY and FY spectra (see thin and thick grey lines in Fig. 4). Spectral distortions due to self-absorption attenuate especially low-energy regions of the investigated spectral range (Tannazi and Bunker, 2005). However, the use of peak height ratios is obviously advantageous precisely because self-absorption may attenuate not only main but also other peaks. Significant linear correlations between main and secondary peak heights for edge jump normalized spectra of certain species ($p < 0.0001$; Fig. 1c) clearly demonstrated that a consideration of peak height ratios was crucial for distinguishing between spectra of different species. However, this differentiation was more reliable for spectra with intense peaks (Fig. 1c), suggesting that self-absorption

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should be efficiently mitigated. Known effects of inappropriate particle sizes may also influence peak height ratios and should therefore be considered during measurements.

Quantitative estimates by bulk XANES measurements require thorough analysis because spectral features of different species overlap (Beauchemin et al., 2003; Doolette and Smernik, 2011). However, due to the poor resolution of bulk XANES analyses in complex matrices even a qualitative detection may not be feasible, e.g. if the species of interest is a minor component. In this case μ XANES analysis, which also allows for X-ray fluorescence mapping of P and other elements at a sub-micron spatial resolution (Brandes et al., 2007; Diaz et al., 2008), appears to be the instrumental method of choice.

The spectral analysis presented in this study also provides recommendations for standard selection and synthesis. For example, only few spectra were available for Ca-P compounds that exhibited relatively broad oxygen oscillations. These spectra included P adsorbed on CaCO_3 and amorphous or poorly crystalline phases, such as ACP or poorly crystalline apatite. Precisely those reference compounds tended to be more unreliable due to co-precipitation. Therefore, these compounds should be additionally verified by other analytical methods or acquired from sources that provide a corresponding product certification.

4 Conclusions

A normalization method was developed in order to identify diagnostic spectral Ca-P features within a compiled library that included 81 P K-edge XANES spectra of various Ca-P species. The normalization method considered relative energy ranges between peaks and peak height ratios between main and secondary peaks. It was found that relative post-edge shoulder heights and relative secondary peak heights provide essential identifying features in addition to the previously reported criterion of a pronounced post-edge shoulder. These relative heights were crucial for improved analyte specificity, specifically because they provided a set of distinctive features (e.g. distinctive species-

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specific peak intensity ranges). A brief spectral breakdown is given in Table 2 in order to summarize determined diagnostic features. From this follows that P K-edge XANES is excellently suited for environmental and biological research on Ca-P, and that the developed method allows for a high degree of differentiation among Ca-P species.

5 Important applications for the method were demonstrated by two examples: (i) application of the method to bone apatite spectra confirmed that bone material likely comprises only poorly crystalline apatite, which suggests direct nucleation of apatite in bone. This direct nucleation in bone is apparently opposed to the well-documented authigenic apatite formation by precursor phases in sediment. (ii) Application of the
10 method to μ XANES spectra of sediment particles provided evidence for the occurrence of OCP in sediment and therefore for the probability of apatite formation by successive crystallization according to the Ostwald step rule. Finally, the presented method also revealed that reference materials were occasionally inconsistent or incorrectly assigned due to apparent contamination. Hence, this study also offered a basis for standard
15 selection and appropriate standard syntheses.

Supplementary material related to this article is available online at
**[http://www.biogeosciences-discuss.net/10/18723/2013/
bgd-10-18723-2013-supplement.zip](http://www.biogeosciences-discuss.net/10/18723/2013/bgd-10-18723-2013-supplement.zip)**

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Table 1. Compiled library of P K-edge XANES spectra for calcium phosphate standards.

