

(RESEARCH ARTICLE)



## A study on amino acid composition and distinctive traits of collagen isolated from the skin and bone of an endemic pearl mullet species, *Alburnus tarichi* (Güldenstädt, 1814)

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### Abstract

In this study, acid soluble collagen was extracted from both the skin (ASC-S) and bone (ASC-B) of pearl mullet *Alburnus tarichi* (Güldenstädt, 1814), and a comprehensive characterization was conducted, which included determining their amino acid profiles. This study represents the first-ever utilization of this particular species as a source of collagen. Notably, both ASC-S and ASC-B, extracted from the skin and bone of pearl mullet, exhibited glycine as the predominant amino acid, along with substantial levels of proline, hydroxyproline, alanine, and glutamic acid. In terms of dry weight percentages, ASC-S yielded 7.7%, while ASC-B yielded 2.5%. Additionally, Fourier transform infrared spectroscopy (FTIR) confirmed that both types of collagens were in their natural integrated state, while X-ray diffraction (XRD) analysis verified that the collagen in both skin and bone maintained their helical structures. UV-Vis spectra showed significant absorptions at 230 nm for the examined collagens. Furthermore, scanning electron microscope (SEM) examinations revealed the porous and fibrous nature of both ASC-S and ASC-B. The amino acid composition, as determined by UV-Vis and FTIR results, categorizes the extracted collagens as type I collagen. Overall, these findings suggest that collagen isolated from pearl mullet has the potential to serve as an alternative source of vertebrate collagens, with promising applications in various industries, including dietary, medical, and pharmaceutical.

**Keywords:** Pearl mullet; ASC; Skin; Bone; Collagen; Nutrition

### 1. Introduction

The pearl mullet (*Alburnus tarichi* Güldenstädt, 1814) is a species of cyprinid fish that belongs to *Cyprinidae* family. The pearl mullet (*Alburnus tarichi* Güldenstädt, 1814) was also named synonymously as *Chalcalburnus tarichi*, Pallas 1811. However, it was listed as *Alburnus tarichi* by the IUCN red list (1). This species is an endemic species that found only in Türkiye's eastern region, where it is the only fish known to inhabit Lake Van (2,3). So, *A. tarichi* is endemic to the Lake Van basin (12,500 km<sup>2</sup> catchment; 3,700 km<sup>2</sup> lake surface area) (2). Additionally, it is known as its very large population which has been declining due to activities in the 12 spawning streams, overfishing, and mining and other forms of pollution. This species was described as a vulnerable species by The International Union for Conservation of Nature's (IUCN) red list of threatened species (1). Pearl mullet is usually caught by locals as a source of food. It among the most preferred fish by local people and can reach about 20 cm and weigh up to 110 g (4).

Collagen, the primary fibrous glycoprotein constituting connective tissue, is a major component found predominantly in the skin, bone, cartilage, tendon, and connective tissues of both mammals and fish (5–7). Collagen makes up from

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25% to 35% of the whole-body protein content and up to 85% of the skin of mammals. However, it makes up from 19% to 38% of the whole-body protein content and up to 96% of the skin of fish (8). A wide range of collagen products are commercially accessible; however, they do not share the same origin, and their suitability for specific dietary needs can vary (9). Many of these collagens are sourced from livestock production in different farms that include cattle, pig, and chicken, while others come from aquatic sources including fish, shellfish, jellyfish, and crustaceans. Cattle are more widely used in collagen production in compare to porcine and fish sources because of its lower price and the abundance of its skin and bones. However, there are increased concerns about the transmission of diseases such as bovine spongiform encephalopathy (BSE) to humans due to consumption of bovine-based products (10,11).

Because of its resistance to stretching and its fibrous composition, collagen contributes to the skin's strength and elasticity, while also playing a crucial role in strengthening blood vessels and promoting tissue development. (12). Collagen's low immunogenicity and high biocompatibility make it a preferred biomaterial in biomedical applications. Besides its industrial uses, there is a great interest in collagen's anti-aging effects in many medical fields such as plastic surgery, burn surgery, and even weight management (13,14). For this reason, many studies have been done to find alternative sources of collagen. In recent times, there has been a growing preference for collagen obtained from aquatic sources. Collagen derived from fish has demonstrated a heightened level of bioavailability, as peptides sourced from fish collagen are established to be easily digested, absorbed, and distributed throughout the body up to 1.5 times more efficiently compared to collagen derived from bovine or porcine sources (15). In many studies, it has been reported that even fish waste constitutes a high potential source of collagen (16–18). Therefore, it has been known that it can be used to supplement the diet.

In the current study, pearl mullet (*Alburnus tarichi* Güldenstädt, 1814) was investigated for its collagen resources and their amino acid profiles. *Alburnus tarichi* (Güldenstädt, 1814) is only found in Türkiye. It is one the most important endemic species found in Lake Van Basin, which is located southeast of Türkiye (19). In addition to its significant biological attributes, it is also a commercially exploited species, with an annual catch totaling between 8,600 – 10,000 tons (20,21). As the pearl mullet ranks among the most commonly consumed freshwater fish in the area, and to our knowledge, no prior research has explored the extraction of collagen from its skin and bones, our study aims to ascertain whether these components can serve as a viable alternative source of collagen.

