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Preliminary Investigation of the Usability of Brown Meagre Otoliths as an Alternative Material in Bone Tissue Engineering

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Abstract

The need for biomaterials is increasing daily due to the increasing number of health problems and the doubling of the population. Especially bone structures are among the most important tissues in terms of human quality of life. In this study, Brown Meagre (BM) fish skull otoliths, which are organic-based calcite structures, were examined and a preliminary investigation of their usability for bone structures was carried out. For this purpose, otoliths taken from BMs speared from the Ordu coast of the Black Sea were cleaned with H_2O_2 and SEM, SEM EDX, XRD, FTIR and ICP MS analyses were performed, and the results were compared with bone structures. In the light of these data, it was determined by analyses that the calcite structure is in the aragonite phase and allows the accumulation of oligoelements. Otolith structures, which have the potential to be alternative raw materials for biomaterial production, also provide the utilisation of organic-based waste materials. It is aimed that otoliths, like coral structures studied in the literature, will contribute to bone tissue engineering studies, which are seen in calcite structures and whose biochemical interactions with enzyme structures are still not completely solved.

1. Introduction

In biomaterials, bone structures are among the most important cell forms. Although the structures compatible with bone structures to date are hydroxyapatites (HA, $Ca_{10}(PO_4)_6(OH)_2$) and production requires alternative studies due to the laborious and costly production process and the difficulties in ensuring compatibility with the body. One of the areas studied to meet this need is biological structures obtained from marine environments [1,2].

Calcite crystal structures differ in each species, even in the habitats of the same species. Although the basic structure appears to be aragonite, otoliths formed in the skull or different parts of fish have different properties due to crystals and oligoelements. In the literature, the organic calcite content of otolith materials could be combined with phosphate structures and used in hydroxyapatite structures [3,4]. Many factors affect the biocompatibility of bones and the most important of which is the pore size and the ability of the cell to allow the tissue to grow, and otoliths will provide an alternative to this gap [5,6].

Loss of limbs in the human body as a result of earthquakes, wars and accidents shows how important it is to improve alternative products in the field of biomaterials in recent years. One of the most important structures that keep the body standing in human life is bones. People experience major problems with bone and cartilage tissue losses. To eliminate these problems, ceramic or ceramic composite materials used in prosthesis and implant applications and showing biocompatibility are defined as bioceramics, and these structures form the materials of the future with various matrix structures and the work of many disciplines [7, 8].

From orthopedic applications to tissue engineering, from dental restorations to bone cements, the search for biomaterials has been developing from ivory prostheses in the 1880s to alumina, hydroxyapatite and glass ceramics today, and the production of biocompatible, low-cost materials is the target of bioceramics studies [9].

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These types of materials provide alternatives to lost or missing organs, with functionality and biocompatibility being the priority, from drugs to titanium and stainless-steel materials. Bone biomaterials are a never-ending need in the field [10]. Bone structures contain both organic and inorganic structures, and the production of materials that provide this harmony is carried out under the control of many factors [11,12].

However, 100% of the bone structure cannot be produced and the control of body reactions to materials has not yet been achieved. For this reason, materials are tested in vitro (outside the body) in environments equivalent to the fluids in contact with the body, and their use is provided with trials after positive results.

Biomaterials are gaining importance day by day and many structures are being studied. In this study, alternative otoliths were studied for structures like structures called 'coral' in the literature and used as biomaterials. These materials, which have special protein structures such as mollusk shells and diatom cell walls, attract attention and are studied. [1,2]

Although there are studies on fish-based products obtained from the marine environment, these studies are insufficient and otoliths create new study opportunities due to their suitability both economically and structurally. In particular, anticancer drug studies show that antioxidant and antihypertensive properties affect biological activity in the production of advanced materials.

When the literature was examined, a preliminary examination was made of the stones (otoliths) extracted from the skulls of fishes known as 'eşkina' fish in Turkish among the public, which are not considered as bioceramics except by scientists working on biology and fish. It is desired to contribute to the literature by studying the usability of these structures especially in bone structures. Literature research conducted on this subject shows that there is not enough work done on the subject and that the publications are insufficient [4, 11, 13].

