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Research Article

Development of a Simple Non-toxic Scale Up Method to Extract Crude Collagen from Yellowfin Tuna (*Thunnus albacares*) Skin

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Abstract

The tuna processing industry generates significant amounts of fish waste with adverse environmental impacts. Therefore, it is important to identify potential ways to reduce waste, such as using waste for industrial-scale production. Thus, the aim of the present study was to develop a simple, innocuous, and scale-up method to extract crude collagen from yellowfin tuna (Thunnus albacares) skin. Collagen extraction was carried out through pre-treatment and soaking in acid to extract acid-soluble collagen. Collagen was extracted by acetic acid-soluble (0.4 M, 0.5 M, 0.6 M) and lactic acid-soluble (0.5 M, as control) methods. Sodium chloride (10% w/v) was used for the precipitation of crude collagen. For the agitation purpose, an especially prepared agitator was used. Then, 10% SDS-PAGE analysis and FTIR analysis were carried out to identify the extracted crude collagen. Collagen from 0.6M lactic acid-treated fish skin had the highest yield of 14.46 ± 0.56 % based on the skin's wet weight ($P < 0.05$). All the crude collagen extracts of different concentrations exhibit Amide A, Amide B, Amide i, Amide ii, and Amide iii, which are the characteristic spectra of collagen in the FTIR spectrum (1700- 1600 cm^{-1}). Based on the SDS-PAGE analysis, the crude collagen extracts have two α bands and one β band. Therefore, they are classified as type I collagen. Thus, this study revealed that tuna skin is an excellent source to extract collagen for commercial uses and the 0.6M lactic acid-treated method was the best method for extracting collagen from yellowfin tuna skin.

Keywords*: Acetic acid, extraction, fish collagen, lactic acid, tuna fish*

1. Introduction

The global market for fish processing has been growing steadily during the last few decades. In terms of value, the fish processing market is expected to reach USD 222.71 million by 2021, at a compound annual growth rate (CAGR) of 3.8% from 2016 to 2021. Factors such as high nutritional value, additional health benefits of processed fish and fish products, development in the aquaculture industry, and increasing health consciousness towards value-added fish products are driving the global fish processing market (Fish Processing Market, 2021). Tunas are among the most important fish commodities globally, and the worldwide demand for tuna is rising. In tropical waters, there are two major species of tuna; namely, *Thunnus albacares* (yellowfin tuna) and *Thunnus obesus* (bigeye tuna) catch on a local and commercial scale. Tuna fisheries are an essential source of food and trade profits for developed and developing countries, supplying the world market with a volume of 5.2 million metric tons and a land value of $11.7 billion (McKinney *et al*., 2020; McCluney *et al*., 2019).

As production increases, fishery waste has received global attention in recent years as it has influenced several biological, technological, and functional factors and socioeconomic drivers (Arvanitoyannis and Kassaveti, 2008). Fish waste management represents an attractive topic because it suggests a possible way to address the environmental impacts of fishery discards. Simultaneously, it provides a tool to exploit them as a food source in a sustainable way. The use of fish waste and by-products allows for the reduction of waste discharged (Caruso, 2015). Several fish processing companies started producing by-products of fish processing waste and processed about 170,000 tons annually. Fishery waste and by-products are an essential source of high-value-added compounds, however, the potential risks associated with the presence of contaminants must be considered before their use (Caruso, 2015). Hence, there is a great scope for using this widely accessible fish processing waste to extract value-added products such as collagen (Kim and Park, 2005).