Calcium phosphate	Mode	Type	Reference for synthesis or source; [deposit]	No.	Reference
HAP-1	FY	N	Excalibur Mineral; [Snarum, Norway]	1	Brandes et al. (2007)
HAP-2	FY	S	Fisher Scientific	2	Shober et al. (2006)
HAP-3	TEY	N/A	N/A	3	Rajendran et al. (2013)
HAP-4	FY	S	Fisher Scientific	4	Peak et al. (2002)
HAP-5	FY	N	National Museum of Natural History, France; [N/A]	5	Giguet-Covex et al. (2013)
HAP-6	FY	N/A	N/A	6	Eveborn et al. (2009)
HAP-7	TEY	S	Clarkson Chromatography Products Inc.	7	Güngör et al. (2007)
HAP-8	FY	S	Sigma Aldrich	8	Hesterberg et al. (1999)
HAP-9	FY	S	Sigma Aldrich	9	Sato et al. (2005)
HAP-10	FY	N	Tennessee Valley Authority; [N/A]	9	Sato et al. (2005)
HAP-11	FY	N	Univ. of Greifswald; [Ehrenfriedersdorf, Germany]	10	Kruse and Leinweber (2008)
HAP-12	FY	SDS	Brandes et al. (2007)	11	Diaz et al. (2008)
HAP-13	FY	S	Chemical supply company	13	Ajiboye et al. (2008)
HAP-14	FY	S	Chemical supply company	15	Beauchemin et al. (2003)
HAP-15	FY	N/A	N/A	16	Toor et al. (2006)
HAP-16	FY	S	Sigma Aldrich	17	Kruse et al. (2010)
HAP-17 (chlorian)	FY	N	Mineral dealer; [Bamle, Norway]	12	Ingall et al. (2011)
OCP-1	FY	S	Christoffersen et al. (1990)	2	Shober et al. (2006)
OCP-2	FY	S	Christoffersen et al. (1990)	6	Eveborn et al. (2009)
OCP-3	TEY	S	Clarkson Chromatography Products Inc.	7	Güngör et al. (2007)
OCP-4	FY	S	Christoffersen et al. (1990)	8	Hesterberg et al. (1999)
OCP-5	FY	S	Christoffersen et al. (1990)	9	Sato et al. (2005)
OCP-6	FY	S	Arellano-Jiménez et al. (2009)	5	Giguet-Covex et al. (2013)
OCP-7	FY	S	Christoffersen et al. (1989)	15	Beauchemin et al. (2003)
OCP-8	FY	S	Christoffersen et al. (1990)	17	Kruse et al. (2010)
FAP-1	FY	N	Excalibur Mineral; [Durango, Mexico]	1	Brandes et al. (2007)
FAP-2	FY	N	Mineral dealer; [Beartooth Mountains, USA]	12	Ingall et al. (2011)
FAP-3	FY	N	Mineral dealer; [Conda, USA]	12	Ingall et al. (2011)
FAP-4	FY	N	Mineral dealer; [Conselheiro Pena, Brazil]	12	Ingall et al. (2011)
FAP-5	FY	N	Mineral dealer; [Ipira, Brazil]	12	Ingall et al. (2011)
FAP-6	FY	N	Mineral dealer; [Renfrew County, Canada]	12	Ingall et al. (2011)
FAP-7	FY	N	Mineral dealer; [Durango, Mexico]	12	Ingall et al. (2011)
FAP-8	FY	N/A	N/A	16	Toor et al. (2006)
CHAP fluorian	FY	N	Mineral dealer; [Snarum, Norway]	12	Ingall et al. (2011)
CFAP-1	FY	N	Mineral dealer; [Brewster, FL, USA]	12	Ingall et al. (2011)
CFAP-2	FY	N	Mineral dealer; [St Just, England]	12	Ingall et al. (2011)
CFAP-3	FY	N	Mineral dealer; [Stoke Climstand, England]	12	Ingall et al. (2011)
CFAP-4	FY	SDS	Brandes et al. (2007)	11	Diaz et al. (2008)
CFAP-5	FY	SDS	Brandes et al. (2007)	11	Diaz et al. (2008)

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Table 1. Continued.