In addition, these structures, which develop in the cell-free environment in the endolymph, are also affected by many factors. In the literature, otolith structures formed in fish are obtained from species such as cynoscion acoupe, croaker drums, etc. which have otolith structures, and are used in different productions. However, the formation stages of these structures (otoliths) in the spontaneously formed stones (otoliths) in Brown meagre skulls used in the study vary in all species.

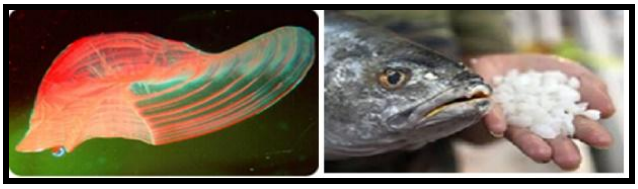


Figure 1. Fish and otolith pictures [14.15].

Otolith is a natural material, and we planning to use in follow-up studies the otoliths as a part of biomaterial for tissue engineering. As is known, bone has a composition with hydroxyapatite structure. The main component of this structure is calcium phosphate. In physiological examinations of bone structure formations, it is seen that ossification occurs with the accumulation of calcium salts in the tissue matrix in the body [16].

When the Brown Meagre otoliths are examined under a microscope in Figure 2, it is seen that the calcium accumulated as the fish grows forms layer-by-layer rings [3,4, 17].

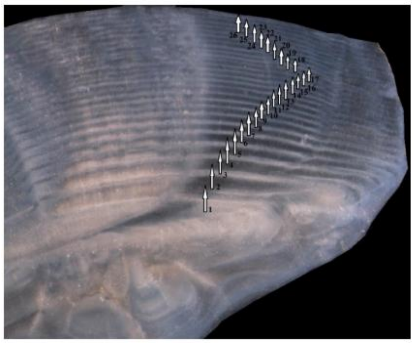


Figure 2. Age rings of Brown Meagre otolith determined by microscope in literature [17].

Otolith structures in marine environments are also the subject of studies in the literature by biologists as well as interdisciplinary fields. However, the formation stages of these structures carry the effects of different habitats in all species. These materials allow new cell migration due to their osteoinducer structure, but they are affected by many factors such as temperature, salinity, pollution, metabolic events in the life cycle, age, and chemical composition.

These structures form scaffolds that change with the effects of their environment and create osteoinductor properties. Many factors such as temperature, salinity, pollution, metabolic events in the life cycle, age and chemical composition affect their formation. These structures, which develop in the cell-free environment within the endolymph, show very different formations under the influence of factors such as high K^+ low Na and changing pH and high CO_2 [4,6,18].

As seen in Figure 1, it shows a formation and provides information about the population dynamics of the fish. The microchemical structure of the otoliths is very complex and some points are still not fully determined and are understood in a limited way. In these structures, otoliths serve as depth and balance sensors, and these features create genetically important and unique data. It was aimed to create an infrastructure by studying these features [3,5,6]. In addition to these studies, when examined in terms of phase structures, it shows that this structure can be used in hydroxyapatite structures, which form the basis for biomaterials. It is seen in the literature that the studies carried out with otoliths, which are mostly examined by expert academics working in the field of fisheries, were carried out to examine the features of this structure from different perspectives [6,17, 19].

In this study, although materials such as coral structures, fish bones, etc. were used, preliminary studies were carried out for the usability of otoliths in the skulls of brown meagre fish, which are not included in the literature, as biomaterials, and in line with the results obtained, a study was conducted on the evaluation of biological waste materials in high-tech materials.

2. Materials and Methods

In this study, brown meagre otoliths were analyzed after being taken from meagre fish caught with spearguns in rocky areas close to the shore in the Black Sea region in Ordu Coasts. In the preliminary study carried out by the authors. The supply of materials (otolith taken from the skulls of fish obtained from the sea by family members were used) and subsequent analysis of the samples obtained. The fish caught (Brown meagre) can be seen in Figure 3.