Collagen is the principal protein in connective tissue and is the most abundant protein in mammals, such as fish (Bae, 2007). Collagen has become very useful in both biomedical and non-biomedical industries in this modern era, with an extended range of usage (Bret *et al*., 2011). Collagen biomaterials have been widely used in industry as cell scaffolds and growth factors for wound dressings, soft tissue augmentation, and dietary supplements due to their biocompatibility, stability, and bioactivity (Ber *et al*., 2005; Tanaka *et al*., 2009). Collagen has been shown to be an important component in the food and beverage industry. It is considered a modern food ingredient in the food industry and is widely used in the food and beverage industries to improve the flexibility, consistency and stability of food and beverage products (Hashim *et al*., 2015). The most widely used is bovine collagen which has been a common source for cosmetic applications today. However, due to the risks of Bovine

Spongiform Encephalopathy (BSE) and Foot-and-mouth disease (FMD) in recent years, the source of collagen must be reconsidered. Therefore, much attention has been focused on the isolation of collagen from skin, scales, and bones, which are byproducts of fish processing that are used as low-value food or simply discarded as waste (Jamilah *et al*., 2011).

Sri Lanka has an entrenched commercial fishing trade with 14 deep-sea fishing harbors. According to available resources, the total fish production in Sri Lanka in 2016 was 530,920 metric tons, of which 17,593 metric tons were exported to other countries. The main fish product categories are Tuna, Sward tail, Marlin and other species. Also, 34 EU certified fish processing plants are well established in Sri Lanka (Fish Processing Market, 2021; Fisheries statistics, 2018). Sri Lanka's tuna fishery is developing rapidly with the development of offshore and deep sea fishing activities (Jayasooriya and Bandara, 2013). Yellowfin tuna has become an important species in Sri Lanka's fisheries with a total production of 38,960 metric tons in 2017 (Fisheries statistics, 2018). A major problem in Sri Lanka is the disposal of fish waste from fish processing industries without causing any environmental impact. Around 6000 Mt of fish waste is estimated to be produced from tuna processing plants in Sri Lanka alone. Currently, fish waste is not entirely used in the manufacturing of value-added goods and products, except for the manufacture of fish meals from part of the island's fish waste (Ariyawansa, 2013). Efficient recovery and use of by-products can minimize environmental pollution and maximize economic benefits for waste (Kim and Mendis, 2006).

A significant number of by-products such as bones, scales, viscera and skin result from the processing of tuna by the fishing industry. In order to increase their economic value and efficient use, these by-products should be processed. Significant quantities of collagen are known to exist in the skin, bones, and fins of fish and it is possible to transform collagen into gelatin (Muyonga *et al*., 2004; Liu *et al*., 2015; Nurilmala *et al*., 2017). On the other hand, the main sources of commercially available collagen are porcine and bovine sources. However, some communities may have problems meeting their religious requirements with such collagen products. In order to solve these problems, fish collagen may thus be an excellent alternative. Previous researches have reported that fish by-products (fish skin) can be used as an alternative material for collagen production in the fish processing industry (Karim and Bhat 2009; Jamilah *et al*., 2013; Wu *et al*., 2014). However, studies regarding the extraction method of collagen at the commercial level are still limited. Therefore, the aim of this study was to develop a simple non-toxic scale-up method to extract yellowfin tuna skin from crude collagen. Furthermore, identify whether tuna skin wastage can be used as an alternative source for generating collagen on a commercial scale by characterizing the extract collagen's biochemical properties.

2. Material and Methods

2.1. Skin samples collection, preparation

Fresh fish skin samples were collected from Ceylon Fresh Sea Food Pvt Ltd and immediately transported to Uva Wellassa University's laboratory under refrigerated conditions (4 ^{o}C) by packing within the Low-Density Polyethylene (LDPE) bags and styrofoam boxes within 7 hours. The fish skin samples were put into polythene bags and they were appropriately sealed and stored under freeze conditions (-20 °C) until they were used for the crude collagen extraction. Before the extraction procedure, skins were manually cleaned by removing scales, bones, and flesh using a filleting knife, and the cleaned skin parts were weighed. After that, the cleaned skin parts were cut into small pieces of dimensions 2 × 2 cm^2 using a scissor and finally washed using distilled water.

