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GREEN CHEMISTRY APPROACHES TO EXTRACTING CALCIUM PHOSPHATE FROM FISH BONE WASTES

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This study explores the extraction of calcium phosphate (CaP) bioceramics from fish bone waste using green chemistry approaches, specifically the alkali treatment method. Fish bones from trout (TF), mackerel (MF), and grey mullet (GMF) were processed to obtain CaP powders, which were characterised using X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Transmission Electron Microscopy (TEM), and X-ray Photoelectron Spectroscopy (XPS). The XRD analysis confirmed the presence of hydroxyapatite (HA) in all the samples, with the TF exhibiting the highest crystallinity. The FTIR spectra revealed characteristic phosphate and hydroxyl groups, indicating the presence of HA. The TEM images showed distinct particle morphologies, with the TF particles aggregated and irregular, the MF particles fibrous, and the GMF particles spherical and uniform. The XPS analysis indicated that the highest elemental compositions with oxygen, calcium, and phosphorus contents were in the TF (47.233 %, 16.187 %, and 9.288 %, respectively). The degradation and ion release studies in phosphate-buffered saline (PBS) showed significant magnesium release, particularly from the TF (32.550 ppm on day 1) and calcium release primarily from the TF (524.89 ppm on day 1). The study demonstrates the potential of fish bone-derived CaP for biomedical applications, such as bone grafts and dental implants, highlighting the environmental and economic benefits of utilising fish bone waste.

INTRODUCTION

The extraction of calcium phosphate (CaP) from fish bone waste represents a significant stride towards sustainable biomaterial production, aligning with the principles of green chemistry. This approach addresses the environmental issue of fishery by-products and contributes to the biomedical field by providing valuable materials for various applications. The focus on utilising fish bone waste is driven by the need to reduce any environmental impact and enhance the resource efficiency, which are core principles of green chemistry.

CaP bioceramics, such as hydroxyapatite (HA) and tricalcium phosphate (TCP), are extensively used in biomedical applications due to their excellent biocompatibility and similarity to human bone minerals. Traditionally, these materials are synthesised from chemical sources; however, the extraction from natural resources like fish bones offers a greener alternative. Fishbone waste, a significant by-product of the fishing industry, presents a sustainable CaP source. The green chemistry approach emphasises a minimal environmental impact and resource efficiency. Techniques, such as thermal calcination, are commonly employed, where fish bones are processed under high temperatures to derive bioceramics with favourable biocompatibility and mechanical properties. This method aligns with green chemistry by reducing the chemical waste and utilising low-value by-products. Moreover, studies have shown that natural-derived materials often contain trace elements like magnesium and strontium, which can enhance the biological properties of the bioceramics [1-3].

The alkali treatment method for extracting CaP from fish bone waste offers a promising green chemistry approach. This process involves soaking fish bones in an alkali solution, such as sodium hydroxide (NaOH), to remove the organic components like proteins and fats. The alkali-treated bones are then rinsed, dried, and milled to obtain a CaP powder. This method has been shown to effectively reduce the fat content and improve the purity of the extracted CaP [4]. Several studies have compared the effectiveness of different extraction methods. For instance, a study by Idowu et al. examined the impact of the alkali treatment on the characteristics of bio-calcium and HA powders derived from salmon bones. The results indicated that the alkali treatment significantly reduced

the lipid oxidation and improved the homogeneity of the particle size distribution [5]. Another study highlighted the use of ammonium bicarbonate solutions for extracting organic (proteins) and inorganic (CaP) phases from fish bones, demonstrating the potential for the simultaneous extraction of valuable compounds. These methods also allow for the recovery of other valuable minerals and elements from the fish bone waste, adding further environmental and economic benefits [1].

CaP derived from fish bone waste has shown promising results in various biomedical applications, including bone graft substitutes, dental implants, and drug delivery systems. The bioactivity and biocompatibility of these materials make them suitable for direct application in the human body, where they can promote bone regeneration and integration. Future research is directed towards enhancing the functional properties of these bioceramics through doping with bioactive ions and developing composite materials that offer improved mechanical properties and biological performance [1, 6, 7]. The environmental impact of extracting CaP from fish bone waste is significantly lower than traditional methods involving mining and processing non-renewable mineral resources. By utilising fish bone waste, the environmental costs of waste disposal are mitigated, and the process also contributes to the circular economy by transforming waste into high-value products. Furthermore, using green chemistry principles ensures that the extraction process minimises the energy consumption and chemical waste, aligning with global sustainability goals [1, 3, 8].