## 2. Material and methods

### 2.1. Materials

Pearl mullet (*Alburnus tarichi* Güldenstädt, 1814) fish weighed (average weight 52 g / each) about 2 kg (approximately 40 individuals) was purchased from the fish market in May 2019, then brought to the laboratory and cleaned, its skin and bones were separated from the body and they were frozen at -20 °C until reuse. Fish skin and bones were thawed in the refrigerator at +4 °C and later was brought to room temperature before application of the extraction procedure.

### 2.2. Methods

#### 2.2.1. Sample Preparation

The preparation of the collagen samples was performed with minor modifications of the method that was previously described by Nagai and Suzuki (2000a) (22). All sample preparation procedures were carried out at not exceeding +4 °C.

#### 2.2.2. Characterization of Collagens

##### Collagen Yields

Collagen yield was calculated based on the dry weight of the material that was initially used (formula is given below).

$$\text{Collagen Yield} \left( \frac{g}{100g} \right) = \frac{\text{Weight of lyophilized collagen}}{\text{Initial weight of lyophilized fish skin}} \times 100$$

##### Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) analysis of collagen samples was performed as previously described by Kittiphattanabawon et al. (2005) (23). Lyophilized collagen samples were gelled with 0.05 M acetic acid at a solid/liquid ratio of 1:40 (w/v) and then stored at +4 °C for two days. Measurements were done by using Mettler Toledo, Model DSC

3, (Schwerzenbach, SWITZERLAND). The samples in gel form, ranging from 5 to 10 mg, were measured in an aluminum pan. Screening was conducted within a temperature span of 10 °C, with an incremental rise of 1 °C per minute. Liquid nitrogen served as the cooling agent, and an empty aluminum container functioned as the reference, with temperature calibration achieved through an indium thermogram. The maximum transition temperature ( $T_m$ ) and total denaturation enthalpy ( $\Delta H$ ) were calculated by use of the DSC thermogram.

#### X-Ray Diffraction Analysis

Crystal structures of lyophilized collagen samples were determined at 0.5 °C/min scan speed and 0.02 °C step interval using an X-ray diffraction (XRD; PANalytical X'Pert High Score Empyrean, 45kV, 40mA) with CuK $\alpha$  ( $\lambda=1.54$ ) radiation in the scanning range of 5 °C to 45 °C.

#### Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of collagen were obtained from 2 mg collagen in about 100 mg of potassium bromide (KBr) under dry conditions. All spectra were performed using a JASCO ATR Pro One Model 6700 FT/IR spectrometer (JASCO International Co. Ltd., Hachioji, Tokyo, Japan) at a data acquisition rate of 4 cm<sup>-1</sup> from 4000 to 600 cm<sup>-1</sup>. Analysis of spectral data was performed using Spectra Manager TM II cross-platform software program (JASCO).

#### UV-Vis Measurement

UV spectra of collagen samples were obtained using a Cary 100 UV-Vis Spectrophotometer (Agilent Technologies). The samples were dissolved in 0.5 M acetic acid at a concentration of 0.2 mg/mL. Readings were made against 0.5 M acetic acid (negative control) in the wavelength range of 200 – 400 nm.

#### Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was performed using the Quanta 650 model, FEI®, (Columbus, Ohio, USA). The surface of the samples was made conductive by coating them with Gold-Palladium (Au/Pd) (approximately 2Å/second). Samples were observed using 30 kV, and EDS technique was used to determine the major compounds of the samples surface regions.

#### Amino Acid Composition

Collagen samples were hydrolyzed under vacuum in 6 N HCl for 24 hours at 110 °C. Amino acid analysis was performed with HPLC (Shimadzu model Nexera-X2 device). Two micro liters of the derivatized sample was injected into the Shimadzu shim-pack XR-ODS II column. Column oven temperature was adjusted to 40 °C. The used mobile phases were (A) KH<sub>2</sub>PO<sub>4</sub> solution (1 mM K<sub>2</sub>HPO<sub>4</sub> in water) and (B) Acetonitrile/Methanol/Water (45/40/15). Amino acids were defined and calculated according to the retention times and peak areas of the standards.

### 2.3. Statistical Analysis

In the current study, the results are expressed as mean  $\pm$  standard deviation. One Way ANOVA was used to determine the differences between groups. Any  $P$  value below 0.05 ( $p < 0.05$ ) were considered as statistically significant.

