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Assessment of acidic bone demineralization process via optical properties

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ABSTRACT

In the present investigation, a variety of strong and weak acids have been chosen for the bone decalcification process. Demineralization of dog cranial bone was carried out using 0.6 M concentration of either hydrochloric, lactic or citric acid. Optical characterization such as: reflectance spectra, tristimulus values and color parameters were employed and related to the degrees of demineralization. The CIE Lab method was used to determine the L (luminosity), 'a' (red–green) and 'b' (yellow–blue) color values of the samples. The study has been extended to include the absorption and extension coefficients of the prepared samples. The variation in the optical parameters proved that the spectral behavior depended on the nature of the bone matrices which resulted from the phase composition of bone. The results indicate that the variation in the optical band gap was derived from Tauc's extrapolation for the demineralizing acids. The data obtained are attributed to the differences of the demineralizing acids and their consequent actions on the products.

KEYWORDS

Demineralization;
Organic/inorganic acids;
Color parameters;
Absorption coefficient;
Extension coefficient.

INTRODUCTION

Research works are concerned with the development of demineralized bone in various formulations and uses. As no studies have reported which acid is more suitable for obtaining tissue engineering (TE) bone constructs, the current study explored the feasibility of several acids to obtain TE bone constructs and follow consequent structural/property relations as a function of demineralization process.

Demineralized bone matrix (DBM) generates an osteoconductive surface and is also a source of osteoinductive factors that prompt bone regeneration. DBM is an approved medical device used in bone defects with a long track record of clinical use in diverse forms[1]. True to its name and as an acid-extracted organic matrix from bone sources, DBM retains much of the proteinaceous components native to bone, with small amounts of calcium-based solids, inorganic phosphates and some trace cell debris. Many of DBM's proteinaceous components [e.g., growth factors (GF)] are known to be potent osteogenic agents[2]. The understanding of the endogenous bone regeneration cascade has inspired the exploration of a wide variety of growth

factors (GFs) in an effort to mimic the natural signaling that controls bone healing. Biomaterial-based delivery of single exogenous GFs has shown therapeutic efficacy, and this likely relates to its ability to recruit and promote replication of cells involved in tissue development and the healing process[3]. The efficacy of a demineralized bone matrix as a bone-graft substitute or extender could be related to the total amount of bone morphogenetic protein (BMP) present. BMPs belong to the transforming growth factor (TGF) super family of proteins[2].

DBM provides a degradable matrix facilitating endogenous release of these compounds to the bone wound sites where it is surgically placed to fill bone defects, inducing new bone formation and accelerating healing. Given DBM's long clinical track record and commercial accessibility in standard forms and sources, opportunities to further develop and validate DBM as a versatile bone biomaterial in orthopedic repair and regenerative medicine contexts are attractive[4].

In 2009 Zhao et al.[5] used demineralized bone matrix (DBM) for tissue reconstruction because its collagen presenting good cell compatibility. Bone replacement biomaterials such as demineralized freeze-dried bone allografts and xenografts have been used recently for sinus augmentation with promising clinical results.

Several mineral and organic acids as well as chemicals were used for the demineralization processes. Among them are HCl, lactic and citric acids[6].

The optical and mechanical properties of cranial bones were reported to depend on the demineralization degree[7]. The amount of hydroxyapatite in the samples falls on an average by 33.9, 47 and 58.6% after 3, 6 and 12 h, respectively. The higher the degree of demineralization of tested bone samples, the more vivid their properties enhancing proliferative potential of culture cells. The obtained results showed that inductive properties of allografts depend on the degree of their demineralization.

Optical measurements of materials vary between appearances to single transmission wavelength. Correlation of translucency evident to human eye, which varies along the visible spectrum, is very seldom. Methods of studying translucency are[8]: (1) direct transmission, (2) total transmission and (3) spectral reflectance.

In the present work, demineralized bovine cranial bone (dog) in a variety of preparations was achieved. Three acids were used, one is the mineral acid, HCl and the other two are organic acids being lactic and citric. Optical analyses were employed to characterize and to reveal the relationship of the degrees of demineralization. Also, the study has been extended to include the changes in the color parameters, absorption and extinction coefficients, and the band tail width and band gap energies for the demineralized bones.