EXPERIMENTAL

Fish Selection and Bone Extraction

Trout fish (TF), Mackerel fish (MF), and Grey mullet fish (GMF) were sourced from a local fishery in Eastern Turkey for this study. The bones were carefully separated from the fresh meat using a knife to minimise any tissue contamination. The extracted bones then underwent a thorough cleaning process, which involved boiling them in tap water for 3 hours to remove any residual organic matter and soften the bone structure. Following this, an alkaline treatment was applied to each type of fish bone. A 2 M NaOH solution was prepared by dissolving 32 grams of NaOH (99 %, Merck) in 400 ml of distilled water at 50 °C. The bones were immersed in this alkaline solution for 6 hours at 50 °C in sealed containers, a process that aids in removing the organic components and enhances the isolation of HA. After the alkaline treatment, the bones underwent further processing. They were washed five times with distilled water to remove any residual NaOH and then dried at room temperature for 72 hours. Finally, the dried bones were crushed into a fine powder using a mortar for 15 minutes, completing the preparation process.

Characterisation Techniques

The extracted powders were extensively characterised using multiple analytical techniques to ensure a comprehensive understanding of their structural and chemical properties. X-Ray Diffraction (XRD, PANalytical Empyrean) was employed to determine the crystalline structure and phase purity of the extracted powders. XRD patterns were collected between the 2θ angles of 20° and 65°, with a step size of 4° and a step time of one minute. A Fourier Transform Infrared Spectroscopy (FTIR, Bruker VERTEX 70v) analysis was used to identify the functional groups and chemical bonds within the CaP structure. Transmission Electron Microscopy (TEM, Hitachi HighTech HT7700) was utilised to examine the particle morphology and size at high resolution. X-ray Photoelectron Spectroscopy (XPS, Specs-Flex) was conducted to analyse the surface chemical composition of the extracted powders. This technique is essential for understanding the surface chemistry, which can influence the material's interaction with biological environments.

Degradation and Ion Release Analysis

A degradation test was conducted to assess the stability and dissolution behaviour of the extracted powders in a physiological environment. This study aimed to simulate conditions similar to those in the human body and understand how the material would behave over time when exposed to bodily fluids. For the test, 150 mg of each type of fish bone-derived HA powder was accurately weighed and placed into separate containers, each incubated in 50 ml of a Phosphate Buffered Saline (PBS) solution at 37 °C, the average physiological temperature. The incubation periods were set at 1, 3, and 7 days to monitor both the short-term and longer-term degradation behaviour. After each incubation period, the filtered solutions were analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7700) to determine the detailed elemental composition, providing precise quantitative data on the concentration of calcium, phosphate, and other potential degradation products.

RESULTS AND DISCUSSION

The XRD pattern illustrates the diffraction peaks for the powders extracted from the GMF, MF, and TF alongside the standard HA pattern (JCPDS No. 09-432) for the reference (Figure 1). The diffraction peaks of GMF, MF, and TF align closely with those of the standard HA, confirming the presence of HA in all three samples. The patterns for TF, MF, and GMF exhibit peaks at similar 2θ positions as the standard HA, indicating that the crystalline structure of HA is present in these

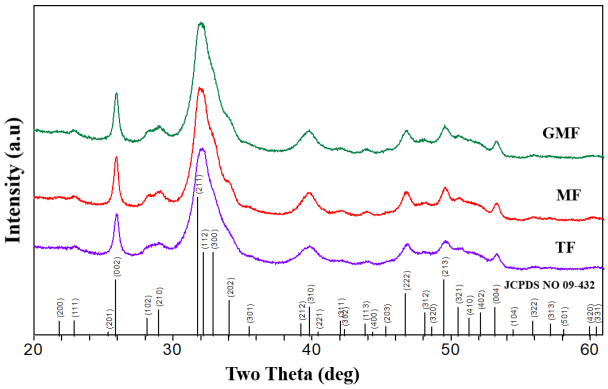


Figure 1. The XRD patterns of the standard HA and the fish-derived HA samples.

samples. The major peaks in the standard HA pattern are observed at 2θ values around 31.7°, 32.9°, and 34.1°, corresponding to the (211), (112), and (300) planes, respectively. These peaks are also prominent in the TF, MF, and GMF patterns, confirming HA's presence. The sharpness and intensity of the peaks provide insights into the crystallinity of the HA. The TF sample shows the most intense peaks, followed by MF and GMF, implying that TF has the highest crystallinity of HA among the three samples [9]. This suggests that the HA in the TF is more crystalline than MF and GMF, possibly due to differences in the biological and environmental conditions these fish species were raised under.