Calcium phosphate	Mode	Type	Reference for synthesis or source; [deposit]	No.	Reference
β -TCP-1	FY	S	Kwon et al. (2003)	2	Shober et al. (2006)
β -TCP-2	TEY	N/A	N/A	3	Rajendran et al. (2013)
β -TCP-3	TEY	S	HiMed Bioactive Materials Resource Center	7	Güngör et al. (2007)
β -TCP-4	FY	S	Sigma Aldrich	9	Sato et al. (2005)
ACP-1	FY	S	Own procedure	6	Eveborn et al. (2009)
ACP-2	TEY	S	HiMed Bioactive Materials Resource Center	7	Güngör et al. (2007)
ACP-3	FY	S	<i>Christoffersen et al. (1990)</i>	9	<i>Sato et al. (2005)</i>
ACP-4	FY	S	<i>Christoffersen et al. (1990)</i>	9	<i>Sato et al. (2005)</i>
DCPD-1	FY	N/A	N/A	6	Eveborn et al. (2009)
DCPD-2	TEY	S	Clarkson Chromatography Products Inc.	7	Güngör et al. (2007)
DCPD-3	FY	S	Sigma Aldrich	8	Hesterberg et al. (1999)
DCPD-4	FY	S	Sigma Aldrich	9	Sato et al. (2005)
DCPD-5	FY	S	Sigma Aldrich	10	Kruse and Leinweber (2008)
DCPD-6	FY	S	Fisher Scientific	2	Shober et al. (2006)
DCPD-7	FY	S	Chemical supply company	13	Ajiboye et al. (2008)
DCPD-8	FY	S	Chemical supply company	14	Ajiboye et al. (2007)
DCPD-9	FY	N/A	N/A	16	Toor et al. (2006)
DCCA-1	FY	N	Non-commercial source; [N/A]	4	Peak et al. (2002)
DCCA-2	FY	N/A	N/A	6	Eveborn et al. (2009)
DCCA-3	TEY	S	Clarkson Chromatography Products Inc.	7	Güngör et al. (2007)
DCCA-4	FY	S	Sigma Aldrich	8	Hesterberg et al. (1999)
DCCA-5	FY	S	Sigma Aldrich	9	Sato et al. (2005)
DCCA-6	TEY	N/A	N/A	3	Rajendran et al. (2013)
DCCA-7	FY	S	J.T. Baker	2	Shober et al. (2006)
DCCA-8	FY	S	Chemical supply company	12	Ingall et al. (2011)
DCCA-9	FY	S	Chemical supply company	13	Ajiboye et al. (2008)
DCCA-10	FY	S	Chemical supply company	15	Beauchemin et al. (2003)
DCCA-11	FY	N/A	N/A	16	Toor et al. (2006)
DCCA-12	FY	N/A	N/A	16	Toor et al. (2006)
DCCA-13	TEY	N/A	N/A	16	Toor et al. (2006)
MCP-1	FY	S	Sigma Aldrich	10	Kruse and Leinweber (2008)
MCP-2	TEY	N/A	N/A	3	Rajendran et al. (2013)
MCP-3	FY	S	Sigma Aldrich	2	Shober et al. (2006)
P on CaCO ₃ -1	FY	S	Own procedure	5	Giguet-Covex et al. (2013)
P on CaCO ₃ -2	FY	N/A	N/A	4	Peak et al. (2002)
Amorphous HAP	FY	N/A	N/A	4	Peak et al. (2002)
Poorly cryst. HAP	FY	N	Mineral dealer; [Mono Lake, USA]	12	Ingall et al. (2011)

FY, TEY: Fluorescence yield, total electron yield; S, N, N/A: Synthetic, natural, not available; Italics: Putative OCP-contaminated ACP; Bold reference numbers: studies not included in Kizewski et al. (2011).

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Table 2. Summary of diagnostic P K-edge XANES features for various Ca-P species.

