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Research Article

Effect of Ultrasonic Amplitude on the Yield and Properties of Barramundi (*Lates calcarifer*) Skin Collagen

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ABSTRACT

Barramundi skin, a by-product of the fish processing industry, has shown potential as an alternative collagen source. However, the commonly used acid extraction method to produce collagen rendered a low yield requires a lengthy time and is not environmentally friendly. As a result, the adoption of greener technology, such as ultrasound, to improve the conventional extraction process is emerging. This study aimed to investigate the effect of different ultrasonication amplitudes on collagen recovery from barramundi skin. The resulting collagens were evaluated for their protein, hydroxyproline and moisture content, colour, molecular weight distribution, and FTIR spectra. Ultrasound-assisted extraction (UAE) was performed at 40 (UAE40), 60 (UAE60) and 80 (UAE80) % amplitude for 20 min. For comparison, acetic acid extraction was also carried out to produce acid-soluble collagen (ASC). UAE increased the yield ($p<0.05$) of collagen from barramundi skin, with UAE80 exhibiting a 7-fold increment compared to ASC. Increasing the ultrasonic amplitude increased the yield considerably but decreased the hydroxyproline content, indicating a reduction in collagen quality. Furthermore, the protein content and SDS-PAGE profile of the extracted collagens revealed that UAE promoted protein degradation. FTIR spectra indicated that despite slightly varying wavenumbers, no detrimental effect on the triple helical structure was seen following UAE with the presence of amides A, B, I, II, and III. Also, the α_1, α_2 and ß-chains were found in all samples, although the band intensity reduced as the amplitude increased. In conclusion, given the right conditions, UAE could improve the extraction yield without influencing the collagen structure.

Keywords: Barramundi skin, Collagen, Physicochemical properties, Ultrasound-assisted extraction

Introduction

The rising demand for collagen over the past decade is driven by its unique properties to be utilised in a broad spectrum of industrial applications. Collagen has been long-established as a foaming agent, emulsifier, stabiliser, microencapsulating agent, and biodegradable film-forming material in the food, pharmaceutical, and biomedical industries [1]. The use of collagen in various industries depends very much on its specific properties, where these properties are primarily related to the source origin and processing method employed. While porcine and bovine collagens are

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commercially manufactured, the scrutiny of fish collagen has been progressing over the past decade due to religious constraints and safety concerns pertaining to mammalian sources [2].

Extraction of collagen from fish sources was proven to be successful with acidic treatment, where acetic acid was commonly reported [3]. Acid soluble collagen is generally of lower yield; hence several proteases were incorporated in the collagen extraction process to address the issue, such as pepsin [4,5], papain [6], trypsin [7], and collagenases [8]. Schmidt et al. [9] stated that the addition of enzymes while boosting collagen solubilisation in an acetic medium does not compromise the collagen triple helix structure. In fact, it has been proposed that proteases hydrolyse some non-collagenous proteins, increasing the purity of collagen [10]. However, different proteases have a distinct cleavage target on the collagen, resulting in varying degrees of hydrolysis at the telopeptide regions [11]. This may require close monitoring and establishment of species-specific collagen extraction by an enzymatic process. Furthermore, the existing extraction procedure is time-consuming, and a significant amount of insoluble collagen is still embedded in the tissue matrix [12].

Of late, few research interests were observed to employ ultrasound-assisted extraction (UAE) in collagen extraction to promote greener and more sustainable processes. Although the use of UAE is considered emerging for collagen extraction, it has been extensively used in extracting protein from various resources. For instance, UAE was found to improve protein recovery and functionality from soybean [13], peanut [14], rice bran [15], meat [16], and egg [17]. The technique offers an advantage in compound extraction as it enhances solvent penetration in the solid matrix, increasing the mass transfer from the solid to liquid phase via cavitation phenomena [18]. The use of UAE has been shown to improve process efficiency by aiding the opening of collagen fibrils during acid and enzymatic treatment, resulting in a considerable reduction in extraction time [19, 20]. However, prolonged UAE time may have detrimental effects on the molecular structure of collagen due to more prolonged exposure to dynamic forces. Ali et al. [12] successfully enhanced the collagen yield from golden carp skin by up to 150 % using UAE compared to the conventional approach. Still, some alterations in the amino acid content and thermal stability of the collagen were found. According to Rodsamran & Sothornvit [21], the efficiency of UAE is greatly affected by ultrasonic power, frequency, solvent, and the matrix to the solvent used. Assessing the impact of these parameters on the extractability and characteristics of collagen is essential for a thorough comprehension of UAE applications, but this knowledge is presently sparse.