The variations in the peak intensities and positions among the samples reflect differences in the purity and crystallinity of HA, which can be attributed to the biological characteristics and environmental factors specific to each fish species. Factors such as the diet, habitat, and bone composition can influence the formation and quality of HA in fish bones. For instance, the sharper peaks in the TF sample might indicate a more uniform and well-ordered HA structure, possibly due to specific dietary or environmental conditions that favour HA crystallisation [10].

The high crystallinity of HA in the TF, in particular, suggests that it could be a superior source for biomedical applications where high purity and crystallinity are critical. Understanding the crystallinity and purity of HA in different fish species can help select the most suitable source for specific applications and optimise the performance and effectiveness of HA-based biomedical products.

The slight variations in the lattice parameters among the TF, MF, and GMF samples compared to the standard HA can influence the crystallinity and structural integrity of the HA (Table 1). The higher crystallinity, as indicated by sharper and more intense XRD peaks, is generally associated with better mechanical properties and stability. With its slightly higher 'a' parameter, the TF sample may exhibit enhanced crystallinity and structural integrity, making it potentially more suitable for applications requiring high mechanical strength [11]. Lattice parameter variations can also impact the bioactivity and biocompatibility of HA. Studies have shown that fish-derived HA can demonstrate superior bioactivity to synthetic HA, forming more new apatite when incubated in simulated body fluid. The minor differences in the lattice parameters among the TF, MF, and GMF samples may influence their interaction with biological tissues, potentially enhancing their osteoconductivity and angiogenic properties. The cell volume and lattice parameters can provide insights into the thermal stability of HA. Smaller cell volumes, as seen in the GMF sample, might suggest a denser packing of atoms, which could enhance the thermal stability [12]. This property is crucial for applications involving high-temperature processing or sterilisation.

Table 1. The lattice parameters for the standard HA and the fish-derived HA samples.

Sample ID	Lattice Parameters			
	a (Å)	b (Å)	c (Å)	Cell Volume (Å)^3
Std HA	9.418	9.418	6.884	528.8
TF	9.424	9.424	6.870	528.4
MF	9.417	9.417	6.870	527.6
GMF	9.407	9.407	6.876	526.9

The provided FTIR spectra of the powders extracted from TF, MF, and GMF show significant similarities with the FTIR spectra of HA (Figure 2). The GMF, MF, and TF spectra show strong peaks around 1080, 1020, and 960 cm^{-1}. These peaks correspond to the phosphate groups (PO_4^{3-}) in HA, which typically show strong absorption bands in these regions. This similarity indicates the presence of phosphate groups in the fish powders, akin to those in HA [13]. A peak around 873 cm^{-1} is also present, indicating the presence of pyrophosphate groups, characteristic of HA [14]. O-H stretching and bending modes were detected at 3575 cm^{-1} and 635 cm^{-1}, respectively. C-O Stretching: Peaks around 1450 cm^{-1} could indicate the presence of carbonate groups (CO_3^{2-}), often found in biological HA due to the substitution of phosphate groups by carbonate [14, 15].

The GMF, MF, and TF spectra match these key features, indicating that the powders extracted from these fish types contain phosphate and hydroxyl groups similar to those found in HA. This similarity suggests that these fish powders could be used as bioactive materials in bone regeneration applications, similar to HA.

The provided TEM images show distinct differences in the shape and size of particles extracted from TF, MF, and GMF, as presented in Figure 3. The TEM image of the powder extracted from TF shows particles that appear to be aggregated and irregular in shape. The particles form a loosely connected network, indicating high porosity. The size of these particles varies significantly,