Ca-P species	Secondary peak height (%) ¹	Post-edge shoulder	Post-edge shoulder height	Oxygen oscillation
FAP	8.5 (<i>n</i> = 8)	broad	very high	narrow
CFAP, CHAP	8.0 (<i>n</i> = 5)	broad	very high	narrow
HAP	7.0 (<i>n</i> = 17)	broad	high	narrow
Synthetic HAP	6.2 (<i>n</i> = 8)	broad	high	narrow
Natural HAP	8.5 (<i>n</i> = 5)	broad	high	narrow
Poorly crystalline HAP	4.2 (<i>n</i> = 1)	narrow	low	broad
TCP	5.9 (<i>n</i> = 4)	broad	medium	medium
OCP	4.0 (<i>n</i> = 8)	broad	medium	narrow
DCPD	3.7 (<i>n</i> = 7) ²	mixed	low	medium
DCPA/MCP	2.5 (<i>n</i> = 16) ³	missing	N/A	broad
ACP	1.6 (<i>n</i> = 2)	narrow	medium	broad

¹ Averaged relative secondary peak height (% of white-line peak height).

² Secondary peak shifted towards main peak.

³ Includes four spectra, which lacked a secondary peak.

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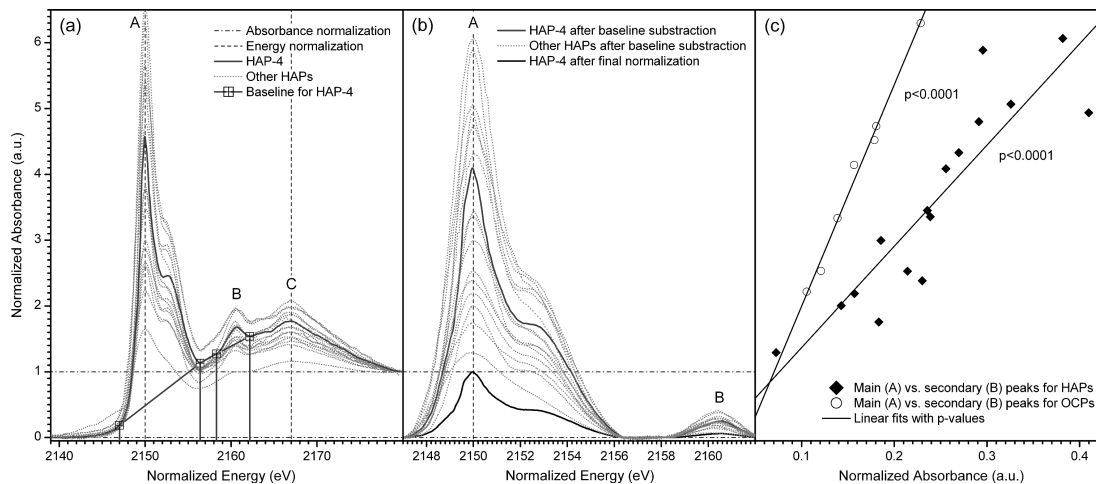


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Fig. 1. Normalization procedure for collected P K-edge XANES spectra as exemplified for HAP-4 and other HAP reference materials. **(a)** Initial absorbance and energy normalization by adjusting absorbance to a unit edge jump at 2180 eV, main peaks (A) to 2150 eV and tertiary peaks (C) to ca. 2167 eV. Baselines for all spectra were operationally defined by linear fits between normalized energies of 2147.05, 2156.4, 2158.25 and 2162.2 eV. **(b)** Spectra after baseline subtraction. These baseline-subtracted spectra were finally scaled relative to maximum main peak intensities of one (see final normalization for HAP-4) to visualize differences among peak intensity ratios (A/B) for reference compounds analysed (Fig. 3). **(c)** As indicated for HAP and OCP reference materials, peak intensities of main (A) and secondary (B) peaks were linearly correlated. Resulting intensity ratios identified species-specific fingerprints (Figs. 3 and 4). See Table 1 for analyte properties.

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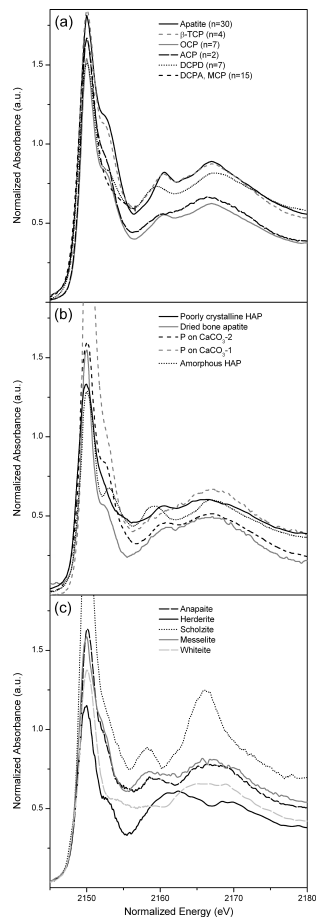