Barramundi (*Lates calcarifer*) is one of the common freshwater fish in Malaysia and has a steady supply in other Southeast Asia countries such as Thailand and Vietnam. Overall, 76.498 MT of barramundi aquaculture production was produced in Southeast Asian countries in 2015, with Malaysia leading the way, followed by Thailand [22]. The skins, which account for roughly 10-12 % of the whole fish discarded after the filleting process, are high in collagen. Earlier studies on collagen from barramundi/seabass skin used acid [1] and enzyme-aided [6] extraction methods, yielding 15.8 % and 44 % collagen recovery, respectively. Using ultrasonication under the right circumstances might be a viable method to increase the extraction efficiency of barramundi collagen associated with high yield while keeping good quality. This work intended to improve the extraction yield and assess the physicochemical properties of barramundi collagen generated using ultrasonic of different amplitude.

Material and Methods

Pre-treatment of barramundi skin

Fresh barramundi skin (*L. calcarifer*) was supplied by Best Seafood Marketing Sdn. Bhd., Johor Bahru, Johor, Malaysia. The skins were cleaned, and the residual meats were removed using a knife. Cleaned skins were cut into small pieces (approximately 1.0 × 1.0 cm^2) and packed into polyethene bags (50 g per bag). The skins were stored at 20°C until usage, with a maximum storage time of 3 months. The skins of barramundi were subjected to pre-treatments for the removal of non-collagenous protein and other unwanted material, as described by Benjakul et al. [23]. Pre-treatments of the fish skin were executed at 4°C in a refrigerated incubator (ST-THZ300C SASTEC, Selangor, Malaysia) with continuous shaking at 150 rpm. The skins were pre-treated for 8 h by soaking in 0.1 M NaOH with a skin/alkaline solution ratio of 1:15 (w/v). Every two hours, the alkaline solution was replaced with a new one. After alkali treatment, skins were washed in cold water

until faintly basic or neutral pH was obtained. Following that, the remaining fat was removed by soaking the skins in 10 % butyl alcohol for 16 h at a skin: solvent ratio of 1:15, with the fresh solvent replaced after 8 h. The defatted skin was washed in cold water to remove the alcohol.

Extraction of collagen from barramundi skin using acid soluble extraction (conventional method)

The conventional process for collagen extraction using acid was performed according to Jamilah et al. [6] with slight modifications. The pretreated skins were suspended in 0.5 M acetic acid at a 1:10 (w/v) skin/acid solution ratio. Using refrigerated incubator shakers (ST-THZ300C SASTEC, Selangor, Malaysia), the mixture was continuously stirred for 48 h. Next, the mixture was filtered through two layers of cheesecloth. After that, the filtrate was salted out with 2.6 M NaCl. A high-speed refrigerated centrifuge was used to centrifuge the precipitate at 2,370 g for 60 min at 4 ℃ (model CR22N Hitachi, Tokyo, Japan). Then, the pellet was resuspended in a minimum volume of 0.5 M acetic acid and dialysed for 24 h in 20 volumes of 0.1 M acetic acid, followed by two days in 20 volumes of distilled water. The collagen was freeze-dried and labeled as ASC.

Extraction of collagen from barramundi skin using ultrasound-assisted extraction (UAE)

Extraction of fish collagen using an ultrasonic process was carried out as described by Ali et al. [12] with slight modifications. Barramundi skin was soaked in 0.5 M acetic acid at a 1:10 (w/v) skin/acid solution ratio. Before ultrasonication, the mixture was first incubated at 4℃ for 30 min to enable the skin swelling partially. The mixture was then preceded with ultrasonication treatment using an ultrasound reactor model Vibra–Cell Processor (VCX 750) (Sonics & Material, Inc, Newtown, CT, USA) with a flat tip probe of 25 mm diameter, single frequency of 20 kHz and a power of 750 W. An ice bath was used to keep the temperature of the mixture at 4℃. The ultrasound was operated in a pulse mode with 5-sec acting and 5-sec resting time to avoid overheating. The treatment was done at different amplitude levels (40, 60, and 80%) for 20 min. Following the ultrasonic treatment, collagen extraction was performed for 48 h with continuous shaking at 4°C (ST-THZ300C SASTEC, Selangor, Malaysia). Collagen recovery was carried out as described for ASC. The resulting collagens after UAE were indicated as UAE40, UAE60, and UAE80, respectively.