MATERIALS AND METHODS

Demineralized matrices

Cranial bovine segments were obtained from sacrificed healthy animals. Muscles, periosteum and adherent soft tissues were removed from the resected specimens using scalpel (without any chemicals). Bones were scrubbed with a stiff brush and bone marrow was discarded[9]. Segments were washed with copious amounts of distilled water for 2 hours, and then dehydrated using absolute ethyl alcohol followed by ether in order to remove lipids, cellular and potentially antigenetic materials. The dehydration process was performed at room temperature to avoid denaturation of bone morphogenetic proteins (BMP). Segments were decalcified by immersion using magnetic stirrer at room temperature for a period of 24 hours and the process of decalcification went out during this period[10]. Three different decalcifying solutions used separately were 0.6 M of each of HCl, lactic or citric acids[11,12]. Treated bone became decalcified with a pliable leathery soft feeling. After completion of the demineralization process, matrices were rewashed with copious amount of distilled water while being agitated by a magnetic stirrer then dehydrated using absolute ethanol.

Optical analyses

The measurements in the visible region from 400 to 700 nm for the prepared demineralized bones were carried out using a UV-3101 PC (UV-VIS-NIR Scanning Spectrophotometer, Schimadzu, Japan), double beam spectrophotometer with standard illuminant C (1174.83) Model V-530 and band width 2.0 nm covers the range 200-2500 nm with accuracy ±0.05%.

Full Paper

The color properties were analyzed using the CIE Colorimetric System, CIE 1931 2-degree Standard Observer[9,10]. The tristimulus reflectance values (x_r, y_r and z_r), the relative brightness (L), the color constants 'a' and 'b', the whiteness index (W), and the color difference (ΔE) were calculated using the CIE relations previously reported[13-15].

The absorption coefficient (α) of the present materials strongly depends on optical transmission, reflection and thickness of film which is evaluated using the relation[16,17]:

$$\alpha = (1/d) - \ln (1-R)^2/T \quad (1)$$

where R is the reflectance, T is the transmittance ($H \approx 10^{-4}$) and d is the thickness of the sample (= 0.01 cm). The optical energy gap (E_g) of the thin films has been determined from absorption coefficient data as a function of photon energy (hυ in eV). According to the generally accepted model proposed by Tauc for higher values of absorption coefficient where the absorption is associated with interband transitions, it yields the power part which obeys the Tauc[18] and Mott and Davis[19] relations as:

$$\alpha h \upsilon = B (h\upsilon - E_g)^n \quad (2)$$

where B is the slope of the Tauc edge called the band tail parameter and n is the type of electronic transition responsible for absorption, being 0.5 for direct transition and 2 for indirect one. In the low absorption region,the absorption coefficient (α) shows an exponential dependence on photon energy (hν) and obeys the Urbach relation[16]:

$$\alpha = \alpha_0 \exp (h\nu / E_b) \quad (3)$$

where α_0 is a constant and E_b is the Urbach energy, interpreted as the width of the tails of localized states in the band gap. The absorption edge (E_e), the band tail (E_b), the direct energy gap (E_d) and the indirect energy gap (E_{ind}) were also calculated from the graphs of: α versus hν, –!n α versus hν, $(\alpha h\nu)^2$ versus hν and $(\alpha h\nu)^{1/2}$ versus hν, respectively.

The extinction coefficient (K) is an important parameter characterizing photonic materials. Value of K can be calculated from transmission and reflection spectra using the relation[17]:

$$K = \alpha\lambda/4\pi \quad (4)$$

where λ is the wavelength in cm and α is the absorption coefficient in cm^{-1}.