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Fig. 2. First-derivative normalized P K-edge XANES spectra of Ca-P reference compounds. **(a)** Averaged spectra for apatites, β -tricalcium phosphate (β -TCP), octacalcium phosphate (OCP), amorphous calcium phosphate (ACP), brushite (DCPD), monetite (DCPA) and monocalcium phosphate (MCP). **(b)** Spectra for poorly crystalline or amorphous apatite, bone apatite and P adsorbed on CaCO_3 . **(c)** Spectra of different non-apatitic Ca-P minerals (Ingall et al., 2011). Oxygen oscillations in c were not adjusted to ca. 2167 eV due to unusual spectral features.

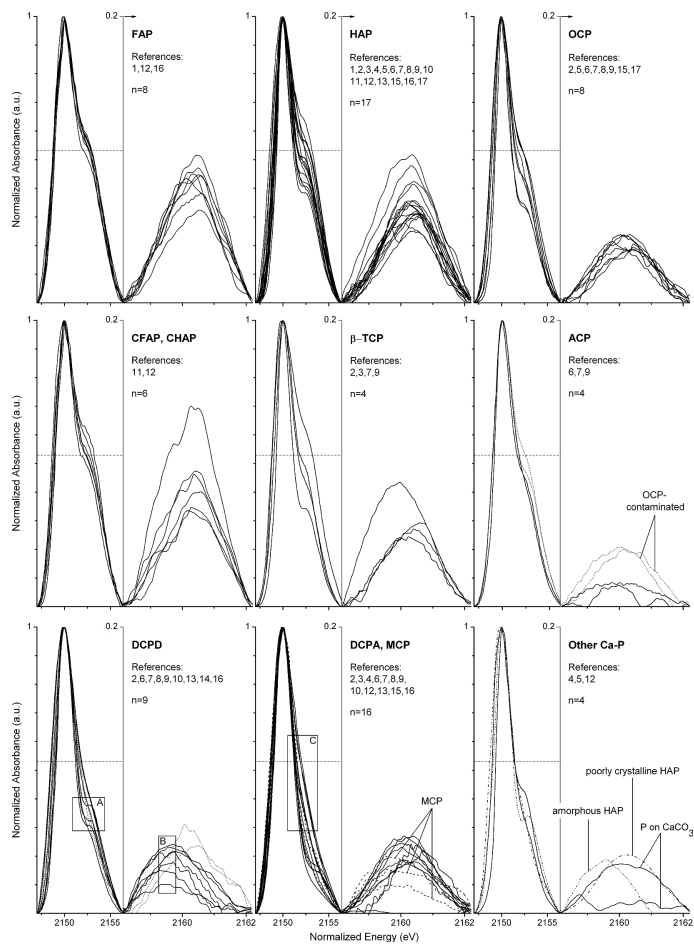


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Fig. 3. Species-specific XANES fingerprints of 74 Ca-P reference compounds identified by normalized secondary peak positions and heights. Secondary peak heights are given as arbitrary units relative to maximum main peak intensities of one. Two ACPs likely contained OCP (dotted curves, text). DCPDs generally showed OCP-like secondary peak heights but their secondary peak positions were at lower energies (B). Two DCPDs with secondary peaks at higher energies (dotted curves) lack OCP-like post-edge shoulders (A). Secondary peaks of DCPAs and MCPs are comparable to those of OCPs, but their main peaks always lack typical post-edge shoulders (C). Reference numbers refer to detailed information in Table 1. Dotted lines indicate the average post-edge shoulder height of apatite ($n = 30$).