Yield of collagen

The yield of collagen extracted was calculated based on the dry weight of fish skin in Eq(1).

$$Yield\ \left(\frac{g}{100}\right) = \frac{Dry\ weight\ of\ collagen\ (g)}{Dry\ weight\ of\ skin\ (g)} \times 100 \quad (1)$$

Hydroxyproline content

Hydroxyproline content was measured using the method by Kolar [24] with a slight modification. Hydroxyproline standard was obtained from Merck (Darmstadt, Germany). Preparation of standard stock solution started with dissolving 60 mg of hydroxyproline with distilled water in a 100 ml volumetric flask. An intermediate solution (6 µg/ml) of hydroxyproline was prepared by mixing 1 ml of stock solution with 99 ml distilled water in a 100 ml volumetric flask. Sample preparation was done according to Grundy [25]. Collagen (0.1 g) was placed into a test tube, and 0.75 mL of 3.5 M sulphuric acid was added to the test tubes. The sample solutions were hydrolysed for 16 h at 105 ˚C using a dry bath and diluted with 11.5 mL of water. Then the sample (2 ml) was added with 1.0 mL of Chloramine-T solution and incubated for 20 minutes at room temperature. One ml of 4-dimethylaminobenzaldehyde solution was later added, and the mixture was set at 60 ˚C for 15 min. The reaction was stopped by incubating the solution in iced water for 5 min. Collagen extract was measured against a hydroxyproline standard curve at 558 nm.

Protein and moisture content determination

The protein and moisture content of the obtained collagens were determined according to AOAC [26] standard procedures. Analyses were carried out in triplicates and calculated on a dry weight basis of the skin.

Colour measurement

Colour measurements were made using Hunterlab Ultrascan Sphere Spectrocolorimeter (Minolta Cr-300 Series, US) and were expressed by brightness (L *), redness (a *), and yellowish (b *)

values. The instrumental colour was measured in triplicates.

Electrophoretic analysis

SDS-PAGE pattern of collagen was determined according to the method described by Laemmli et al. [27] using 10 % resolving gel and 4 % stacking gel. The samples were dissolved in 0.02 M sodium phosphate containing 1 % sodium dodecyl sulphate (SDS) and 3.5 M urea (pH 7.2). The mixtures were then centrifuged at 8500 g for 5 min at room temperature to remove undissolved debris. Solubilised samples containing 20 µg/µl of protein were mixed at 1:1 (v/v) ratio with the sample buffer (0.5M Tris-HCl, pH 6.8, containing 4 % SDS, 20 % glycerol). Gels were casted in Mini Protein unit (Bio-Rad Laboratories Inc., Richmond, CA, USA) and samples were loaded on the gel and a constant current of 15 mA/gel was passed through for 75 min. After electrophoresis, the gel was stained with 1 g/L Coomassie brilliant blue R-250, dissolved in water, methanol and trichloroacetic acid (5:4:1), and de-stained using a solution containing methanol, distilled water, and acetic acid at a ratio of 5:4:1. Protein ladder (Sigma, St. Louis, MO, USA) with molecular weight ranges from 35 to 250 kDa (Sigma, St. Louis, MO, USA) were used as a marker for α-chain and β-component mobilities and to estimate the peptides molecular weight. For comparison, type 1 collagen from calf skin (Sigma, St. Louis, MO, USA) was also loaded.

Fourier transform infrared spectroscopy (FTIR) analysis

FTIR analysis was carried out in accordance with the modified approach of Sinthusamran et al. [1]. A 4 mg sample of dried collagens were mounted into the sample compartment. Temperature was set at 25°C and the spectra reading were in the range between 4000 – 500 cm^{-1} (Shimadzu IRTracer-100, Japan). All spectra were collected by adding 32 scans at a resolution of 4 cm^{-1} against a background spectrum recorded from the clean empty cell at 25°C.