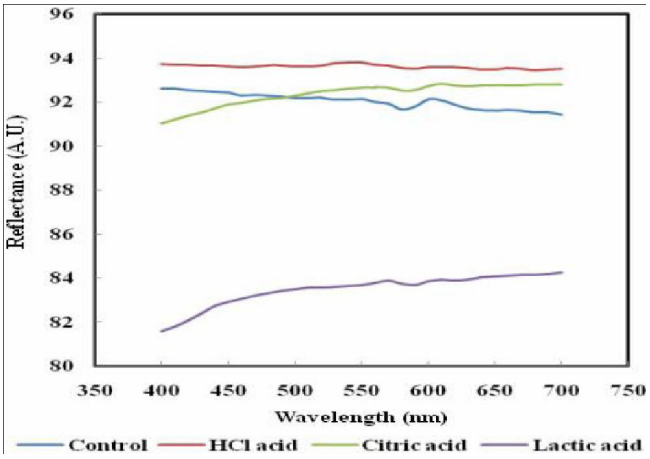

Figure 1 : The change in reflection percentage (R%) spectra of bone/matrices pre- and post-demineralization using different acids

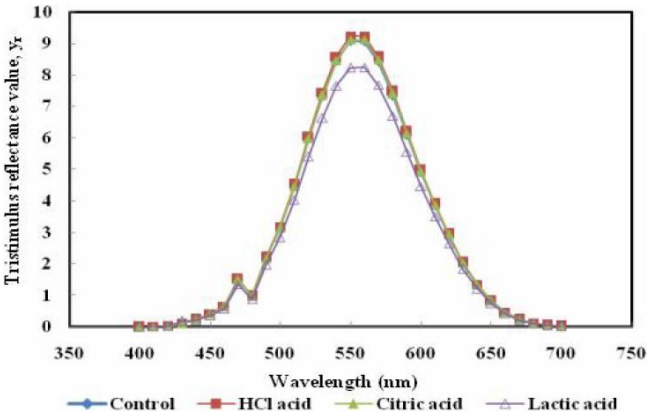

Figure 2 : Variation of the tristimulus reflectance value (y_r) with wavelength of bone/matrices pre- and post-demineralization using different acids

RESULTS AND DISCUSSION

Reflection measurements

The dependence of the reflectance (R%) of control and demineralized matrices in the wavelength range from 400-700 nm is illustrated in Figure 1. From the values of R for the different matrices, the tristimulus reflectance value or the relative brightness (y_r) are calculated and plotted as a function of wavelength (Figure 2). Values of y_r revealed that the lactic/matrix exhibits lowest relative brightness. On the other hand, values of y_r for HCl and citric/matrices almost coincide with that of control/matrix. The samples have similar behavior as negligible change in the peak position (about 555 nm) could be detected (Figure 2).

TABLE 1 : The x_r, y_r and z_r tristimulus reflectance values of bone/matrices pre- and post-demineralization using different acids

Demineralizing agents	x_r		y_r	z_r
	λ = 445 nm	λ = 590 nm	λ = 555 nm	λ = 460 nm
Control	3.6768	8.2482	9.0593	19.0778
HCl acid	3.7238	8.4018	9.2224	19.3275
Citric acid	3.5975	8.3156	9.1114	18.9643
Lactic acid	3.2890	7.5196	8.2458	17.1151

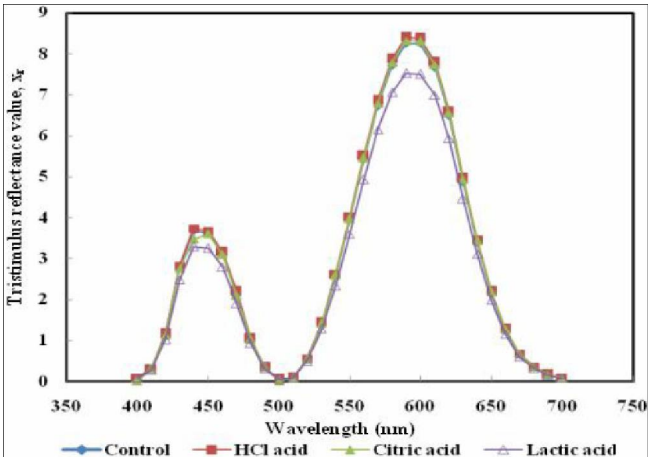

Figure 3 : Variation of the tristimulus reflectance value (x_r) with wavelength of bone/matrices pre- and post-demineralization using different acids