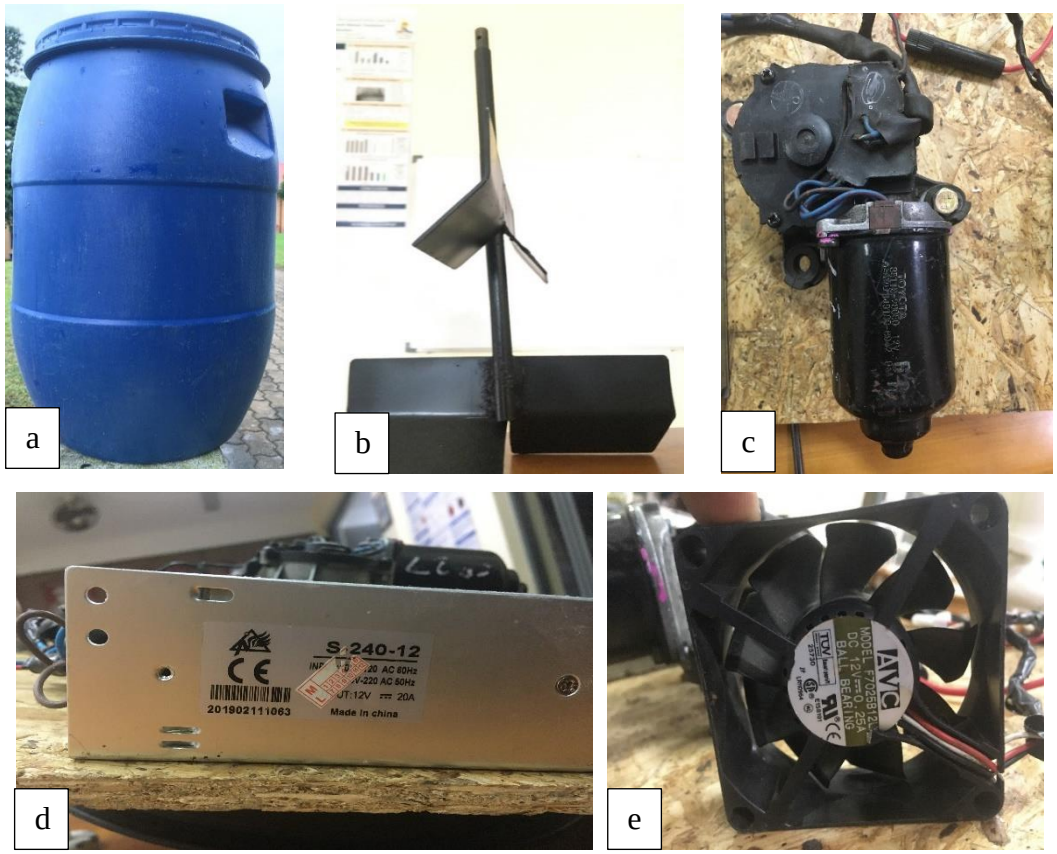

Figure1: Instruments used for the preparation of special agitator (a) 30 L Container, (b) metal fin, (c) 12 V Toyota Dolphin van viper Motor, (d) AC-DC convertor power pack Model Number S240-12, and (e) cooling fan (12 V, 20 A).

2.2. Preparation of special agitator

A special agitator developed by using 12 V Toyota Dolphin van viper Motor, AC-DC convertor power pack (Model Number S240-12, 20 A and 12 V-220 V), 30 liters container, axel (0.37 m) with a plate (0.1 m×0.25 m) and a cooling fan (12 V,20 A). All the items were properly assembled to complete the agitator as shown in Figure 1.

2.3. Pre-treatment of fish skin

During the extraction process, the pre-treatment process was followed based on the method described by Nagai and Suzuki (2000) with slight modifications. First, 15 L of 0.1 N NaOH solution was prepared. Then, 3 kg of cleaned skin was weighed and put into the agitator and it was filled using the prepared NaOH solution at a ratio of 1:5. All experimental steps were carried out at room temperature and the mixture was continuously stirred for two days and the NaOH solution was changed in every 24 hours.

