2015 International Nuclear Atlantic Conference - INAC 2015
São Paulo, SP, Brazil, October 4-9, 2015
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: **978-85-99141-06-9**

DETERMINATION OF Ca/P MOLAR RATIO IN HYDROXYAPATITE (HA) BY X-RAY FLUORESCENCE TECHNIQUE

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ABSTRACT

Hydroxyapatite (HA) is a mineral composed of calcium phosphate employed for endodontics, restorative dentistry and other applications in orthopedics and prosthesis. Additionally, this biomaterial is an inexpensive but efficient adsorbent for the removal of heavy metals and other unwanted species of contaminated liquid effluents. This is especially interesting when low-cost effective remediation is required. A Ca / P molar ratio of 1.667 is consistent with the theoretical Ca / P ratio for calcium hydroxyapatite with a compositional formula of $Ca_{10}(PO_4)_6(OH)_2$, which properties are well discussed in the literature. The aim of this work was implementing and to validating a methodology for simultaneous determination of major and minor constituents in the hydroxyapatite (HA) as well as providing the Ca / P molar ratio. To accomplish these achievements, wavelength dispersive x-ray fluorescence spectroscopy (WDXRF) was applied. This is a non-destructive technique that requires no chemical treatment, enabling fast chemical analysis in a wide variety of samples, with no hazardous waste being generated as a result of the process of determination. A standard reference material from NIST (SRM 1400 – Bone Ash) was used to validate the methodology for the determination of magnesium, phosphorus, potassium, calcium, iron, zinc, strontium and the Ca / P ratio in HA samples by WDXRF. The Z-score test was applied as a statistical tool and showed that the calculated values were of less than 1.8 for all the measured analytes.

1. INTRODUCTION

Hydroxyapatite (HA) is a naturally occurring mineral that can be chemically synthesized via several processes. The quality of synthesized material depends on the parameters of the chosen process and the purity of the reagents used. HA has an excellent biocompatibility and presents a similar chemical composition to human bone mineral phase. These characteristics allow it to be used as a biomaterial for the replacement and regeneration of bone tissue [1].

Because of its application as regenerative material and bone replacement, hydroxyapatite has been a key material for decades in the fields of biomaterials science and biomechanical engineering. The slow ratio to which hydroxyapatite is reabsorbed and biodegraded in the body motivated the use of other calcium phosphates with similar properties [2].

Some of the calcium phosphates used as biomaterials are shown in Table 1, with their respective chemical formulas and Ca / P molar ratios. The Ca / P molar ratio is a parameter that allows evaluating the solubility of calcium phosphates compounds. In general, the bigger the Ca / P molar ratio, the lower the solubility [2, 3].

Table 1: Calcium phosphates, chemical formulas and Ca / P molar ratios.

Calcium phosphate	**Chemical formula**	Ca/P
Tetracalcium phosphate (TTCP)	$Ca_4O(PO_4)_2$	2.0
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Amorphous calcium phosphate (ACP)	$Ca_3(PO_4)_2.nH_2O$	1.5
Tricalcium phosphate (α, α', β, γ) (TCP)	$Ca_3(PO4)_2$	1.5
Octacalcium phosphate (OCP)	$Ca_8H_2(PO_4)_6.5H_2O$	1.33
Calcium monohydrogen phosphate (DCPD)	$CaHPO_4.2H_2O$	1.0
Dicalcium phosphate (DCP)	$CaHPO_4$	1.0
Calcium Pyrophosphate (CPP)	$Ca_2P_2O_7$	1.0
Calcium pyrophosphate dihydrate (CPPD)	$Ca_2P_2O_7.2H_2O$	1.0
Heptacalcium phosphate (HCP)	$Ca_7(P_5O_{16})_2$	0.70
Tetracalcium dihydrogen phosphate (TDHP)	$Ca_4H_2P_6O_2$	0.67
Monocalcium phosphate monohydrate (MCPM)	$Ca(H_2PO_4)2.H_20$	0.50
Calcium metaphosphate (α, β, γ) (CMP)	$Ca(PO_3)_2$	0.50

X-ray Diffraction (XRD) is a widely used analytical technique for the identification of calcium phosphates and Ca / P molar ratios. Complementary analytical techniques such as, Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), Neutron Activation Analysis (NAA) and others, are also used to quantify toxic chemical elements in calcium phosphates biomaterials. These contaminants may be incorporated during the manufacturing processes and their quantification is required to ensure the quality of the final product [4, 5, 6].

X-ray Fluorescence Spectrometry (XRF) is a non-destructive technique that allows, not only to screen the elements present in a sample (qualitative analysis), as well as to establish the proportion (concentration) at which each element is present in the sample. In XRF, a source of high energy radiation (gamma or X rays) excites the atoms present in the substance of interest. When an atom in the ground state is exposed to an external power source (eg: an X-ray), it absorbs energy, promoting the electrons to higher energy levels. In these states, the atom is at an unstable situation, called "excited state". In nature, all matter tends to seek its most stable configuration, thus, excited atoms naturally tend to return to its ground state by emitting a photon with a certain energy. This energy is characteristic of each chemical element, allowing their identification and quantification [7].