Statistical analysis

The results were analysed statistically by ANOVA using Microsoft Excel data analysis tool. The value of $p < 0.05$ indicates significant differences between samples.

Results and Discussion

Yield of collagen

The yield (dry weight basis) of collagen from barramundi skin extracted using ultrasonication at different amplitudes is shown in Figure 1. The incorporation of UAE notably increased the collagen yield (20.1 – 56.61 %) as compared to the conventional acid extraction (ASC) (8.32 %) method. With an increase in ultrasonic amplitude as high as 80 %, the yield rose sevenfold in comparison to the ASC. The rate of collagen solubilisation during acid extraction depends on the electrostatic swelling of collagen molecules caused by the

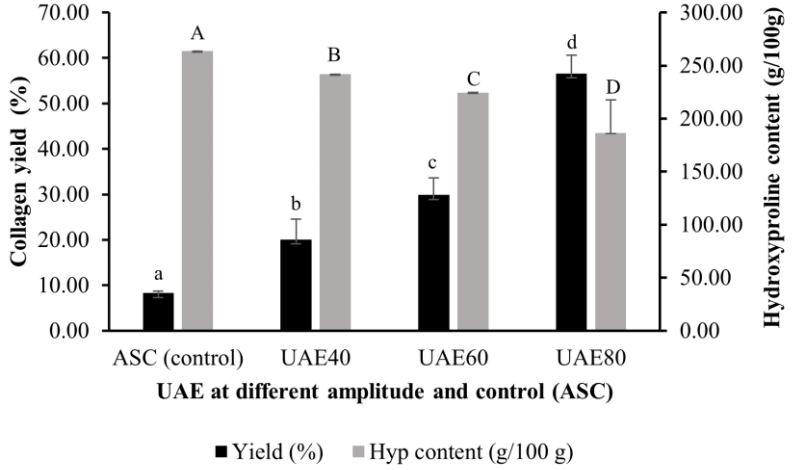

Figure 1. Extraction yield and hydroxyproline content of collagen from barramundi skin extracted using ultrasound-assisted process at different amplitude. Control – extraction without ultrasound (ASC). Bars represent the standard deviation (n=3). Different letters indicate significant differences ($p < 0.05$) for different extraction conditions.

repulsion of hydronium (H_3O^+) ions [28].The increase in repulsion between polypeptide chains (α-helix) will lead to solubilisation. Higher amplitude generated more energy due to high-intensity dynamic forces and cavitation to loosen the skin matrix, thus enabling greater penetration of acetic acid into the skin matrix to enhance collagen swelling [20].

At the same amplitude (80 %) and shorter time (20 min), the extractability of collagen from barramundi skin (56.61 %) is comparable to that reported for collagen extracted from clown featherback (*Chitala orna*ta) skin (57.35 %) using UAE for 30 min [20]. Akram & Zhang [19] suggested that collagen extraction is assisted by employing an adequate ultrasound treatment duration. At 36 min of UAE, they obtained the highest collagen yield (15.47 %) from chicken sternal cartilage. However, the extended ultrasonic treatment (36 min) caused considerable disruption of the secondary structure of the collagen. Interestingly, the use of UAE in collagen extraction from barramundi skin revealed in this work demonstrated a considerable improvement in yield when compared to papain-aided extraction (44%), as reported by Jamilah et al. [6]. Because enzymatic collagen extraction is more expensive than conventional chemical extraction, the reproducible, safe, ecologically friendly, and reliable features of ultrasound during acid extraction may provide a more affordable manufacturing option [9].

Hydroxyproline content

In contrast to the collagen yield, the hydroxyproline content of collagen extracted from barramundi skin employing UAE at increasing amplitudes exhibited a declining trend (Figure 1). The hydroxyproline content of ASC was substantially higher ($p<0.05$) (263.65 g/100g) than that of collagens treated with ultrasound. The hydroxyproline content gradually decreased as UAE was administered at 40 % (241.95 g/100 g) and 60 % (224.59 g/100 g) amplitudes, thereafter dramatically as the amplitude was increased to 80% (186.62 g/100 g). In principle, hydroxyproline is a unique amino acid that can only be found in collagen; consequently, it is commonly used to determine the collagen content of foods [11]. Collagen yield and hydroxyproline content are frequently used to evaluate the efficiency of the extraction process and the quality of the extracted collagen. Petcharat et al. [20] proposed measuring hydroxyproline content as a benchmark for collagen purity. The results of this study agreed with their findings, which showed that the hydroxyproline content decreased with increasing amplitude level for collagen extraction from clown featherback (*Chitala ornata*) skin. It was postulated that high levels of ultrasonic power resulted in more significant disruption of the skin matrix [29]. This may result in protein component leaching and a reduction in the hydroxyproline content of the extracted collagen.