2.4. Extraction of crude collagen from fish skin

Acid soluble collagen was extracted using the method described by Nagai (2000). The prepared skin samples were washed thoroughly using distilled water and divided into equal portions. Each part was put into a plastic bottle for further extraction.

Acid soluble collagen (ASC) was extracted by Acetic acid-soluble and Lactic acid-soluble extraction methods. In the Acetic acid-soluble extraction method, an equal mass of fish skin was reconstituted into plastic bottles of equal dimensions at a ratio of 1:4 (w/v) of fish skin to acetic acid (0.5 M) and the replicates were prepared. Then, the lid was closed and kept for 3 days for the extraction of collagen. After 3 days, the bottle's content was filtered using two layers of cheesecloth and fish skin parts were discarded and the filtrate was used for further processing. After filtering, each bottle's filtrate was centrifuged at 4300 rpm for 30 minutes at 4 ^{o}C and the supernatant was collected and the precipitate was discarded. Then, for the precipitation of collagen, 10% Sodium chloride solution was added to the supernatant and kept for three days. After that, the weight of the crude collagen was taken out.

In lactic acid-soluble extraction, the equal mass of fish skin samples was divided and put into plastic bottles with equal dimensions in the ratio of 1:4 (w/v) of fish skin to Lactic acid replicates. The lactic acid replicates were prepared with (0.4 M, 0.5 M, and 0.6 M) concentrations. Then, the lid was closed and kept for three days for the extraction of collagen. After three days, the bottle's content was filtered using two layers of cheesecloth and fish skin parts were discarded and the filtrate was used for

further processing. After filtering, each bottle's filtrate was centrifuged at 4300 rpm for 30 minutes at 4 °C and the supernatant was collected and the precipitate was discarded. Then, for the precipitation of collagen, 10% Sodium chloride solution was added to the supernatant and kept for three days. After that, weight of the crude collagen was taken out.

2.5. Analysis of crude collagen

2.5.1. SDS-PAGE analysis

SDS-PAGE gels (10%) were prepared according to the method described by Sambrook and Russel (2006). The molecular weight of peptides was determined by comparing the bands of the sample to the marker.

2.5.2. Fourier transform Infrared spectrum (FTIR) analysis

For the FTIR analysis, Collagen samples (Samples of commercial collagen, reference standard and lyophilized collagen) were ground with a mortar and pestle. For FTIR-KBr discs, pellets were prepared using a press by adding 1–3 mg of ground collagen with about 100 mg of KBr. Background noise was corrected with pure KBr data. The samples' FTIR spectra were obtained with 32 scans, ranging from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} (Thompson *et al*., 2009).

2.6. Statistical Analysis

All the data were analyzed by using Minitab 17.0 version statistical software package and Microsoft Office Excels software package. Data were expressed as means with standard deviation with a minimum of three independent measurements. One-way ANOVA and Tukey test were used to analyze the data in the analysis of functional properties and statistical significance was considered at $p<0.05$. Three replicates were used for each experiment**.**

3. Results and Discussion

3.1. Special agitator for the agitation of the tuna skin

The agitator was prepared as shown in Figure 2, which was used for all agitation steps throughout the extraction procedure of crude collagen from tuna skin. The agitator is having 30 liters capacity, 12 voltage, and 50 rpm rotational speed.