In this context, the aim of this work is to provide an analytical method using Wavelength Dispersive X-ray Fluorescence (WDXRF) for simultaneous determination of major and minor constituents in hydroxyapatite and calcium phosphates applied as biomaterials, as well as the Ca / P molar ratio.

The advantages of the proposed methodology compared to those that employ other analytical techniques are: direct and non-destructive analyzes; preservation of the sample for future analysis; absence of time-consuming chemical procedures in sample preparation, resulting in no waste generation; fast analyses, taking approximately one hour to identify and quantify a wide range of elements, from F to U, in a wide range of concentrations (from μg g^{-1} to %); precision and accuracy comparable to the mentioned techniques.

2. EXPERIMENTAL

2.1. Sample preparation

Dried samples in powder form, with particle size of 74-105μm, were pressed with a spatula on an aluminum cylinder of 25mm diameter and 0.5mm height, supporting approximately 1.5g sample. Subsequently, holder/sample set was covered with a polypropylene film (thin film for XRF - SPEX®) 5 μm thick and subjected to spectrometer under predetermined instrumental conditions.

2.2. Instrumental parameters

The experiments were carried out using a WDXRF spectrometer RIGAKU Co, model RIX 3000, with a Rh anode X-ray tube, a 75 μm Be window and a 60 kV maximum acceleration voltage, a scintillation detector NaI(Tl) and a flow-proportional counter. The Fundamental Parameters (FP) method was applied for correction of the absorption/excitation effects. Parameters such as excitation, emission line, divergence slit, diffracting crystal, detector, scan counting time, and Bragg's positions are shown in Table 2

Table 2: Measurement conditions for WDXRF - Excitation: 50 kV x 50 mA

Elements	Emission line	Divergence slit (μm)	Diffracting crystal	Detector	Step (°) / Time (s)	Bragg's positions (°)
Na	Na-Kα	560	TAP	FPC	0.05 / 1.0	52.000-58.000
Mg	Mg–Kα	560	TAP	FPC	0.05 / 1.0	42.000-48.000
Al	Al-Kα	560	PET	FPC	0.05 / 1.0	140.000-147.000
Si	Si-Kα	560	PET	FPC	0.05 /0.4	106.000-112.000
P	P-Kα	560	Ge	FPC	0.05/0.4	138.000-144.000
S	S-Kα	560	Ge	FPC	0.05/0.4	108.000-114.000
Cl	Cl-Kα	160	Ge	FPC	0.05/1.0	90.000-96.000
K	K-Kα	560	Ge	FPC	0.05/0.4	60.000-64.000
Ti-U	Kα	560	LiF(200)	SC	0.02/0.2	5.000-90.000

TAP thallium acid phtalate, PET pentaerythritol, LiF lithium fluoride, Ge germanium, SC scintillation detector, NaI(Tl): FPC flow-proportional counter

2.3. Fundamental Parameters Method

The X-ray fluorescence technique employs many methods for quantitative chemical analysis such as Linear Regression method, Internal Standard, Compton method, and Fundamental Parameters Method (FP).

The Fundamental Parameters (FP) approach to calibration in X-ray fluorescence is based on mathematical algorithms to correct effects of coexisting elements (absorption and enhancement of X-rays and overlap of peaks) through the measure of the intensity of each element's emission line and the tabulated values of the main fundamental parameters, such as the primary spectral distribution (source), the photoelectric and the mass absorption coefficients, fluorescence yields and others.

The calculations involve two underlying steps: calibration and prediction. In the calibration step, the FP equation is employed to predict the characteristic intensities of the calibration standards' emission lines. The calculations are performed specifically for the instrument through which the measurements are obtained, since the PF's equation considers geometry aspects, source and instrumental conditions. The calibration takes into consideration the theoretically calculated intensities and the measured intensities, which are corrected for each characteristic line. This correction is acquired through the angular coefficient, experimental net intensities versus the calculated relative intensities, which correspond to the proportional factor used in the correction.

In the predicting step, it is necessary to estimate the approximate composition of the sample first. Typically, the relative intensity of the detected emission lines, corresponding to each element in the matrix, is considered, assuming that the total emission pertains to a composition of 100% (or another total value, if the minor constituents are ignored) [8, 9, 10]. From this equation, the intensities that should be observed for the assumed composition are software-calculated. They are compared to the measured values, then the assumed composition is adjusted and a new set of expected intensities is calculated. This interaction process is automatically repeated until the assumed composition provides an X-ray intensity that corresponds to the measured value at a significance level of 0.05. The composition that successfully meets this relationship becomes the result of the analysis [10, 11].