Table 1. Physicochemical properties of collagen from barramundi skin extracted with ultrasound-assisted process at different amplitude

Physicochemical properties	ASC	UAE40	UAE60	UAE80
Moisture content (%)	14.76^{a}±0.77	14.87^{b}±2.82	14.69^{c}±0.31	13.64^{d}±0.14
Protein content (%)	81.89^{a}±1.35	77.30^{b}±0.25	76.29^{c}±0.30	75.33^{d}±2.20
Hunter colour value				
*L**	82.27^{a}±0.04	77.34^{b}±0.82	73.61^{c}±0.22	72.45^{d}±0.06
*a**	4.40^{a}±0.10	4.29^{b}±0.04	5.16^{c}±0.05	5.75^{d}±0.05
*b**	1.90^{a}±0.04	-1.56^{b}±0.29	6.16^{c}±0.02	7.39^{d}±0.11
Molecular weight of the peptides (kDa)				
ß-chain	202.78	203.27	205.72	206.71
α1-chain	135.83	138.13	139.46	139.46
α2-chain	122.51	125.49	126.70	127.00

Remarks: *Mean values with the different superscripts within each row were significantly different at $p < 0.05$. ASC: acid-soluble collagen, UAE40: ultrasound-assisted extraction at 40% amplitude, UAE60: ultrasound-assisted extraction at 60% amplitude, UAE80: ultrasound-assisted extraction at 80% amplitude

Protein and moisture content

The protein and moisture content of collagens from barramundi skin extracted using UAE at different amplitudes are shown in Table 1. Collagen extracted using conventional method had a high protein content (81.89 %), as typically reported for ASC in previous study [6]. The impacts of ultrasonication amplitude on protein content is in tandem with the hydroxyproline content, where greater UAE amplitude reduced the protein content in collagen. The protein content for UAE40 (77.3 %), UAE60 (76.29 %) and UAE80 (75.33 %) were significantly lower ($p<0.05$) than that of ASC, despite the fact that protein content of more than 70 % in collagen is regarded high [19]. The total protein content indicates the efficiency of the collagen extraction process to successfully recover protein from the skin matrix and is often used to characterise the collagen specification in commercial manufacturing. The data demonstrated that, while higher power of ultrasonication considerably increased the amount of collagen recovered, some protein denaturation occurred along the process, which may have an impact on the overall quality of collagen. On the other hand, a work focusing on the different UAE times (6 – 36 min) at the same amplitude (ϕ10) for collagen from chicken sternal cartilage found that the protein increased the time extended [19]. Collagens extracted from barramundi skin had moisture content ranging from 13.64 – 14.87 %, with significant differences ($p<0.05$) between samples. Although the moisture content obtained in this study was higher than that of other reported collagens [19,30], it was still lower than the collagen specification value of 15% [31]. The high moisture content was most likely owing to the spongey-like and hygroscopic nature of the resultant collagen after freeze-drying.

Colour properties

Hunter colour values are used as an aesthetic component to describe the collagen criteria. The colour of barramundi collagens as affected by the different extraction conditions are shown in Table 1. In general, all collagens exhibited a good visual whiteness. The *L* value, which reflects lightness for ASC, UAE40, UAE60, and UAE80 showed a significance difference ($p<0.05$), of which the control was the lightest (81.89). Increased ultrasonic amplitude resulted in collagen with darker appearance, as evidence by the decreasing *L* values (Table 1). The presence of skin pigments during the extraction phases is linked to the low *L* value [6]. UAE with a higher intensity may shear faster, releasing more pigments. Moreover, collagen extracted at greater amplitudes (60 and 80 %) had a yellowish characteristic, represent by the *b* values (6.16 and 7.39, respectively). The colour of lyophilised collagen has no effect on its functional properties; nonetheless, consumers typically prefer lighter-coloured foods, and colourless collagen is easily included in diversified product formulations [32].