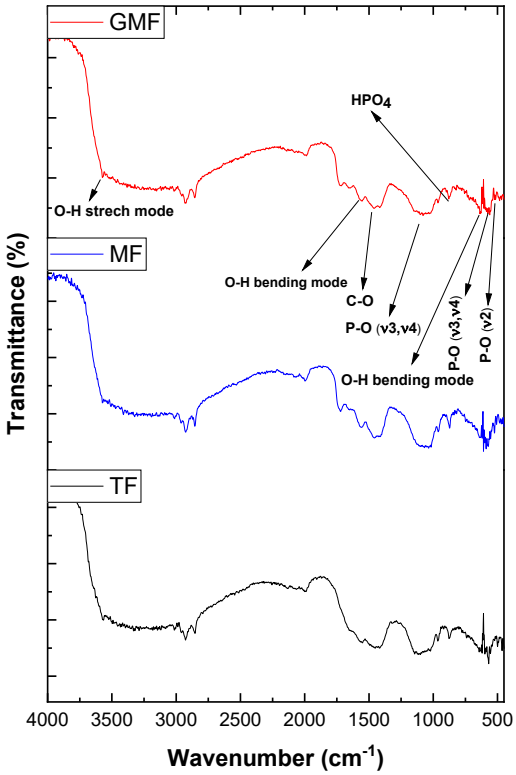
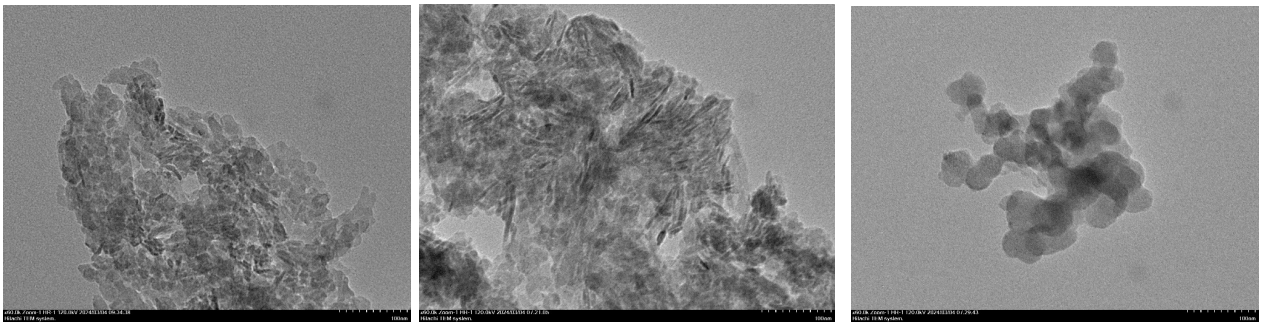


Figure 2. FTIR spectra of the fish-derived HA samples.

with some regions showing smaller clusters and others larger, more extended structures. The TEM image of the MF powder reveals a more fibrous and layered structure than the other samples. The particles appear elongated and somewhat aligned, forming a dense network. This morphology indicates the presence of fibrous proteins and other structural components typical of mackerel fish tissue. The size of these fibres can be seen to range from tens to hundreds of nanometres in length, with the width being relatively consistent. The fibrous nature of the particles suggests a potential for forming strong, interconnected networks, which could be beneficial for applications requiring structural integrity. The TEM image of the GMF powder shows particles that are more spherical and less aggregated compared to the TF and MF powders. These particles appear to be relatively uniform in size, with diameters typically in the range of tens of nanometres. The spherical shape and uniform size distribution suggest that the GMF powder might have undergone a different preparation process or that the inherent properties of grey mullet tissue led to the formation of such particles.

The differences in the particle shape and size among the TF, MF, and GMF powders can be attributed to the inherent structural differences in the fish tissues and the methods used for their preparation. The fibrous nature of MF particles aligns with findings from studies on mackerel oil particle formation, which show a tendency for fibrous and elongated structures under certain preparation conditions [16]. Similarly, the spherical and uniform particles observed in the GMF are consistent with observations in studies on grey mullet tissue, which highlight the presence of rounded and uniform extracellular vesicles and other particulate matter [17, 18].

The XPS analysis of the powders extracted from TF, MF, and GMF reveals significant insights into their elemental composition and chemical states, which can be compared with the HA (Figure 4). The binding energy, a crucial parameter in XPS, indicates the energy required to remove an electron from a specific element within the material. For instance, in the context of HA, calcium (Ca) typically exhibits a binding energy around 347 eV for Ca 2p3/2, while phosphorus (P) in the form of PO_4^{3-} shows a binding energy near 133 eV for P 2p. Oxygen (O) can display various binding energies depending on its chemical state, such as around 531 eV for O 1s in hydroxyl groups (OH^-) or slightly lower for lattice oxygen (O^{2-}). Carbon (C), often present due to organic matter or carbonate substitutions, typically shows a binding energy of around 285 eV for C 1s. Comparing these values with those from fish-derived powders, slight shifts in the binding energies can indicate the presence of trace elements, carbonate substitutions, or organic components, which are common in biological materials. These shifts can affect the material's properties, such as its bioactivity, mechanical strength, and biodegradability.



a) TF b) MF c) GMF

Figure 3. TEM images of the fish-derived HA samples.