## 3. Results

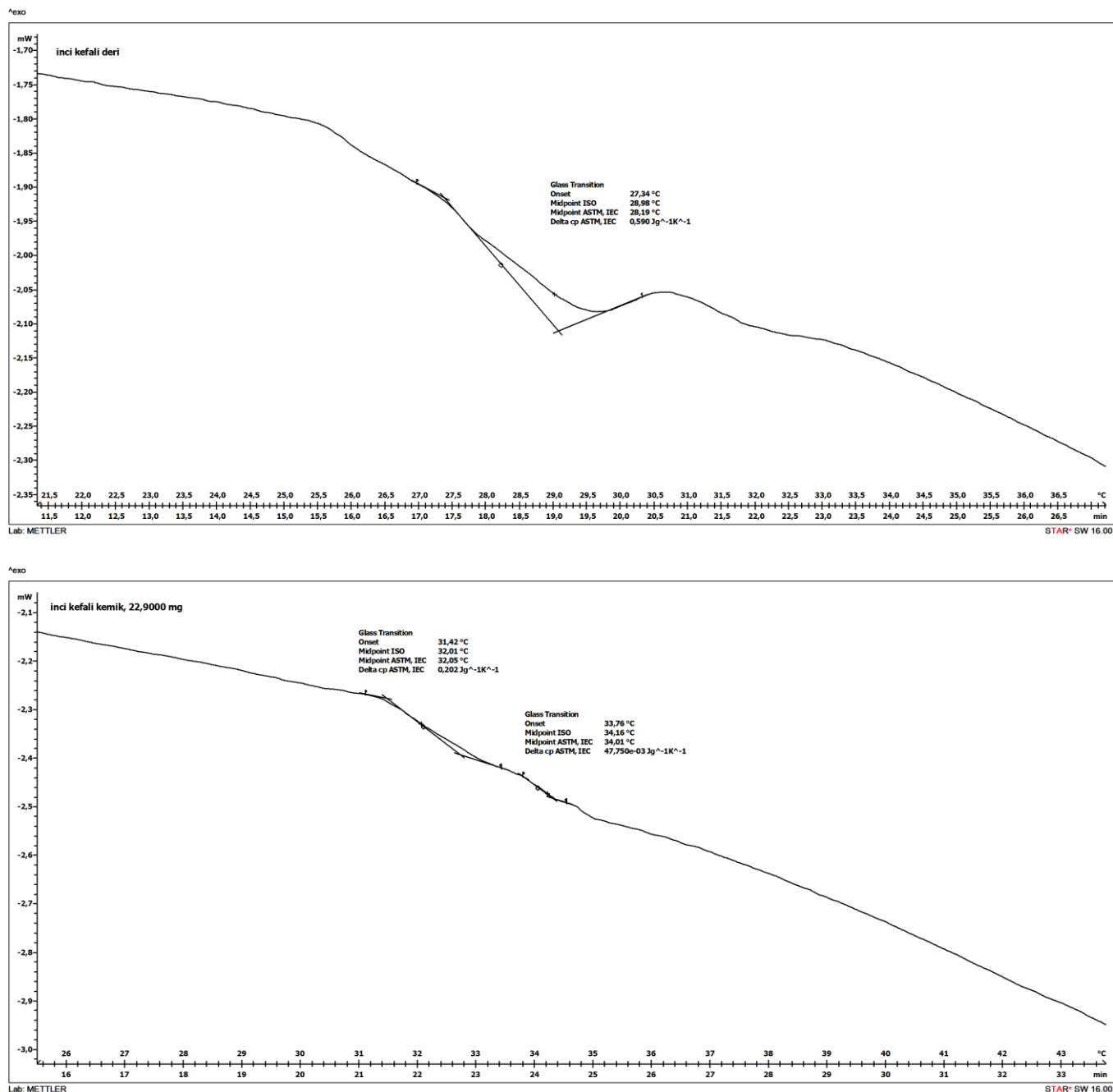
### 3.1. Collagen Yield

Based on the wet weight, the yields of acid-soluble collagens extracted from pearl mullet skins (ASC-S) and bones (ASC-B) were 49.09% and 22.95%, respectively. Collagen yield obtained from the pearl mullet skins was found to be higher than their bones ( $p < 0.05$ ).

### 3.2. Thermal Stability of Collagen by Differential Scanning Calorimeter (DSC)

The maximum transition temperatures ( $T_{max}$ ) of acid-soluble collagen extracted from template skin and bones dissolved in 0.5 M acetic acid are shown in Figure 1.  $T_{max}$  and enthalpy ( $\Delta H$ ) value of ASC-S was found as 28.98 °C, 0.590 J/g. However, there were two different  $T_{max}$  and enthalpy ( $\Delta H$ ) values for ASC-B and they were found as 1<sup>st</sup> 32.01 °C, 0.202 J/g and 2<sup>nd</sup> 34.16 °C, 0.04775 J/g. The thermal stability of collagen is affected by its amino acid composition, especially imino acids constitution. While proline and hydroxyproline provide the spatial structure of collagen with pyrrolidine rings, hydroxyproline increases the thermal stability of collagen by forming inter-chain hydrogen bonds that stabilize the triple helical structure of collagen (24). Therefore, the  $T_{max}$  value has a positive relationship with the