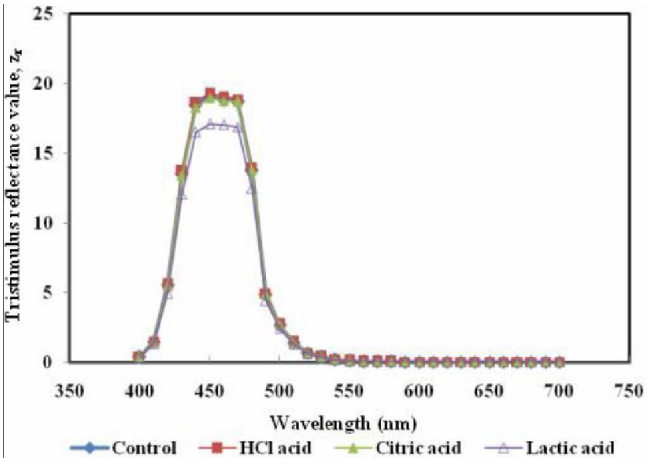

Figure 4 : Variation of the tristimulus reflectance value (z_r) with wavelength of bone/matrices pre- and post-demineralization using different acids

Figure 3 and Figure 4 show the variations of the tristimulus reflectance values x_r and z_r, respectively, with wavelength in the range 400-700 nm for control and demineralized matrices. It is clear from the figures that the behaviors for each x_r and z_r for all the matrices are similar and also show no change in their peak positions. TABLE 1 illustrates the values of x_r, y_r and z_r at the peak positions for control and demineralized matrices. It is noticed from the table that the lactic/matrix has the lowest tristimulus reflectance values x_r, y_r and z_r.

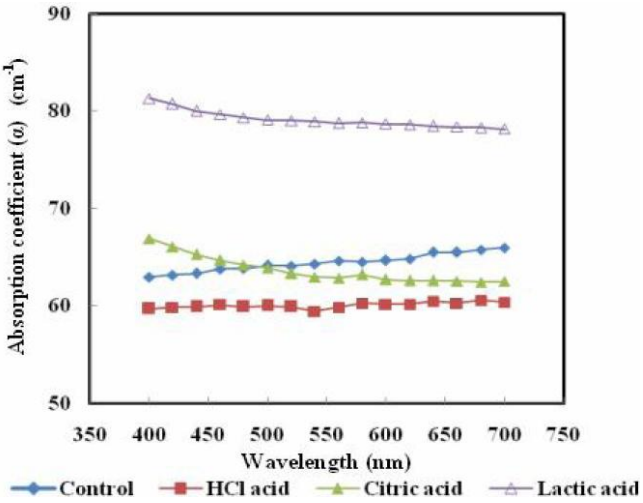

Figure 5 : The absorption coefficient (α) of bone/matrices pre- and post-demineralization using different acids.as a function of wavelength

TABLE 2 : The variations in color parameters and their % changes of bone/matrices pre- and post-demineralization using different acids

Color parameters	Demineralizing agents			
	Control	HCl acid	Citric acid	Lactic acid
L	5.6216	5.6261	5.7593	5.6361
ΔL%	-	0.08	2.45	0.26
a	-0.0086	-0.0122	-0.0318	-0.0156
Δa%	-	-40.16	-466.23	-79.44
b	-0.0125	0.0031	0.4898	0.0395
Δb%	-	124.34	4007.59	415.44
W	-2.5242	-2.5332	-2.8147	-2.5539
ΔW%	-	-0.36	-11.51	-1.18
Ye	-0.5085	-0.0578	14.7908	1.0551
ΔYe%	-	-88.64	3008.49	307.47
ΔE	-	1.3067	40.3462	4.2297
ΔC	-	-0.0027	0.4755	0.0273
ΔH	-	1.3067	40.3434	4.2296