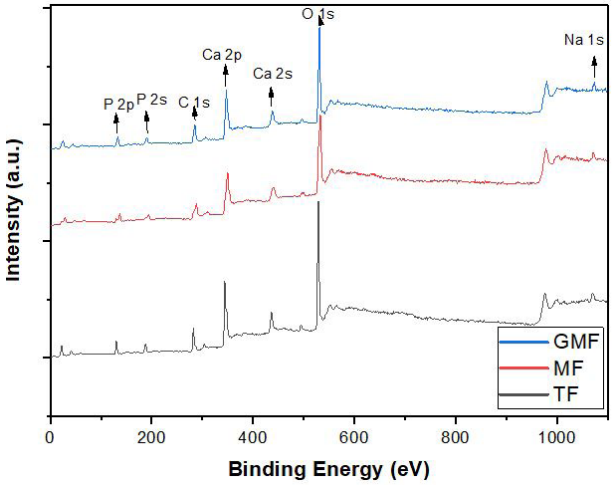


Figure 4. XPS spectra of the fish-derived HA samples.

For example, a higher binding energy for calcium might suggest the presence of additional ionic interactions or substitutions within the HA lattice [19]. Studies have shown that fish scale-derived HA often has a Ca/P ratio close to synthetic HA, but with slight variations due to trace elements and organic matter [20]. The presence of carbonate groups and trace elements such as sodium (Na), magnesium (Mg), and silicon (Si) in the fish-derived HA samples can be confirmed through the XPS analysis, which shows distinct binding energy peaks for these elements. These trace elements can improve the biological performance of fish-derived HA samples by promoting cell adhesion, proliferation, and differentiation [21]. Additionally, the higher bioactivity of the fish-derived HA samples compared to synthetic HA has been demonstrated in studies where fish-derived HA samples formed more new apatite in simulated body fluid (SBF) and showed higher osteoblast-like cell adhesion and proliferation [22].

The atomic percentages of O, Ca, and P are relatively consistent across the three fish species, suggesting a similar overall composition. However, there are some notable differences (Table 2). The oxygen content is the highest in the TF (47.233 %), followed by MF (46.403 %) and GMF (45.520 %). The calcium content is the highest in the TF (16.187 %), followed by GMF (15.484 %) and MF (14.671 %). The phosphorus content is the highest in the MF (10.264 %), followed by GMF (9.968 %) and TF (9.288 %). The Ca/P ratio is a crucial parameter for evaluating the quality and composition of HA. The stoichiometric Ca/P ratio for pure HA is 1.67. Comparing the Ca/P ratios, TF has a ratio of 1.74, MF has a ratio of 1.42, and GMF has a ratio of 1.55. The Ca/P ratio of TF (1.74) is slightly higher than the standard HA, while MF (1.42) and GMF (1.55) are lower. These variations suggest differences in the calcium phosphate phases present in each sample [23]. The presence of O, Ca, and P in proportions similar to the standard HA indicates that these fish-derived powders contain HA-like structures. This aligns with previous studies showing that fish scales and bones are rich sources of HA [24]. The differences in the Ca/P ratios among the samples and compared to the standard HA suggest the presence of other calcium phosphate phases or impurities. TF may contain calcium-rich phases with a higher ratio, while MF and GMF might have more phosphate-rich phases or deficient HA [25]. The relatively high carbon content (24-27 %) in all the samples likely indicates the presence of organic matter, which is typical for biologically-derived HA [26]. This organic content could influence the biocompatibility and osteoconductivity of the material. The presence of sodium (Na) and silicon (Si) in small quantities could potentially enhance the biological performance of these materials. Sodium is known to play a role in bone metabolism, while silicon can improve the bioactivity of HA [27]. The variations in the Ca/P ratios and the presence of additional elements suggest that these materials may have unique properties that could be advantageous for specific biomedical applications. Further studies on the crystallinity, particle size, and biological performance of these fish-derived HA powders would be beneficial to assess their potential as biomaterials fully.

Table 2. Chemical compositions of the fish-derived HA samples.