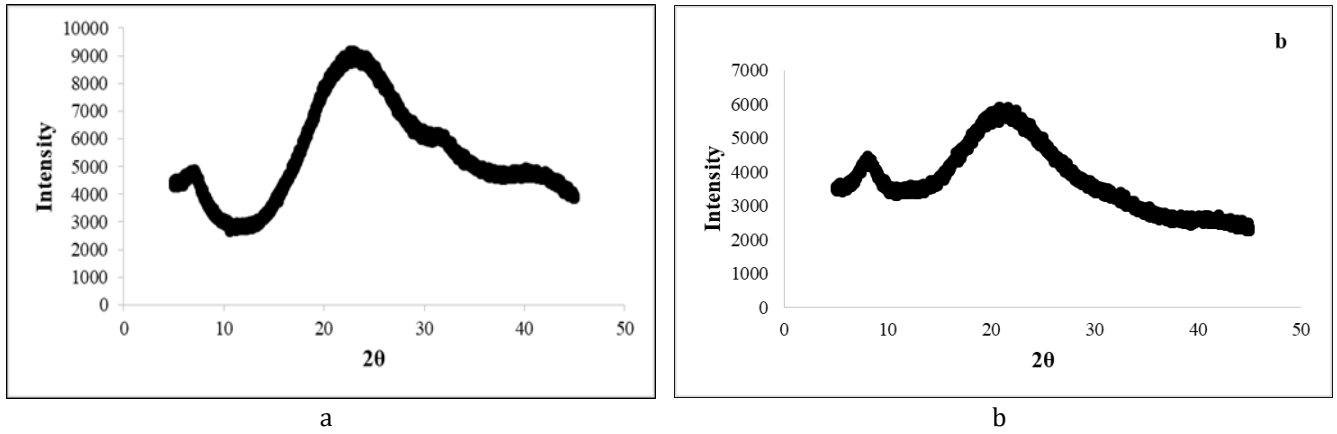
imino acid content. In fact, the reason why the  $T_{max}$  value of cattle skin collagen is higher than the value we obtained at 40.8 °C is the higher amount of imino acid it contains (25).



**Figure 1** DSC thermogram of ASC-B (a) and ASC-S (b) from the skin and bone of pearl mullet dispersed in 0.05 M acetic acid.

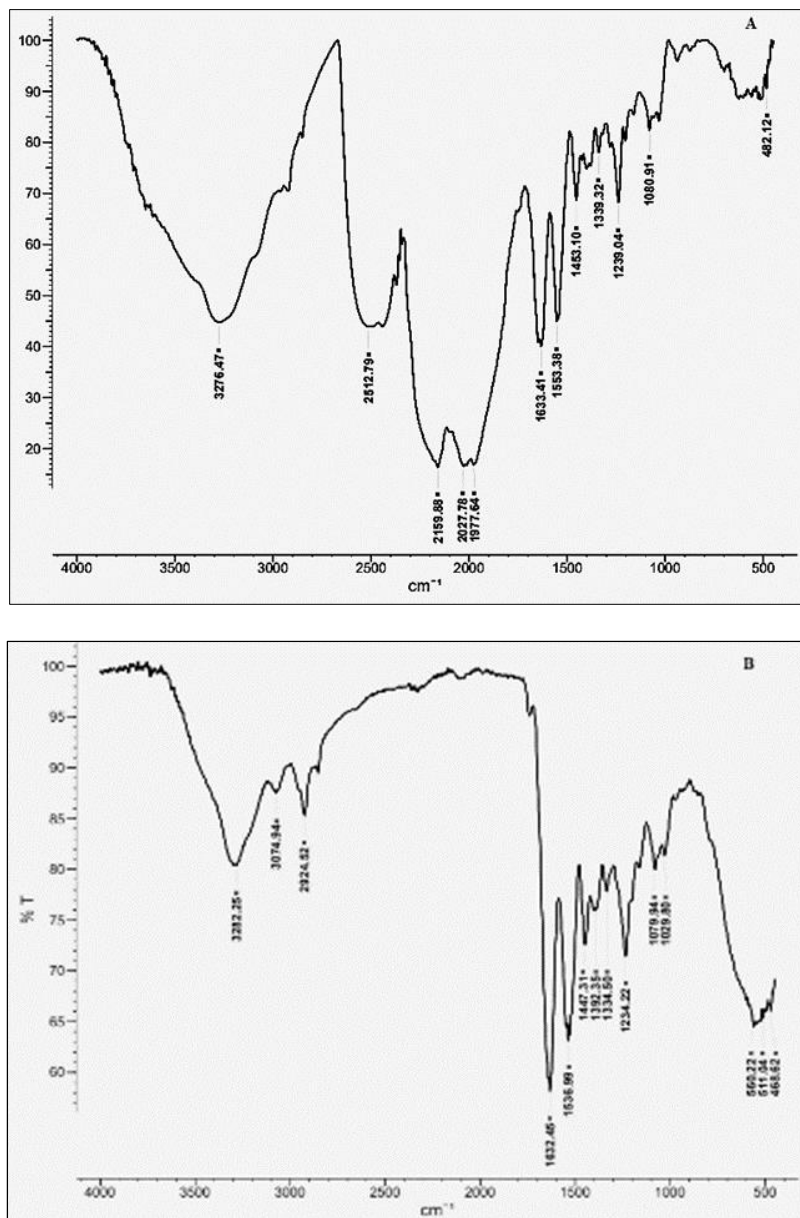
### 3.3. X-Ray Diffraction (XRD)

XRD is often used to analyze the crystal structure of polymers. When X-ray encounters crystalline particles, refraction occurs and the position and density of the diffraction peak reflects the structural properties of the crystals (26). As shown in Figure 2, the XRD curve for both ASC-S and ASC-B has characteristic two break peaks at diffraction angles ( $2\theta$ ) of approximately 6.91° and 22.90° for ACS-S and 8.04° and 22.96° for ASC-B. The first sharp peak is related to the triple helix structure of the collagen, while the second large peak indicates the distance between the chains. These results confirm that both of the collagens preserve the triple helix structure and is not denatured.