Figure 3. Brown Meagre (Sciaena umbra) with otoliths removed from the skull

The area where the otoliths taken from the otoliths and skull bones are located in Figure 4.

Figure 4. Brown Meagre (Sciaena umbra) with otoliths removed from the skull

The otoliths were taken from the skull of Brown Meagre, cleaned and dried at 30ºC. The weight of the stones used in the study varies between 15 g and 25 g. They were then subjected to agate grinding, treated with H_2O_2 and dried (Figure 5). Otoliths' microchemical structure is very complex and some points are still not fully determined and understood in a limited way. In these structures, otoliths act as depth and equilibrium sensors and these features constitute important and unique data from a genetic point of view.

Figure 5. Powder Preparation from otoliths with agate morter and drying after treatment H_2O_2

SEM and SEM EDX analysis were performed with a Hitachi SU 1510 Brand Model device at 15 kV current and 750 magnification, and an X-ray diffraction analysis was performed with Rigaku MiniFlex XRD. Afterward, FTIR analysis was performed using Fourier transform infrared spectrometer (FTIR) (Perkin Elmer Spectrum Two). Detection of oligoelements by ICP-MS analysis was done by preparing the material in an oven with H_2O_2 using the Thermo iCAP RQ device.

3. Results and Discussion

The results of the analysis applied to otolith structures provide extensive information about these structures and reveal that the structure varies in terms of oligoelements and phase formations as well as calcite.

Characterisation techniques are important in materials science and in this preliminary study, information about the potential of the material was obtained by making an examination from macrostructure to microstructure and an attempt was made to contribute to the literature to transform waste materials into high value-added products.

3.1 SEM, SEM-Mapping, SEM-EDX Analysis

These structures, which develop in the cell-free environment in the endolymph, show very different formations under the influence of factors such as high K^+ low Na and changing pH and high CO_2. When the otoliths used in the study are analyzed by SEM and SEM EDX analysis, it is seen that they show a layer-by-layer formation [19,20].

This structure was hardly disintegrated during agate grinding for SEM analysis and it was observed that the layered structure in the microstructure affected the macrostructure.

Figure 6 below shows the SEM and SEM-EDX analysis applied to otoliths. This analysis shows that the structure develops layer by layer. The literature shows that the stone grows according to the size of the fish and these otoliths develop over time by growing layer by layer. For this reason, larger stones occur in larger fish. [6,17, 19].