2.4. Methodology evaluation

The methodology was validated in terms of precision and accuracy through statistical tests suggested by INMETRO [12]. The data were obtained for 3 replicates of each sample and 7 measurements for each replicate, adding up to a set of 21 measurements for each element of NIST's 1400 standard reference material (SRM), *Bone Ash*.

The Chauvenet test was applied for the detection of the outliers, Eq. 1 [13]

$$|Xi - \bar{X}| > Kn * s \quad (1)$$

Where Xi is a measured value, $\bar{X}$ is the average value, Kn is the Chauvenet's coefficient and s is the standard deviation.

The limit of quantification (LoQ) for the certified elements was calculated for a 95% confidence level, according to Eq. 2 [14].

$$LoQ = 2 * \sqrt{\frac{\sum_{m=1}^{n}(C_m - \bar{C})^2}{n-1}} \quad (2)$$

Where, C_m is the measured value, $\bar{C}$ is the average value and n is the number of replicates.

The precision was evaluated through the relative standard deviation (RSD) and the acceptability was verified using the equation of Horwitz, in which the HORRAT's (HO_R) values fit more accordingly Eq. 3 [12].

$$HO_R = \frac{DPR\%_{experimental}}{DPR\%_{previsto\ da\ equação\ de\ Horwitz}} \quad (3)$$

The accuracy was evaluated through the Z-score test, according to Eq. 4 [12]

$$Z = \frac{(\bar{X}_{Lab} - \bar{X}_{CRM})}{\sqrt{U_{Lab}^2 + U_{CRM}^2}} \quad (4)$$

Where, Z is the test value, $\bar{X}_{Lab}$ is the experimental average, $\bar{X}_{CRM}$ is the CRM's true value, U_{Lab}^2 is the experimental variance and U_{CRM}^2 is the variance obtained for the CRM.

3. RESULTS AND DISCUSSION

In Table 3, certified and determined values, RSD %, HO_R, RE %, LoQ, and Z-score for SRM 1400 from NIST are presented.

Table 3: Certified and determined values, RSD %, HO_R, RE %, LoQ, and Z-score for SRM 1400

Elements	Xcert ± σ	Xdeter ± σ	RSD (%)	HO_R,	RE (%)	Z	LoQ
Ca (%)	38.18±0.13	38.4±0.6	1.6	0.8	-0.6	-0.4	1.12
P (%)	17.91±0.19	17.8±0.3	1.7	0.8	0.6	0.3	0.67
Mg (%)	0.684±0.013	0.634±0.025	3.9	1.4	7.3	1.8	0.04
*Na (%)	0.6	0.64±0.05	7.8	2.0	*	*	0.02
*Si (%)	0.13	0.125±0.004	3.2	1.1	*	*	0.02
*Al (μg g^{-1})	530	761±57	7.5	1.9	*	*	11
K (μg g^{-1})	186±8	195±15	7.7	1.9	-4.8	-0.5	3
Fe (μg g^{-1})	660±27	659±39	5.9	1.5	0.2	0.02	8
Zn (μg g^{-1})	181±3	165±12	7.3	1.8	8.8	1.3	3
Sr (μg g^{-1})	249±7	248±19	7.7	1.9	0.4	0.05	2

* ≡ Noncertified Values

The precision, estimated by relative standard deviation (RSD), ranged between 1.6 and 7.8%. The acceptability calculated by the HORRAT's test (HO_R) showed that all the values are ≤ 2, indicating that the precision is satisfactory [12].

The accuracy, evaluated by the relative error (RE), ranged from 0.2 to 8.8%. The acceptability, calculated by the Z-score test (Z), showed ≤ 1.8 values. Thus, accuracy can also be considered satisfactory [12].

The LoQ (limit of quantification) for the major elements showed the following values: Ca: 1.12; P: 0.67; Mg: 0.04; Na: 0.02; Si: 0.02 %; and for the trace elements: Al: 11; K: 3; Fe: 8; Zn: 3; and Sr: 2 $\mu g\ g^{-1}$.

The certified value for the Ca / P molar ratio is 1.65 ± 0.01, whereas the measured result was 1.67 ± 0.02. The comparison of results using Z test revealed no statistically significant difference. Thus, the chosen methodology can be considered effective for Ca / P molar ratio determination in calcium phosphates. Additionally, it is also possible to predict the chemical formula, according to Tab. 1, which, for the obtained value, corresponds to hydroxyapatite: $Ca_{10}(PO_4)6(OH)_2$.

4. CONCLUSIONS

The results showed that the proposed methodology is suitable for the determination of major and minor constituents in calcium phosphates with a satisfactory accuracy and precision. It al so proved to be effective to determine the Ca / P molar ratio, which allows estimating the mol ecular formula of the compound of interest.

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