**Figure 2** X-ray diffraction spectra of pearl mullet's ASC-S (a) and ASC-B (b)

### 3.4. Fourier Transform Infrared (FTIR)

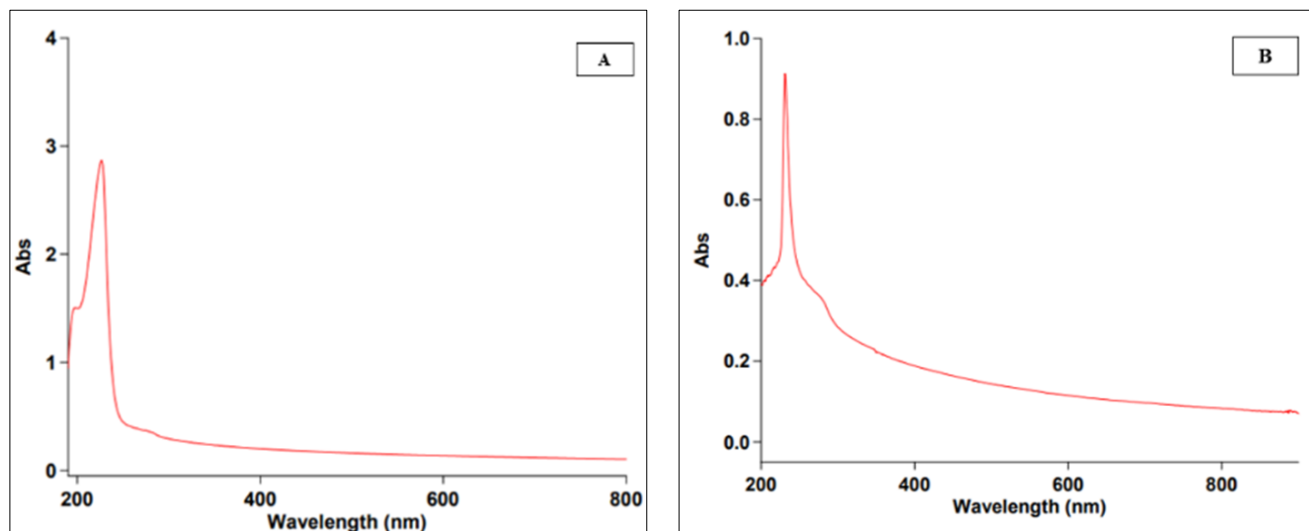


**Figure 3** The FTIR spectra of acid-soluble collagens from skin (A) and bone (B) of pearl mullet

FTIR spectra of collagen extracted from pearl mullet skins and bones are shown in Figure 3. Collagen showed similar spectral properties with five characteristic collagen absorption bands (amide A, amide B, and amide I, II, and III), indicating the presence of high proline and hydroxyproline amino acids in the collagen molecule, these are typical bands for collagen and they mean that the obtained collagen is type I collagen. Amide A absorption peaks of ASC-S and ASC-B were found to be 3276.47 and 3282.25  $\text{cm}^{-1}$ , respectively.

### 3.5. Ultraviolet and Visible Light (UV-Vis) Absorption Spectroscopy Analysis

UV-Vis spectroscopy is a tool that can be employed to evaluate the purity of collagen (27). ASC-S and ASC-B UV-Vis measurement results are shown in Figure 4. There is a single absorption peak and showed maximum absorbance at 230 and 232 nm, respectively.