Protein patterns

Figure 2 depicts the protein patterns of collagen extracted from the skins of barramundi using conventional and UAE extraction methods with varying amplitudes. Generally, all extracted collagens exhibited identical protein patterns to the type 1 bovine standard collagen. ASC and those collagens extracted using UAE consist of two different α-chains (α1- and α2-chains) and its dimer ß-chain. These major components were similar to the electrophoretic patterns of other reported studies for fish collagen [4,5,10]. In addition, the presence of γ-chain (trimer) indicated that collagen had high cross-links [4]. The molecular weight distribution is related to protein quality, while the occurrence of α and β components indicate collagen purity [19].

The molecular weight (MW) of peptides detected for ASC, UAE40, UAE60 and UAE80 are summarised in Table 1. No major differences in peptide size of the ß, α1-, and α2-chains of all samples, indicating all treatments managed to preserve the integrity of collagen structure. However, the intensity of the bands, especially the ß-chain decreased when the amplitude level increased at the same ultrasonication time (20 min). Higher degradation with less retained α- and ß-chains was obviously found in collagen extracted with UAE at 60 and 80 %. Similar observations were reported for collagen from clown featherback skin obtained using UAE at 80 % amplitude for 30 min [20]. Nonetheless, apparent peptides with MW range below 100 kDa were observed only for ASC and UAE40. When UAE was imposed up to 60 and 80 % amplitude, intense degradation of protein structure that occurred may liberate peptides of MW lower than 37 kDa, hence are not detected in this study. Hydrodynamics forces of high pressure generated during microbubbles collapse may

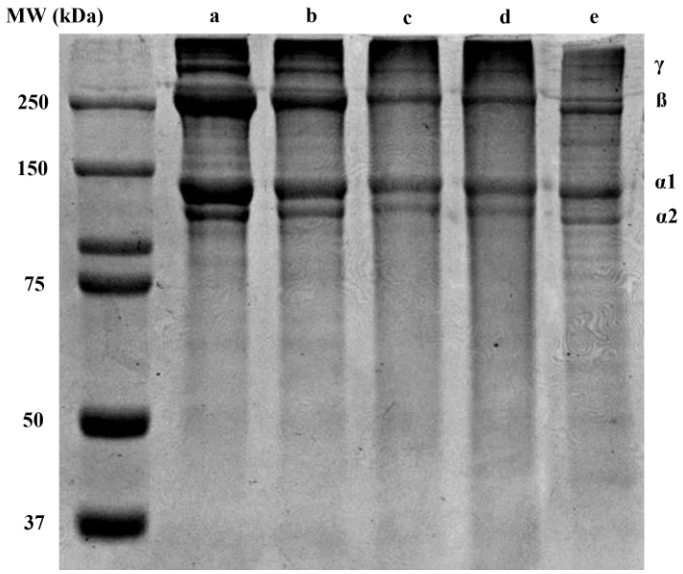

Figure 2. Protein pattern of collagens from barramundi skin extracted using conventional and ultrasound-assisted process. (a) ASC, (b) UAE40, (c) UAE60, (d) UAE80 and (e) type I bovine collagen standard.

cause collagen macromolecules fragmentation and protein migration which accelerates proteolysis, resulting in the releasing of short and low MW peptides [33].

Fourier transform infrared chromatography

The IR spectra recorded for collagens from barramundi skin extracted using acid extraction (ASC) and ultrasound-assisted extraction (UAE) show typical bands for collagen type I: Amide A, Amide B, Amide I, Amide II and Amide III (Figure 3). The N-H stretching vibration-related Amide A absorption spectrum emerged between 3400 and 3440 cm^{-1}. When the N-H group of a peptide forms a hydrogen bond, the position transitions to lower frequencies [3]. The Amide A peaks of ASC and UAE were identified at 3292 cm^{-1} and 3307 cm^{-1}, respectively, indicating that more N-H groups were implicated in hydrogen bonds, which hold the collagen triple-helical structure together [34]. The higher wavenumber of Amide A for UAE suggested that ultrasonication may have resulted in increased number of low MW peptide, which may have liberated more free amino group [4]. The peak absorption of Amide A for ASC in this study was similar to that reported for ASC from seabass (*Lates calcarifer*) skin (3292 cm^{-1}) [1]. The amide B bands, associated with an asymmetrical stretch of CH_2 [34], were found at a wavenumbers of 2922 and 2923 cm^{-1} for ASC and UAE, respectively. The lower band intensity of Amide B for UAE might be due to the destructive effect of ultrasound waves on N-H bonds [35].