The observed changes in the color parameters

(L, a, b, W, Ye, ΔE, ΔC and ΔH) calculated from the reflectance curves (Figure 1) of bone/matrices pre- and post-demineralization with different acids under investigation are tabulated in TABLE 2. As it is well known, L parameter measures the brightness of the sample and varies from 100 for perfect white to zero for black. It is that the control/matrix is darker in color than the other demineralized matrices and citric acid/matrix acquires the brightest color. The color constant 'a' varies from green for negative value and red for positive one. It is noticed from TABLE 2 that 'a' values decrease for all matrices and the lower value of 'a' is that for citric acid/matrix which indicates that it has more green coloration. In considering the color constant 'b', it was found that a considerable increase in 'b' values for all demineralized matrices indicating the tendency towards yellow color instead of the blue one. By following the values of whiteness (W), it is noticed that W shows nearly the same behavior as the color constant 'a'. While by following the values of yellowness (Ye), it is observed that Ye shows nearly the same behavior as the color constant 'b'. Moreover, the results indicate that variations in color differences between samples occurred by the presence of different dimeneralization agents with the control/matrix.

From the obtained results, it can be concluded that, the change in the tristimulus reflectance values (x_r, y_r and z_r) shows the trend of HCl > citric > lactic/matrices which is in good agreement with the same order of the effect of acids on the demineralization process. Moreover, the percentage changes in color parameters proved that the demineralizing acids generate disorder in the system as well as changes in the molecular configuration which lead to the formation of color centers. Such distorted microstructure is responsible for the dielectric differentiation of the treated matrices.

Optical absorption measurements

The total absorption spectral response (α) for the demineralized cranial bone (dog) under investigation is calculated in the visible wavelength range from 400 to 700 nm and in the photon energy (hν) range 3.10-1.77 eV. Figure 5 shows the relation between the absorption coefficients (α) as a function of wavelength in the visible range (400-700 nm) for bone/matrices pre- and post-demineralization using different acids. It is clear from the figure that the absorption coefficient (α) values of lactic/matrix sample revealed the highest values in comparison with the other matrices. Also, the values of absorption coefficient for citric and lactic matrices decrease with wavelength and behave in opposite direction as shown by the control/matrix. On other hand, the behavior of absorption coefficient of HCl/matrix is nearly independent on wavelength. The obtained variations in the absorption coefficient may be due to the change in the chemical bonds which in turn lead to the formation of new color centers, i.e., preferential light absorption at particular wavelength.

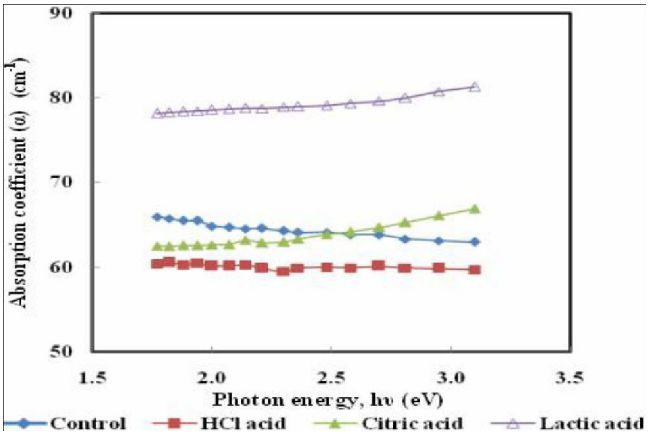

Figure 6 : The absorption coefficient (α) of bone/matrices pre- and post-demineralization using different acids as a function of photon energy (hυ)

Figure 6 illustrates the variation in the absorption coefficient (α) with photon energy (hν) for bone/matrices pre- and post-demineralization using different acids. It is clear that for control/matrix the absorption coefficient (α) values decreases with increasing photon energy. The trend of absorption coefficient for citric and lactic matrices shows opposite behavior and the values of α increase with increasing photon energy.