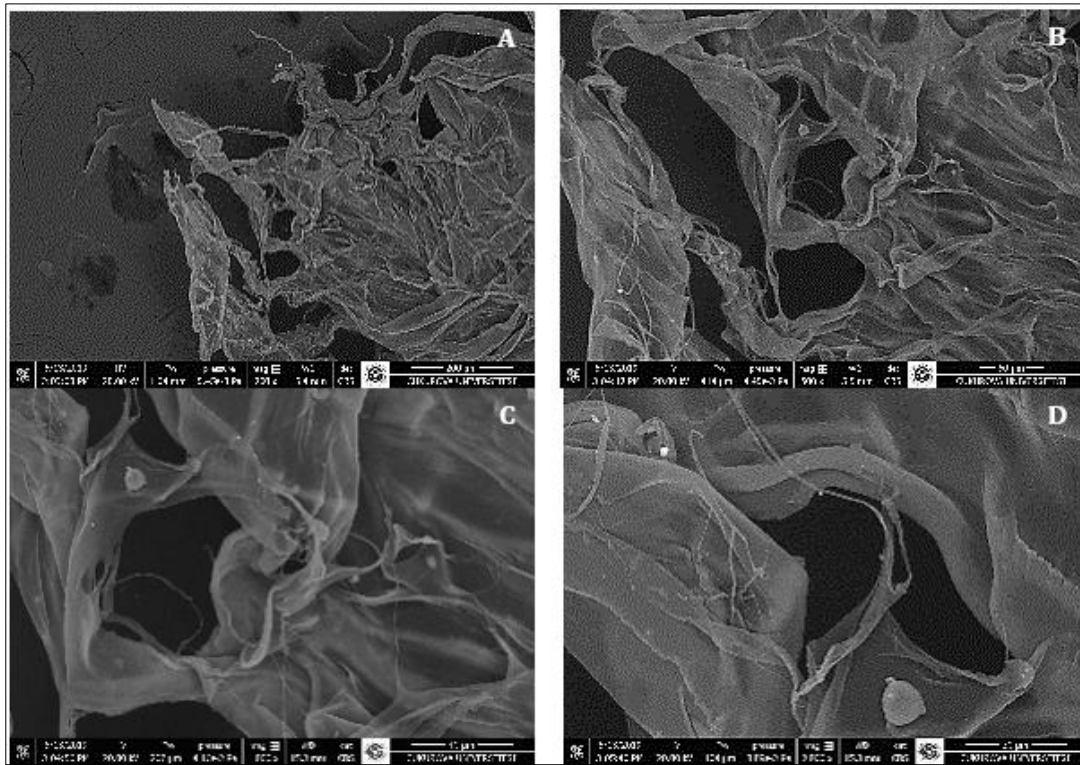


**Figure 4** UV- Spectra of acid-soluble collagens from skin (A) and bone (B) of pearl mullet

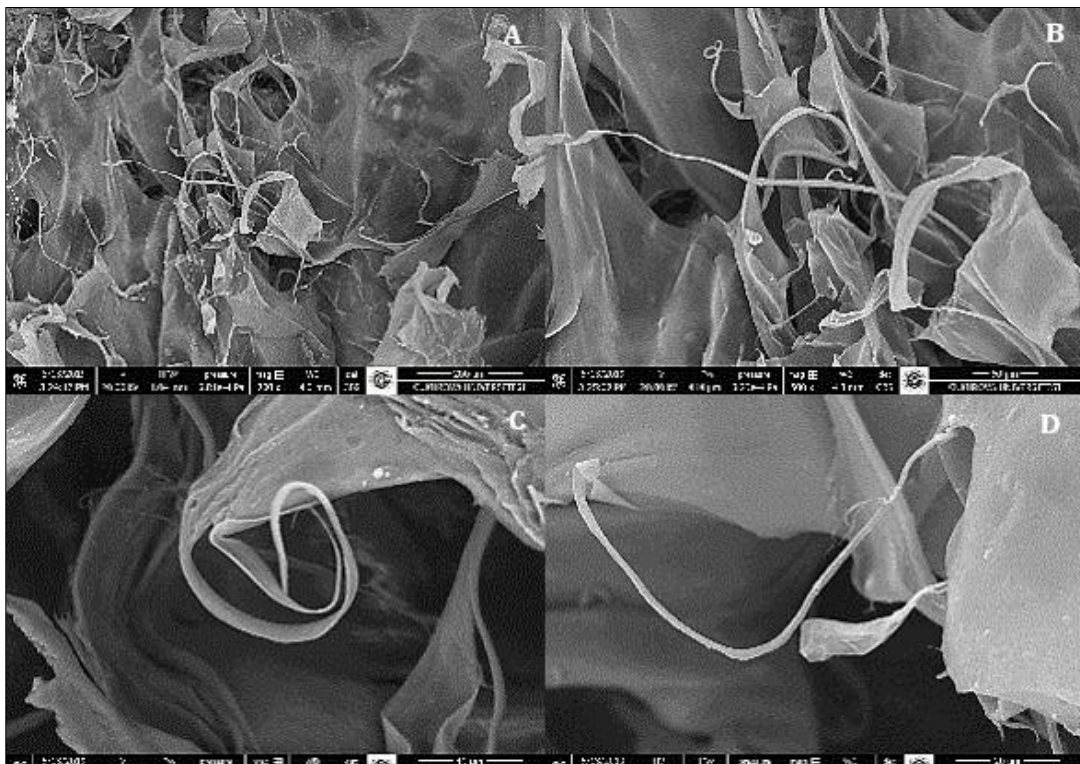
### 3.6. Scanning Electron Microscopy (SEM)

The morphological structures of the extracted lyophilized (freeze dried) collagens (ASC-S and ASC-B) were visualized by scanning electron microscopy (SEM) under three different magnifications  $\times 200$ ,  $\times 500$ ,  $\times 1000$  and  $\times 2,000$  (Figure 5 and Figure 6). Both of the lyophilized collagens were seen as soft, white, and spongy with a porous structure when naked eye observations were made. However, when the observation was done with SEM, both of the collagens were found to have a dense, irregular, and partially wrinkled surface image bound by randomly wrapped filaments. This is probably due to dehydration during lyophilization. Likewise, some similar results have been reported by various research such as the observation of collagen obtained from *Amur sturgeon* skin (28) and *Istiophorus platypterus* skin (29).

In this study, both of the studied collagens were alike in many ways. They represent poor organization, intersecting fibers, entangled bundles, and some fibrils have been found to have intricate meshes in contact with others. The fibrils of different thickness of both collagens were intertwined throughout the porous matrix. As a result, the SEM images of the collagens support that they have type I collagen with fibrillar structure.