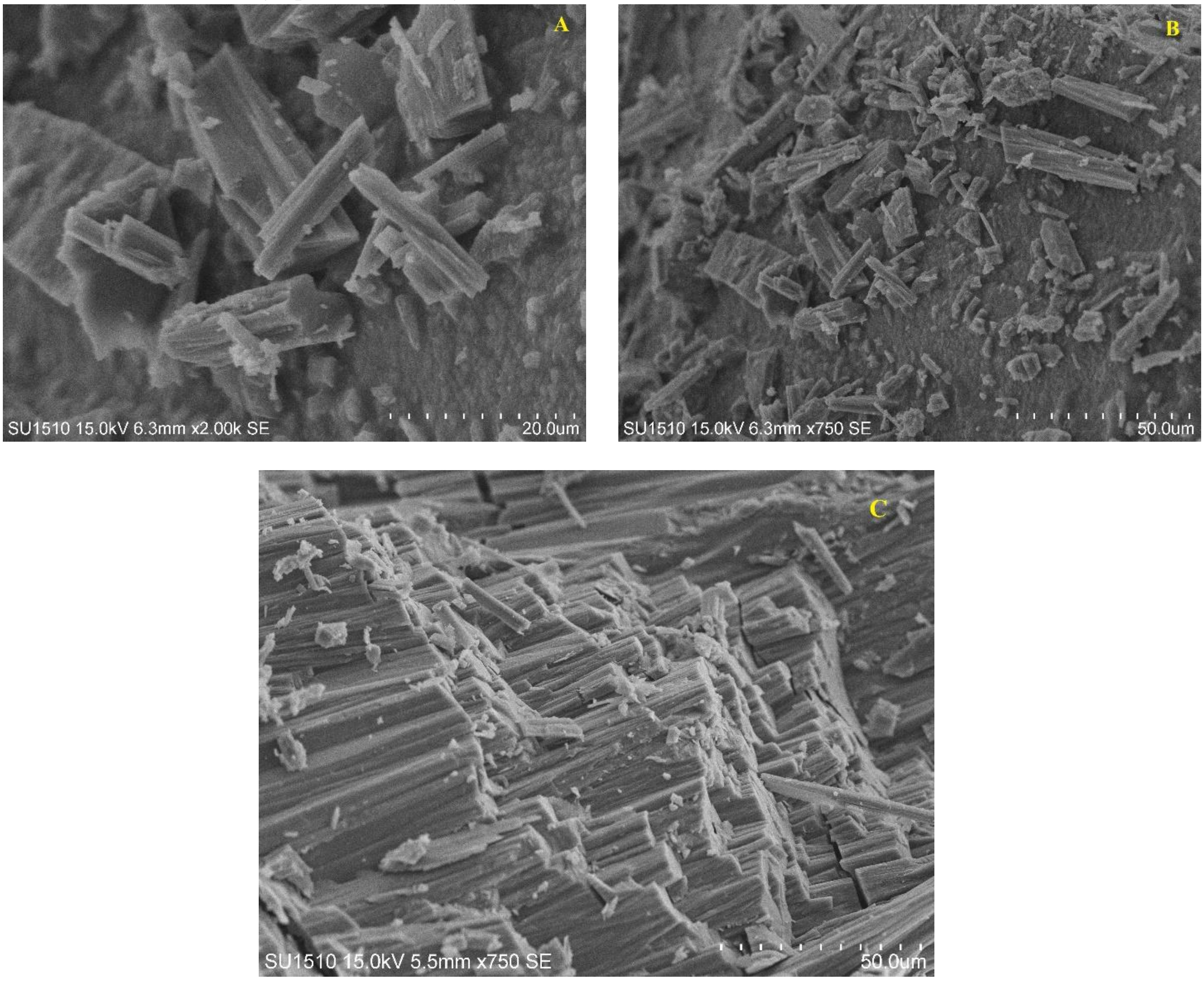


Figure 6. SEM analysis of otoliths (A, B and C).