TABLE 3 : Values of band tail energy (E_b) and direct energy gap (E_d) of bone/matrices pre- and post-demineralization using different acids

Demineralizing agents	E_b (eV)	E_d (eV)
Control	-0.028	1.42
HCl acid	-0.005	1.27
Citric acid	0.071	1.77
Lactic acid	0.035	1.74

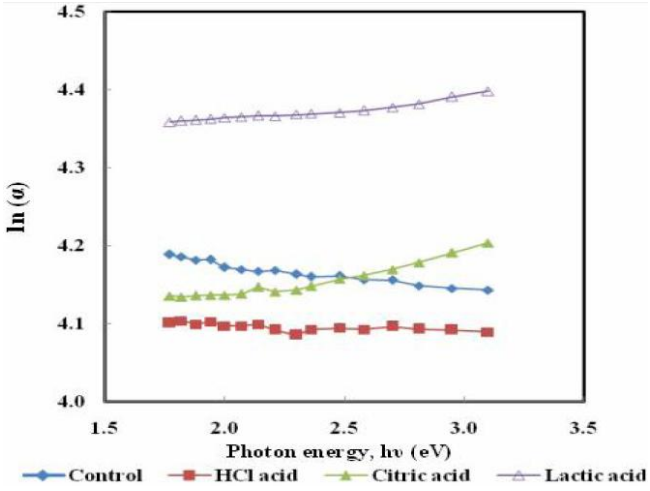

Figure 7 : Urbach law plots for bone/matrices pre- and post-demineralization using different acids

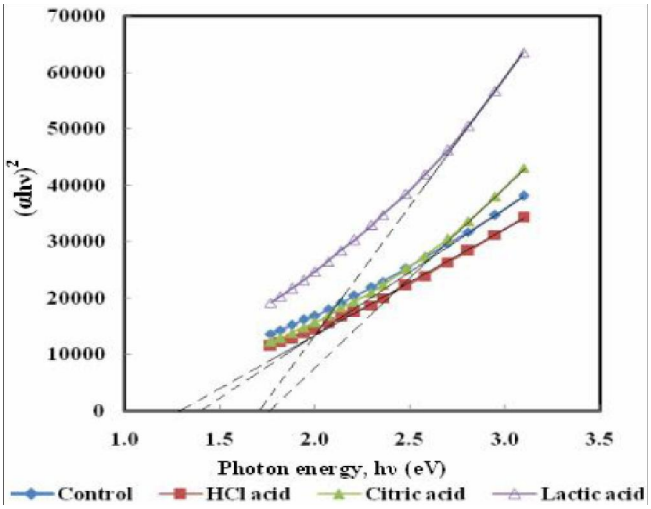

Figure 8 : The variation of $(\alpha h\upsilon)^2$ for bone/matrices pre- and post-demineralization using different acids as a function of photon energy (hυ)

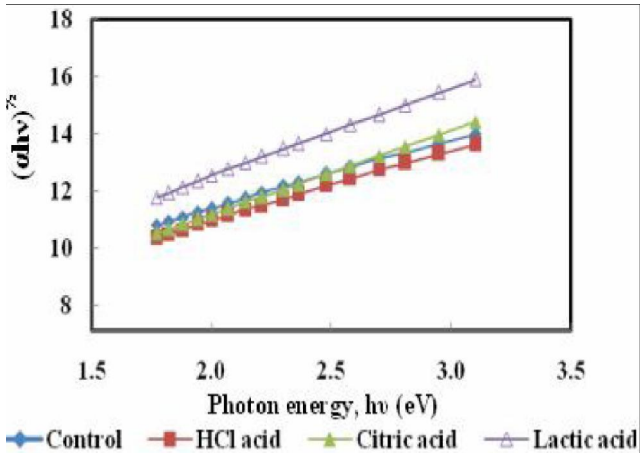

Figure 9 : The variation of $(\alpha h\upsilon)^{1/2}$ for bone/matrices pre- and post-demineralization using different acids as a function of photon energy (hυ)

Figure 7 shows the relation between –!n α and hυ for bone/matrices pre- and post-demineralization using different acids. It clear from the figure that nearly straight lines are obtained started with photon energy equal about 2.2 eV. This suggests that the absorption may follow the quadratic relation for inter-band transitions given.