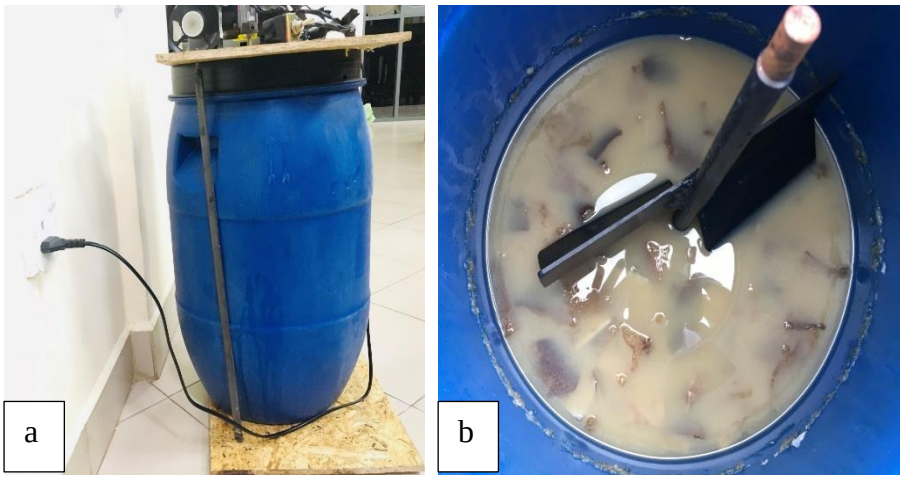

Figure 2: (a) Overall finished look of the agitator, (b) inside view of the agitator during operation.

3.2. Crude collagen extraction

According to the results, higher concentrations of lactic acid gave higher collagen yield ($p<0.05$). Yields of collagen with lactic acid extraction were 2.14 ± 0.08, 9.48 ± 0.17 and 14.46 ± 0.53% at lactic acid concentrations of 0.4, 0.5 and 0.6 M, respectively. The collagen yield with acetic acid extraction was 3.30 ± 0.30% for 0.5 M. The skin of this fish was subjected to consecutive 3 days of agitation with NaOH for the pre-treatment. To convert insoluble native collagen to soluble collagen, it involves treatment to destroy the tertiary, secondary and to some extent primary structure of native collagen by breaking non-covalent bonds (See *et al*., 2013). Disruption of non-covalent bonds should ideally occur without breaking any peptide bonds with cleavage of intra and intermolecular covalent cross-links. Controlled hydrolysis is therefore needed for this conversion (Ockerman and Hansen, 1988). NaOH pre-treatment is essential to completely remove the non-collagenous proteins in the fish skin to obtain an accurate yield of collagen (Singh *et al*., 2011). After pre-treatment, fish skins were agitated with acetic acid and lactic acid for 3 days. All impurities were then dissolved and highly viscous solutions were obtained. The first stage of solubilization was the hydration of fibrous collagen on exposure to acids. It has been known that the proteins are denatured below pH 2.0. Therefore, digestion with strong acid for a long time had led to partial chemical hydrolysis of protein (Muralidharan *et al*., 2013). Lactic acid was reported to completely solubilize the collagen within 24h of extraction (Skierka and Sadowska, 2007). It was the best solvent for collagen extraction in terms of its higher yield, as it could completely

solubilize the collagen in a shorter time, as evidenced by Skierka and Sadowska (2007).

The yields of these collagens are mentioned in Table 1. The low yield of collagen obtained using some acids was due to incomplete solubility and it was suggested that intermolecular cross-links still exist in collagen (Sivakumar *et al*., 2000). Previous studies confirm with lactic acid concentration ranging from 0.5 to 0.6M gives a higher yield of collagen in the laboratory scale (Wang *et al*., 2008) and our study proves that it is the same with the large-scale production.

Table 1: Percentage yield of extracted crude collagen

Type of Acid	**Lactic Acid**			**Citric acid (Standard method)**
Concentration (M)	0.4	0.5	0.6	0.5
Collagen yield (%)	2.14 ± 0.08	9.48 ± 0.17	14.46 ± 0.53	3.30 ± 0.30

3.3. Analysis of functional properties of crude collagen

3.3.1. SDS-PAGE analysis

The molecular weight of the obtained collagen was estimated with 10% SDS-PAGE as shown in Figure 3. As reported by previous researchers, the electrophoretic patterns of collagen extracted using acid showed similar patterns of β (supposed to be dimerized collagen), α1, and α2 chains. (Jamili *et al*., 2019, Ariyawansa *et al*., 2017). The basic structure of collagen consists of a triple helix with α1 and α2 chains, and the collagen produced from fish basically types I (Silvipriya *et al*., 2015). The higher acid concentration resulted in thinner bands of collagen and some other bands disappeared, suggesting partial degradation of proteins, as shown in Figure 3.