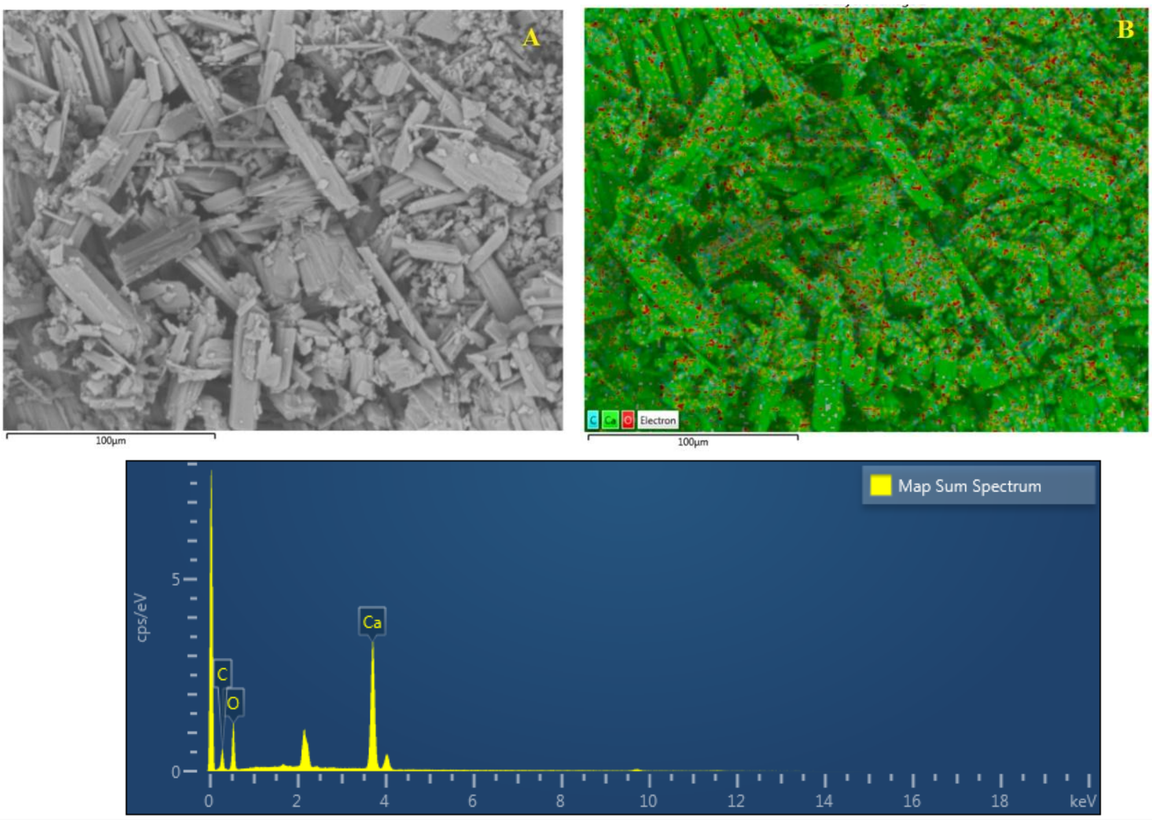
SEM EDX analysis was performed in the form of mapping of the image taken from a general region, and SEM, SEM Mapping and EDX analysis are given together in Figure 7. When the sem analysis given in Figure 6 is examined in detail by Mapping and EDX, Ca, C, and O, which are the basic elements in the structure, are clearly seen. However, a more detailed examination allows us to obtain information about oligoelements. As in Figure 6, needle and rod-like structures represent the durable structure that accumulates over time.

When apatites found in bone structures are examined in the literature, they are determined to be nanostructured, granular crystals by SEM and TEM analyses [21,22].

If SEM analyses are compared with the biological structure given here, it is seen that it has a similar structure. When EDX analyses were examined, it was found that carbonates were present in the structure and the studies on the presence of these $(CO_3)^{-2}$ structures had an important effect on the formation, physical structure and transformation of apatite [23].

And it has been observed that some carbonate species constitute the basic type of biological apatite.

Calcite, especially with its crystal structure, gives positive results in the production of alternative biomaterials to bones. Although $CaCO_3$ structures in organic or inorganic structures are called calcite, the orientation of these structures in the crystal plane can change, which enables the formation of different bonds with body fluids, tissues and enzymes [24,25].



Map Sum Spectrum				
Element	Line Type	Weight %	Weight % Sigma	Atomic %
Ca	K series	47.01	0.38	25.19
O	K series	44.74	0.42	60.06
C	K series	8.25	0.24	14.75
Total		100.00		100.00

Figure 7. SEM (A), SEM Mapping(B) and SEM-.EDX analysis of otoliths.

3.2. FTIR Analysis

The main components of bone structure are Ca, C, P and O, but it also contains other minor elements. These components start from the age of the person, diet, poisoning, diseases, etc. It carries many effects on it. By analysing the working principles of these structures, solutions against diseases are investigated. For this reason, FTIR analyses were performed on the main raw material to be used in the study and the starting material intended to be used in bone structures was examined. Ca, C and O bonds are important in these structures and FTIR analysis was performed to understand the bond structures of the crystals structures and is given in Figure 8. When all these data are verified, it is clear that the organic phase is present. Considering that all Ca is present as $CaCO_3$, this means that 7.2 % C and 29.89 % O remain for the other (non-crystalline) components (organic matter + H_2O).