Element	Atomic % of major elements			Standard HA
	TF	MF	GMF	
O 1s	47.233	46.403	45.520	---
C 1s	24.977	26.928	26.608	---
Ca 2p	16.187	14.671	15.484	---
Na 1s	1.907	1.734	1.380	---
P 2p	9.288	10.264	9.968	---
Si 2p	0.408	---	1.040	---
Ca/P ratio	1.74	1.42	1.55	1.67

The degradation and ion release study of the powders extracted from the TF, MF, and GMF in PBS over 1, 3, and 7 days are summarised in Table 3. The results reveal interesting patterns compared to the HA. All the fish bone samples showed significant magnesium release, with TF exhibiting the highest initial release (32.550 ppm on day 1). This is substantially higher than typical HA, which contains minimal magnesium [22]. The high magnesium content and release from fish bones can benefit bone regeneration, as magnesium plays a crucial role in bone metabolism and stimulates osteoblast proliferation [3].

The calcium release was observed primarily in the TF (524.89 ppm on day 1) and, to a lesser extent, in the GMF (19.56 ppm on day 7). This release pattern differs from synthetic HA, which typically shows more sustained calcium release over time [28]. The initial

Table 3. Release of ions in PBS at various times.

Sample ID	Immersion Time	Concentration of minerals (ppm)					
		Na	Mg	P	K	Ca	Sr
Pure PBS	0 Day	3354.79	<0.000	124.80	167.35	<0.000	<0.00
TF	1 Day	3495.32	32.550	107.81	183.96	524.89	1.874
	3 Days	3965.31	2.634	147.12	221.33	<0.000	0.018
	7 Days	3579.57	0.530	96.61	179.73	<0.000	<0.000
MF	1 Day	3477.84	0.613	107.48	180.86	1.41	0.063
	3 Days	3574.30	0.733	102.02	183.40	2.14	0.072
	7 Days	3468.35	0.588	90.84	179.43	<0.000	0.015
GMF	1 Day	3564.27	1.689	117.51	184.68	4.89	0.158
	3 Days	3530.14	1.186	95.64	183.11	0.370	0.053
	7 Days	3564.37	7.511	138.05	189.86	19.56	0.480

burst release from TF could be advantageous for rapid bone mineralisation. Interestingly, the phosphorus concentrations fluctuated across all the samples, with some showing lower levels than pure PBS at certain time points. This suggests a complex ion exchange process between the fish bone powders and the PBS solution, which is not typically observed with synthetic HA [29]. Small amounts of strontium were released from all the fish bone samples, with GMF showing the highest release (0.480 ppm on day 7). Strontium enhances bone formation and reduces bone resorption, making it a valuable component not typically found in synthetic HA [30]. Slight increases in the sodium and potassium concentrations were observed across all the samples, likely due to ion exchange processes. This is similar to what occurs with HA in physiological solutions [31].

The degradation patterns observed in these fish bone powders suggest a more dynamic and potentially bioactive behaviour compared to synthetic HA. The high initial magnesium release, particularly from TF, could enhance the osteogenic properties [32]. The varying release profiles of different ions indicate that these materials may offer a more biomimetic environment for bone regeneration than pure HA [33]. However, the rapid initial release of ions, especially in the TF, may lead to faster material degradation. This could be advantageous for short-term applications but may limit any long-term structural support compared to more stable HA scaffolds [34].

CONCLUSIONS

This study successfully demonstrated the extraction of calcium phosphate (CaP) bioceramics from fish bone waste using green chemistry approaches, specifically the alkali treatment method. Fish bones from trout (TF), mackerel (MF), and grey mullet (GMF) were processed to obtain CaP powders, which were extensively characterised using X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Transmission Electron Microscopy (TEM), and X-ray Photoelectron Spectroscopy (XPS). The results confirmed the presence of hydroxyapatite (HA) in all the samples, with TF exhibiting the highest crystallinity. The FTIR spectra identified characteristic phosphate and hydroxyl groups, confirming the presence of HA. The TEM images showed distinct particle morphologies: the TF particles were aggregated and irregular, the MF particles were fibrous, and the GMF particles were spherical and uniform. The XPS analysis indicated elemental compositions with TF's highest oxygen, calcium, and phosphorus contents. The degradation and ion release studies in phosphate-buffered saline (PBS) demonstrated a significant magnesium release, particularly from TF (32.550 ppm on day 1) and a calcium release primarily from TF (524.89 ppm on day 1). These findings suggest that fish bone-derived CaP materials have the potential to promote bone regeneration and integration due to their bioactive ion release profiles. The environmental and economic benefits of utilising fish bone waste for CaP extraction are substantial. This approach mitigates the environmental costs associated with waste disposal and contributes to the circular economy by transforming low-value by-products into high-value biomedical materials.

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