Collagen contains two α chains (α1 and α2 chains), and one β chain mentioned in Figure 3. Based on these specific bands, this collagen was classified as type I collagen. Collagen can contain high molecular weight components, including β chains, indicating cross-linkages in collagen molecules (Gómez-Guillén *et al*., 2002). However, the cross-linking of collagen in fish skins was generally low, and the high cross-linked molecules (γ chain) are rarely found.

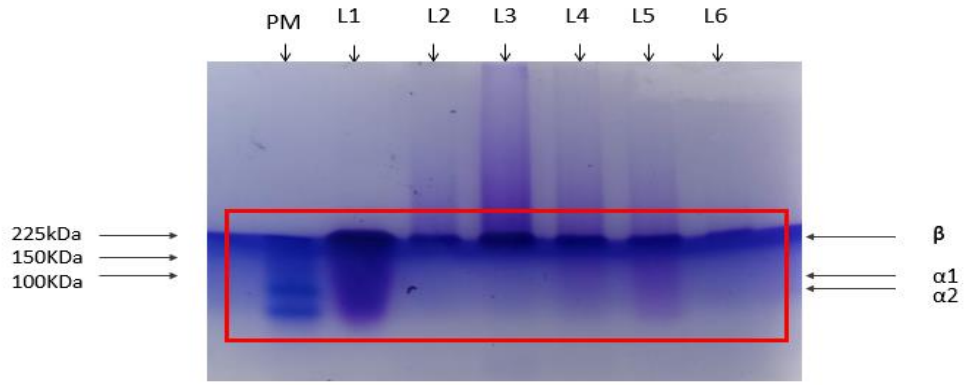

Figure 3: SDS-PAGE gel image of the extracted crude collagen and commercial

3.3.2. FTIR analysis

Functional groups of collagens can be detected by FTIR spectroscopy. Highly polymerized IR spectral data are usually interpreted as vibrations of a structured unit. Polypeptide and protein units generally provide nine characteristic absorption bands, namely amides A, B, and I–VII (Kong and Yu, 2007). This study's results were similar to previous reports (Jamili *et al*., 2019, Ariyawansa *et al*., 2017) as shown in Figure 4. According to the published papers, the collagens from *Thunnus albacares* skin showed nine characteristics of FTIR absorption band, namely A, B and I, VII could be observed in a typical IR spectrum of which amide I band (1700 – 1600 cm^{-1}) was most sensitive and widely used in studies of protein secondary structure.

The spectra of both extracted collagen and commercial collagen showed an amide A band (associated with N–H stretching). The amide B band of extracted collagen was observed as a specific peak in the spectrum (Figure 4 a-d). Polypeptide backbone C–O stretching vibration was found in the range of 1600 - 1700 cm^{-1}. The hydrogen bond formation between N–H stretch and C–O of the fourth residue was confirmed by the absorption peak around 1600 cm^{-1}. N–H in-plane bend and the C–N stretching vibrations were noted around 1500 cm^{-1}. Amide II and amide III were found at the same wave number around 1240 cm^{-1}, further confirming the crude collagen's hydrogen bonding (Wang *et al*., 1994). Therefore, the IR spectra of both the following extracted collagen and commercial collagen show similar characteristics.