When the materials with natural calcite content are examined, the main bands of the structure show 1400-1640 cm^{-1} FTIR values, while CO_3^{2}structures have band values of 711 and 870 cm^{-1} [26,27].

The graph given in Figure 8 is compared with XRD and SEM analyses, it is seen that the desired bone main component group is similar to the desired bond structures

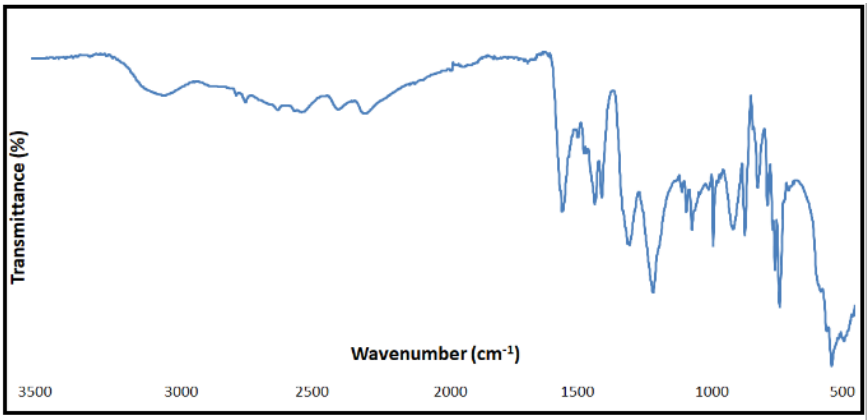


Figure 8. FTIR analysis of otoliths.

3.3. XRD Analysis

When brown meagre otoliths are examined by XRD analysis, it is thought that they have a calcite-aragonite structure and therefore, it is thought that the crystal structure will provide an advantage to obtain hydroxyapatite structure by combining stoichiometrically with phosphate structures in biomaterial production. [3-6].

The presence of carbonate groups in apatite structures plays an important role in the formation, physical properties and morphological structure. In the literature, it has been determined that carbonate apatite structures can be formed by substitution as hydroxyl group (type A) and phosphate group (type B). The nanoscale crystals of these formations vary and this affects the mechanical properties of the bone. The oligoelements on the surfaces of these nanocrystals are also important [22].

Studies on long bones of animals have shown that the c-axes of apatite crystals are preferentially orientated to withstand stress. Because the organic part of the bone plays a role in the metabolic activity of the body by harbouring different ions and cations. These data form the basis for understanding calcium phosphate structures and properties. In the literature, it has been determined that CO_3 ions affect the solubility and biological activity in regions A and B. XRD analyses show that the otolith is in the aragonite phase and ICP MS analysis results show that the aragonite phase is the thermodynamically stable and least water-soluble form of calcite and that the aragonite phase allows the deposition of oligoelements due to the orthorhombic and trigonal crystal structures of aragonite and calcite [20,22].

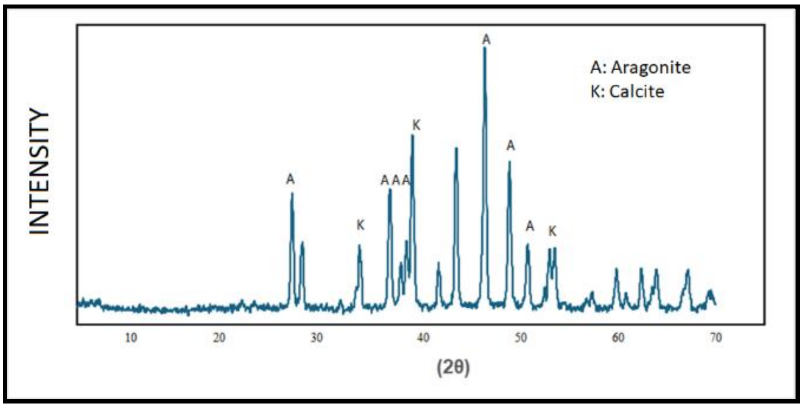


Figure 9. XRD analysis of otoliths.