The values of band tail energy (E_b) can be deduced from the slopes of the straight lines (TABLE 3). The E_b values increase with demineralizing agents and this change could arise from the random fluctuations of the internal fields associated with the structure of control/ matrix.

Figures 8 and 9 show the dependence of $(\alpha h\upsilon)^2$ and $(\alpha h\upsilon)^{1/2}$ on photon energy (hυ) for bone/matrices pre- and post-demineralization using different acids, respectively. It can be observed that the allowed direct energy gap (E_d) is determined by extrapolating the linear parts of the curves to zero absorption and the values are given in TABLE 3. It is noticed from these intercepts that the values of E_d decrease with HCl acid matrix by about 11% while increases with organic acids i.e., citric and lactic acids by about 25 and 23%, respectively. Therefore, the obtained values for E_d show the dependence on demineralizing agents. Such decrease or increase could be due to the number of ions per unit length available for conduction and the change in molecular configuration in the bone matrix.

Extinction coefficient

The extinction coefficient (K) describes the prop-

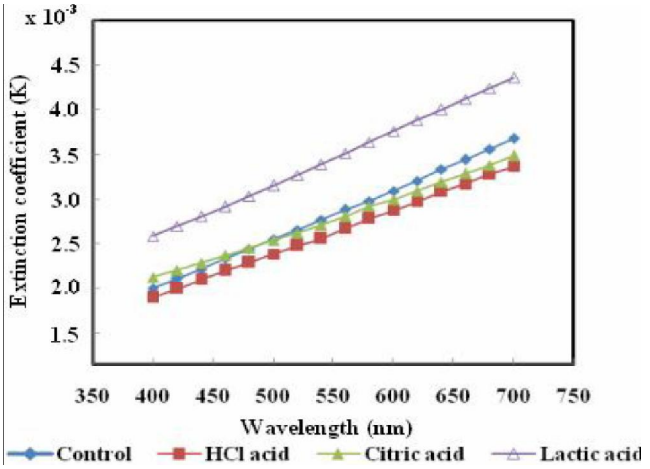

Figure 10 : Variation in the extinction coefficient (K) as a function of wavelength (λ) for bone/matrices pre- and post-demineralization using different acids

erties of the material to light at a given wavelength and indicates the amount of absorption loss when the electromagnetic wave propagates through the material, i.e., represents the damping of an EM wave inside the material. Figure 10 shows the variation in the extinction coefficient with wavelength of bone/matrices pre- and post-demineralization.

It is clear that similar behavior for all samples are observed and the values of K are found to be small in the order 10^{-3} throughout the studied wavelength range (400-700 nm) proving that the samples under investigation are considered to be semi-insulating materials at room temperature. Also, the lactic/matrix indicates the highest value of K through the whole range of wavelength. Additionally, the behavior of the absorption coefficient is preserved for all samples near the absorption edge.

CONCLUSIONS

From the present study and the obtained results, it may be concluded that:

1. Various degrees of the efficiency of demineralization process of the cranial bone (dog) were achieved. Three acids one mineral and two organic acids, all at similar concentration, were employed to characterize and reveal the consequent resulting matrices. Also the study was extended to include color parameters as functions of the demineralization processes.
2. The percentage changes in color parameters proved that the demineralizing acids generated disorder in the system as well as changes in the molecular configuration which lead to the formation of color centers.
3. The trend of absorption coefficient of HCl/matrix is independent on wavelength while they increase with increasing photon energy for the other two organic acids with lactic/matrix revealing the highest values.
4. The absorption followed the quadratic relation for inter-band transitions.
5. Optical analyses relationship was successful in assessing the degrees of demineralization.
6. The values obtained for E_d proved the dependence on the demineralizing agents where the band tail energy (E_b) and direct energy gap (E_d) of the bone/matrices revealed highest values for the citric/ matrix followed by lactic while the mineral acid came at the end.
7. The matrices under investigation are considered to be semi-insulating materials at room temperature.

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