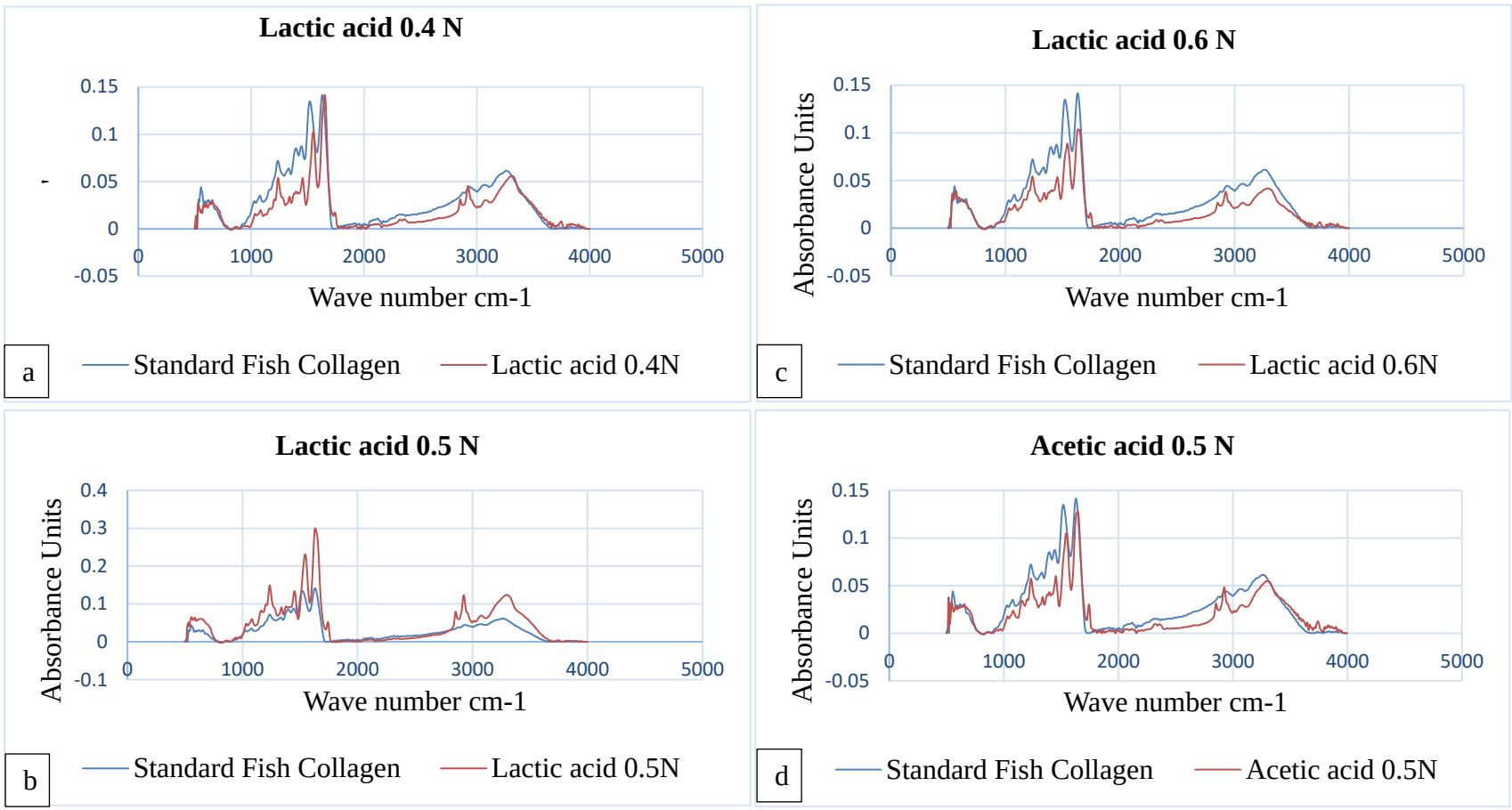

Figure 4: FTIR spectrum of standard fish collagen versus (a) Lactic acid 0.4 N, (b) Lactic acid 0.5 N, (c) Lactic acid 0.6 N and (d) Acetic acid 0.5 N.

4. Conclusion

The results of this study revealed that simple non-toxic scale-up method could be develop to extract crude collagen from Yellowfin Tuna (*Thunnus albacares*) skin and 0.6 M lactic acid was most effective. SDS-PAGE and FTIR-AR analysis confirms that the extracted crude collagen is similar to commercial fish collagen. Furthermore, physical, chemical and enzymatic changes in marine collagen structure should be studied to provide suitable methods for collagen extraction from marine resources.

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