3.4. ICP-MS Analysis

Bone structure is not only composed of calcium phosphate structure, but also contains oligoelements and these elements affect the phase transformation of calcium phosphate compounds. These structures play an inhibitory role and can change the crystal formation of the structure. In addition to examining the structure in nanostructure, it also provides a connection with the properties of organic structure, namely collagen structures, together with the oligoelements on the surface, and these relationships are still not fully explained by researchers and affect biological activity. When XRD and ICP MS analysis in Figures 9 and 10 are examined, it is seen that the aragonite structure required for the HAP structure and the presence of elements such as Sr, Fe and Cr, which are important for bone structure in oligoelements, are present and form a correct infrastructure as a biomaterial [20].

Sample Code	Numune kons., ng/mL, ppb	Element, ng/mL, ppb												
		Mg	Mn	Fe	Co	Ni	Cu	Zn	As	Sr	Cd	Ba	Pb	Cr
Calcite aragonite phase	46560	3,354	0,177	79,954	0,02	0,127	0,018	0	0	82,788	0	0,532	0,217	2,717

Sample Code		% Element												
		Mg	Mn	Fe	Co	Ni	Cu	Zn	As	Sr	Cd	Ba	Pb	Cr
Calcite aragonite phase		0,007204	0,00038	0,171723	4,3E-05	0,000273	3,87E-05	0	0	0,177809	0	0,001143	0,000466	0,005835

Sample Code		Element, mg/kg, ppm												
		Mg	Mn	Fe	Co	Ni	Cu	Zn	As	Sr	Cd	Ba	Pb	Cr
Calcite aragonite phase		72,03608	3,801546	1717,225	0,429553	2,727663	0,386598	0	0	1778,093	0	11,42612	4,660653	58,35481

Figure 10. ICP-MS analysis of otoliths.

The elements in human bone structure have two types of structuring as abundance and trace amounts. While the abundant elements in the structure constitute the basic tissue, trace amounts are required for body functions. Elements such as F, Cu, Mg, Mn, Zn, Co, B, Cr, I, Fe, Mo, Ni, S, Sn, V, Sr and Ba are taken into the bone structure through a balanced diet. It is important that the crystal structure of the bone and oligoelements should be in a way to allow their settlement. Because these elements fulfil the role of cofactors by activating the catalytic sites of enzymes. Fe, which is one of the most important elements in the structure, is recognised by proteins and increases the reserve by binding and transports it to the required regions. However, the specific biochemical behaviour of some elements is still under investigation [28,29].

ICP-MS analysis results give significant values in bone structures and this analysis result shows that brown meagre otoliths are suitable for use in hydroxyapatite structures.

4. Conclusions

Calcite formations that form otolith structures can allow the phase change of different elements in the crystal structure cavities, and the oligoelements in the structure show this change. These surface interactions and crystal structure changes are important in bone structures where tissue-implant interactions are important.

For this reason, otoliths are promising in terms of obtaining structures like these structures, whose formation stages are still not clearly resolved by studying different biological sources, and it is aimed to use these structures in the production of hydroxyapatite material in the continuation of the study. It is thought that otoliths have the potential to create the opportunity to make different designs for various applications and can be involved in many studies, such as tissue regeneration and allowing the development of different tissues.

As a result of the data obtained from the analyses applied to the otoliths used in the study, it was determined that the material with rod-like and needle-like microstructure is calcite. Calcite forming the main structure is in the form of aragonite and this structure is promising especially for carbonate-containing apatite structures. The deposition of oligoelements in the crystal structure depends on this main structure, but the abundant and scarce elements are of great importance in the development of the properties of the bone structure. The fact that different organic structures have different developments shows that brown meagre otoliths can be an alternative in the field of bone tissue engineering biomaterials.

Declaration of Ethical Standards

The authors of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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