**Figure 5** SEM images of acid-soluble collagen from skin of pearl mullet A:  $\times 200$ , B:  $\times 500$ , C:  $\times 1,000$ , D:  $\times 2,000$



**Figure 6** SEM images of acid-soluble collagen from bone of pearl mullet A:  $\times 200$ , B:  $\times 500$ , C:  $\times 1,000$ , D:  $\times 2,000$

### 3.7. Amino Acid Composition

The amino acid compositions of acid-soluble collagen extracted from pearl mullet skin (ASC-S) and bones (ASC-B) are shown in Table 1. Both ASC-S and ASC-B collagens have been determined to have similar amino acid compositions. As expected, the ASC-S and ASC-B samples were high in glycine (Gly), proline (Pro), and hydroxyproline (Hyp) due to the characteristic (Gly-Pro-Hyp) triple-helix repeats of all collagens. Tryptophan and cysteine were not detected. Proline and hydroxyproline found in both ASC-S and ASC-B, which are important imino acids that ensure the structural integrity of collagen. The total amount of imino acid (Pro + Hyp) is 18.82% and 18.77% for ASC-S and ASC-B, respectively, and is statistically similar ( $p > 0.05$ ).

**Table 1** Amino acid profiles (g/100g protein) of skin (ASC-S) and bone (ASC-B) collagens of pearl mullet

Amino Acid	ASC-S	ASC-B
Aspartic acid	5.23±0.29	5.25±0.47
Glutamic acid	8.58±0.26	8.67±0.18
Serine	5.13±0.11	5.17±0.28
Glycine	28.54±0.23	28.81±0.18
Threonine	3.08±0.04	3.20±0.01
Arginine	7.17±0.03	7.72±0.12
Alanine	9.88±0.04	9.94±0.08
Tyrosine	0.35±0.04	0.44±0.01 <sup>b</sup>
Cysteine	0	0
Valine	1.03±0.08	0.89±0.02
Methionine	0.68±0.02	1.32±0.11
Tryptophan	0	0
Phenylalanine	2.27±0.14	2.24±0.22
Leucine	2.04±0.02	2.37±0.05
Lysine	4.61±0.12 <sup>a</sup>	4.33±0.22 <sup>b</sup>
Hydroxyproline	9.47±0.51	9.52±0.08
Proline	9.35±0.22	9.25±0.11
Total imino acid (Hydroxyproline + proline)	18.82±0.29	18.77±0.18

±, represents standard deviations. The superscript letters <sup>a</sup> and <sup>b</sup> indicate to the statistical differences ( $P < 0.05$ ) between groups within the same line.

## 4. Discussion

### 4.1. Collagen Yield

Similar to the current study, Kittiphattanabawon et al. (2005) was found the yield of collagen obtained from *Priacanthus tayenus* bone (1.6%) to be lower than the yield of collagen obtained from its skin (10.9%) (23). The authors also suggest that their ASC-B yields were lower than the current study, whereas their ASC-S yields were higher. Additionally, the obtained results of this study suggested that the collagen yield of this study is higher than the yield of collagen extracted from carp bone (1.06%) (30) by over than 3.5 folds. Unlikely, Dođdu et al. (2019) extracted collagen from silver cheeked pufferfish *Lagocephalus sceleratus* skin and the collagen yield was found to be 50.9%, which is much higher than the current study (31). In another study, Benjakul et al. (2010) was found the collagen yield (7.7% and 7.1%) extracted from *Priacanthus tayenus* and *Priacanthus macracanthus* skin, both of these results represented very close collagen yield to the current study (32). However, Wei et al. (2019) isolated and characterized collagen from sturgeon fish and reported that the collagen yield was 5.73% (33). The current study's results represent more collagen yield than Wei et al. (2019)'s study.



#### 4.2. Thermal Stability of Collagen by Differential Scanning Calorimeter (DSC)

The pearl mullets' imino acid content in ASC-S was 18.82%, while the ASC-B was 18.77%. This value was found to be lower than the collagen obtained from many cold climate fish (34). This explains why collagen isolated from subtropical and tropical fish have better thermal stability (35).

#### 4.3. X-Ray Diffraction (XRD)

As it mentioned above, Figure 2 shows the XRD curve for both ASC-S and ASC-B has characteristic two break peaks at diffraction angles ( $2\theta$ ) of approximately  $6.93^\circ$  and  $22.78^\circ$  for ACS-S and  $7.68^\circ$  and  $22.17^\circ$  for ASC-B. The first sharp peak is related to the triple helix structure of the collagen, while the second large peak indicates the distance between the chains. These results confirm that both of the collagens preserve the triple helix structure and is not denatured. Similar results have been obtained by several studies include carp scale collagen study by Zhang et al. (36), *Oreochromis niloticus* skin collagen (37), *Gadus macrocephalus* skin collagen (Sun et al. 2017b), Atlantic cod and Atlantic salmon skin collagen (38), and skins and bones of *Arabibarbus grypus* (39) and *Luciobarbus esocinus* (40).