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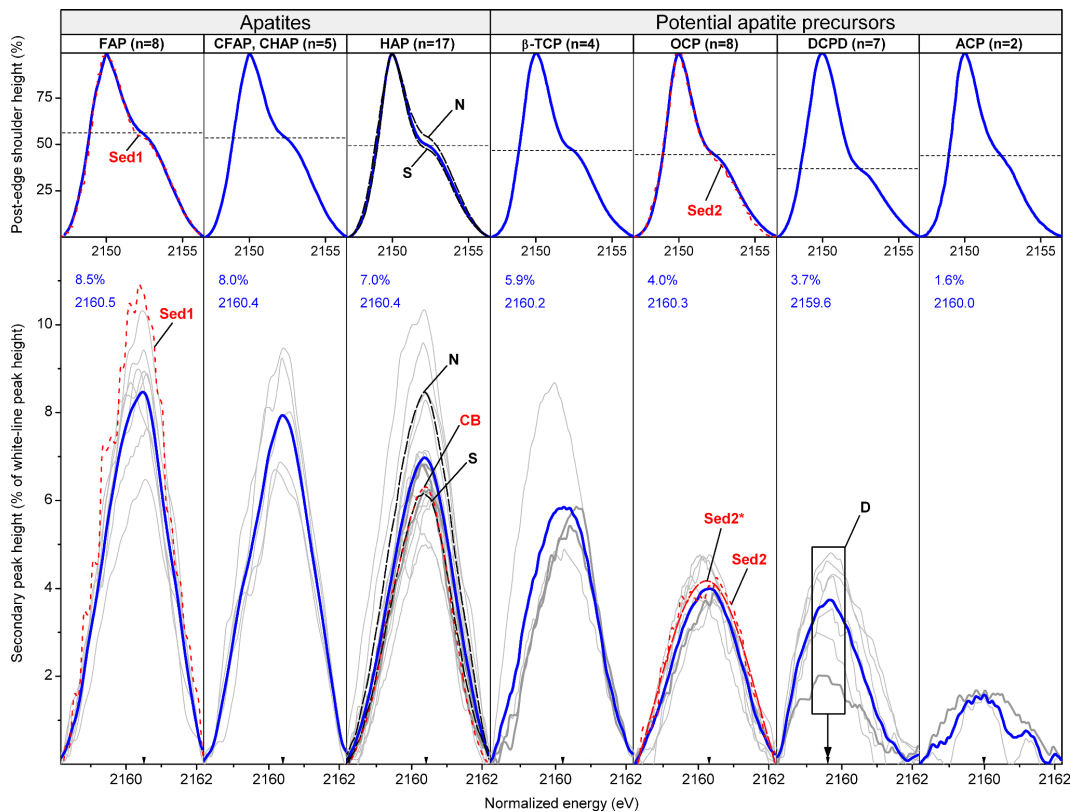


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Fig. 4. Secondary peak heights and post-edge shoulder heights in percentages of white-line peak heights for P K-edge XANES spectra of different Ca-P species. Peak height percentages of μ XANES data of sediment particles (Brandes et al., 2007) indicate apatite (Sed1) and OCP (Sed2). The apparent OCP peak is different from DCPD due to its different peak position as indicated by a Gaussian Fit (Sed2*; cf. position with D). Individual spectra collected in fluorescence yield and total electron yield mode are shown for secondary peaks by thin and thick grey lines, respectively. Thick blue lines indicate averages for multiple standard curves (n), for which average secondary peak height percentages and average secondary peak positions (eV) are indicated. Multiple natural (N; average of 5 spectra) and synthetic (S; average of 8 spectra) HAP specimens contribute to the total average of the secondary HAP peak ($n = 17$; precise source of 4 HAP specimens unknown). Secondary peaks of averaged spectra for 6 calcined bone apatites (CB; not included in the total HAP average) and synthetic HAP (S) almost perfectly match. FAP: Fluorapatite; CFAP: Carbonate fluorapatite; CHAP: Carbonate hydroxylapatite; HAP: Hydroxylapatite; β -TCP: β -tricalcium phosphate; OCP: Octacalcium phosphate; DCPD: Dicalcium phosphate dihydrate or brushite; ACP: Amorphous calcium phosphate.