The degree of molecular order seen in collagen and the formation of its triple helical conformation are both attributed to the amide I, II, and III bands. The amide I region with characteristic frequencies ranging from 1600 to 1700 cm^{-1} of collagen represents C=O stretching vibration along the polypeptide backbone. Meanwhile, the amide II (1550-1600 cm^{-1}) and III (1230-1240 cm^{-1}) regions featured C-N stretching and N-H bending, respectively [4]. The amide I and II regions of proteins are indications of secondary structure, whereas the amide III region is a unique collagen molecule fingerprint [36]. The peak wavenumber of amide I for UAE (1651 cm^{-1}) shifted to a higher value compared to that of ASC (1634 cm^{-1}), suggesting the greater loss of molecular order due to thermal uncoupling of inter-molecular cross-link as a result of the application of UAE [37]. Amide II bands for both ASC and UAE were found at wavenumbers 1548 cm^{-1}. ASC and UAE displayed wavenumber of amide III at 1238 cm^{-1} and 1235 cm^{-1}, respectively, representing hydrogen bonds involved in maintaining the native structure [20]. The amide III is a dynamic vibrational mode consisting of C-N stretching vibrations with N-H in plane bending from amide linkages. It also exhibits large absorptions from wiggling vibrations from CH_2 groups on the backbone of glycine and on the side chains of proline [37].

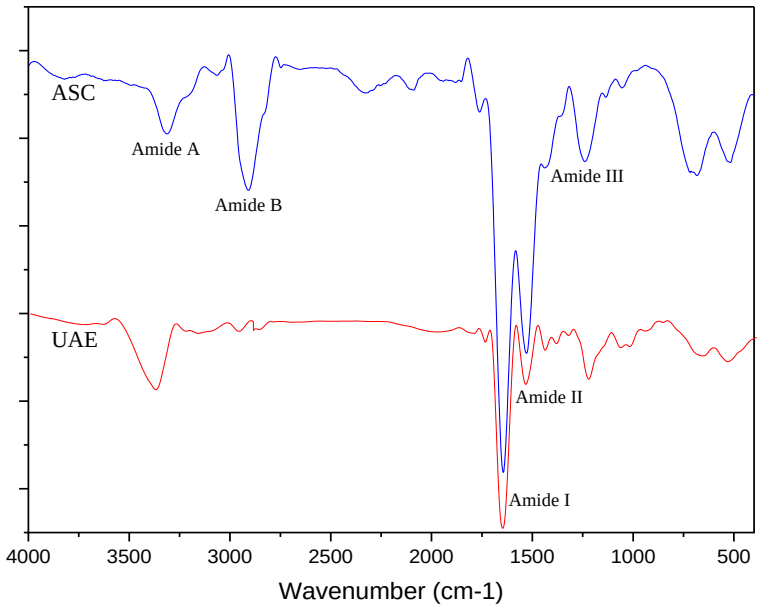

Figure 3. FTIR spectra of collagen from barramundi skin extracted using conventional (ASC) and ultrasound-assisted process (UAE), showing the presence of Amide A, B, I, II and III.

Conclusion

The yield of collagen extracted from barramundi skin could be significantly increased by using ultrasound-assisted extraction with higher amplitude (80%). However, the reduced hydroxyproline and protein content indicated a decline in quality. The triple-helical structure of the collagens isolated from the skin of barramundi using ultrasound was preserved at the molecular level, though the functional group or interaction between chains of ASC and UAE may somewhat differ. UAE at high amplitudes may perform more efficiently in less time, minimising adverse impacts on collagen structure integrity. Consequently, optimising UAE settings for collagen extraction may provide a deeper understanding of the application of ultrasound as a viable approach for obtaining a high yield and high-quality collagen from barramundi skin.

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