#### 4.4. Fourier Transform Infrared (FTIR)

FTIR spectra of collagen extracted from pearl mullet skin and bones showed that Amide A absorption peaks of ASC-S and ASC-B were  $3219.58$  and  $3290.93$   $\text{cm}^{-1}$ , respectively. According to Sai and Babu (2001), Amide A band generally originates from N-H stress vibration and occurs in the wavelength range of  $3400$ - $3440$   $\text{cm}^{-1}$  (41). However, Doyle et al. (1975) mentioned that when the NH group of a peptide is involved in the hydrogen bond, the position can shift to a low frequency, usually around  $3300$   $\text{cm}^{-1}$  (42). Therefore, the shift of amide A towards lower wavelengths, as observed in this study, indicates that hydrogen bonded hydroxyl groups are present in both skin and bone collagens. Amide I band of ASC-S and ASC-B were  $1637.27$  and  $1632.45$   $\text{cm}^{-1}$ , respectively. These results are consistent with the  $1625$ - $1690$   $\text{cm}^{-1}$  range that is the position of the general amide I bands of collagen. Similar results were acquired by Shalaby et al. (43). Amide II band was found to be  $1541.81$   $\text{cm}^{-1}$  for ASC-S and  $1548.56$   $\text{cm}^{-1}$  for ASC-B, amide II band is generally seen at wavelengths of  $1550$ - $1600$   $\text{cm}^{-1}$  (44), its shift to lower wavelengths represents the formation of hydrogen bond. The triple helix structure of collagen can also be presented by the ratio of the density between the absorption peak of amide III and the absorption peak of  $1450$   $\text{cm}^{-1}$ . In our study, the Amide III absorption peaks of ASC-S and ASC-B were  $1231.33$  and  $1236.15$   $\text{cm}^{-1}$ , respectively. The ratio of the density between the absorption peak of Amide III and the absorption peak of  $1450$   $\text{cm}^{-1}$  was  $1.18$  (ASC-S/ASC-B). Matmaroh et al. (45) stated that a value approaching  $1.0$  indicates that collagen still has a triple helix structure.

#### 4.5. Ultraviolet and Visible Light (UV-Vis) Absorption Spectroscopy Analysis

This spectroscopy is usually used in order to assess the purity of collagen (27). As presented in Figure 4, there is a single absorption peak and showed maximum absorbance at  $230$  and  $232$  nm, respectively. This range is the distinctive absorbance of type I collagen. Generally, the highest protein absorbance is observed at  $280$  nm; however, our results have shown maximum absorbance at  $230$ - $232$  nm due to the absence of tryptophan amino acid and low tyrosine amino acid content in both ASC-S and ASC-B.

#### 4.6. Scanning Electron Microscopy (SEM)

The morphological characteristics of the extracted and freeze-dried ASC-S and ASC-B were noted as soft, white, and spongy with a porous structure when observed without magnification. However, upon examination of the SEM results, both types of collagens displayed a dense, irregular, and partially wrinkled surface image surrounded by randomly entwined filaments. This observation may be attributed to the dehydration process during lyophilization. Some similar results have been reported by various research groups, the observation results of collagen obtained from, *Luciobarbus esocinus* (40), *Arabibarbus grypus* (39), *Salmo salar* L. (46), *Amur sturgeon* (28) and *Istiophorus platypterus* skins and/or bones (Tamilmozhi et al. 2013).

#### 4.7. Amino Acid Composition

The amino acid compositions of ASC-S and ASC-B collagen of pearl mullet presented results as expected. As with other collagens, tryptophan and cystine were not detected (47–49). Proline and hydroxyproline found in both ASC-S and ASC-B are important imino acids that ensure the structural integrity of collagen. In the current study, the total amount of imino acid (Pro + Hyp) were found  $18.82\%$  and  $18.77\%$  for ASC-S and ASC-B, respectively, and they are statistically similar ( $p > 0.05$ ). This value is similar to the values reported for *Oreochromis niloticus* ( $19.8$  –  $19.4\%$ ) (50), *Luciobarbus esocinus* ( $19.33$  –  $19.18$ ) (40), *Arabibarbus grypus* ( $19.28$  –  $19.55$ ) (39), and Carp ( $19.4\%$ ) (51); higher than the values reported for tilapia, ( $17.75\%$ ), grass carp ( $17.90\%$ ) and silver carp ( $17.78\%$ ) (52); lower than the value reported for

tilapia (25.4%) (53). The variation in imino acid content among distinct species can be attributed to the differences in their habitats, particularly the variations in temperature (54).

## 5. Conclusion

In summary, the extraction and characterization of collagens from pearl mullet skin and bone were successfully achieved. Both obtained collagens were identified as type I collagen, exhibiting a standard amino acid composition. FTIR and XRD analyses confirmed the preservation of their triple helical structure throughout the extraction processes. Both extracted collagens showed maximum absorption at 230-232 nm with no absorption at 280. The SEM images of both collagens exhibited interconnective pores with lace-like fibers. given the favorable attributes demonstrated by the collagens extracted in this study, there is significant potential for their utilization as a valuable alternative in dietary, medical, and pharmaceutical applications (can be used extensively in various medical applications). For example, the strength and flexibility of the collagen may help in the repair and regeneration of skin and nutraceuticals industries.

## Compliance with ethical standards

### Disclosure of conflict of interest

Authors declares no conflicts of interests

### Author Contributions

Concept: Yasemen Yanar, Mustafa Göçer, and Muhsin Aydın; Sample Collection: Mustafa Göçer; Methodology and applications: Yasemen Yanar and Mustafa Göçer; Literature review, data collection or processing: Yasemen Yanar, Mustafa Göçer, and Muhsin Aydın; Writing: Mustafa Göçer and Muhsin Aydın.

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