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# Isolation of threadfin bream *(Nemipterus japonicus)* waste collagen using natural acid from calamansi *(Citrofortunella microcarpa)* juice

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**Abstract**

Collagen was isolated from threadfin bream *(Nemipterus japonicas)* waste (mixture of scale and fin) by using 0.5 M citric acid or calamansi juice *(Citrofortunella microcarpa)* for 12 and 24 hrs at 4°C. The physico-chemical characteristics of the collagens were then compared with the commercial collagen. Shorter extraction time (12 hrs) and extraction using calamansi juice resulted in higher yield. The yield was 22% (12 hrs) and 20.37% (24 hrs) for collagen extracted using calamansi juice and 8.3% (12 hrs) and 6.9% (24 hrs) for collagen extracted using citric acid. Collagen extracted using calamansi juice were light yellow (L = 93.70, a = -1.84, b = 13.44) while citric acid collagens were white (L = 94.82, a = 0.31, b = 0.20). Sensory evaluation on odor recognition test showed that collagen extracted with calamansi juice has a pleasant natural fragrance which is sweet citrus. Electrophoresis profile indicated that the collagen were of type I comprising of α1 and α2 chains. Threadfin bream collagen contained higher amount of imino acids proline (254.72 to 275.50/1000 residues) and hydroxyproline (7.56 to 13.50/1000 residues) than commercial collagen which is 21.25 and 5.16/1000 residues, respectively. Maximum transition temperature ($T_{max}$) falls within a close range for all the collagens ranging from 24.81 to 25.91°C. Calamansi juice collagens were more viscous compared to others. The extraction of threadfin bream collagen for 12 hrs using calamansi juice generally leads to collagen characterised by pleasant odor, reasonably high yield and more viscous. Therefore, natural source such as calamansi juice could be an alternative medium for collagen extraction.



## Introduction

Bovine and porcine are the main source for collagen extraction, however, collagens from chicken, duck, fish, starfish, sea cucumber, eel and cuttlefish have also been extracted and studied (Nagai and Suzuki, 2000; Cheng *et al*., 2009; Duan *et al*., 2009; Balti *et al.*, 2011; Zhu *et al*., 2012; Tan *et al*., 2013; Huda *et al*., 2013; Veeruraj *et al*., 2013; Kaewdang *et al*., 2014; Simoes *et al*., 2014). Parts of the animals used for collagen extraction include bone marrow, horn, hoof, bone, skin, feet, cartilage and testis (Zhao *et al*., 2011; Zhang *et al*., 2013; Simoes *et al*., 2014). For collagen extraction from fish, the swim bladder, cartilage, skin, bones, fin and scales have been used (Nagai and Suzuki, 2000; Kittiphattanabawon *et al.*, 2010; Liu *et al.*, 2012; Kaewdang *et al*., 2014).

Acid solubilization process is widely used in the preparation of acid-soluble collagen by extracting collagen using acid solution (Bae *et al*., 2008; Matmaroh *et al*., 2011; Yan *et al*., 2012; Sinthusamran *et al*., 2013; Tamilmozhi *et al*., 2013; Minh Thuy *et al*., 2014). Commonly used acids are from organic acid such as acetic, citric and lactic acid whereas inorganic acid such as hydrochloric acid has also been used occasionally (Palpandi *et al*., 2010). Generally, collagen extraction can be achieved by using 0.5 M organic acid treatment at 4°C for 24 to 48 hrs (Nalinanon *et al*., 2008; Kiew and Mashitah, 2013).

*Citrofortunella microcarpa* is known as calamansi or calamondins which is a hybrid between sour mandarin and kumquat (Allen, 1975). The fruit is small and protected by a thin skin. The flesh is yellow-orange and taste sour but the juice is not bitter. In Malaysia, calamansi juice is commonly constituted into drinks such as ice tea and syrup to increase sourness. Furthermore, calamansi is popular as condiment in meals such as curry noodles and 'laksa'. In food industries, the fruit is dried salted or fermented into pickles while the skin is used in marmalade production. Calamansi is a citrus fruit where the juice contains organic acid from citric acid that can be used as substitute for lime and lemon. Medicinally, the fruit is known as anti dandruff and helps relief cough. The seed's oil has been characterised and the essential oils from the rind has also been studied for its potential use as an anti-

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protozoan agent (Mojica *et al*., 2004; Manaf *et al*., 2008)

Threadfin bream scale and fin are part of waste materials discarded from threadfin bream processing in food industry (Nalinanon *et al*., 2011). In daily cooking, the scale and fin are usually removed before consumption. The flesh is highly demanded for surimi production (Benjakul *et al*., 2003). The collagen and gelatin in this fish act as protein additive and a good binding agent in surimi (Montero *et al*., 2001).

Nowadays, there are increasing attentions for alternative sources of mammalian collagen especially from seafood by-products such as from fish (Kittiphattanabawon *et al*., 2005; Nalinanon *et al.*, 2011). Collagen extracted from mammals have been associated with disease outbreak such as mad cow and foot and mouth disease while the use of porcine and bovine by-products are restricted in certain religion (Yan *et al.*, 2012). Collagen extraction using standard organic acid requires significant time which varies from few days to weeks (Duan *et al*., 2009; Kittiphattanabawon *et al.*, 2010; Matmaroh *et al*., 2011). Calamansi juice is easily obtained and economical, therefore its utilization could reduce collagen production cost and perhaps shorten the production time. In this study, threadfin bream waste collagen was extracted using calamansi juice or citric acid where the extraction time was varied. The characteristics of the collagens were compared with the commercial collagen.

## Materials and Methods

*Materials*

Calamansi fruit *(Citrofortunella microcarpa)* was purchased from a local orchard in Kulim, Kedah, commercial collagen from Toujours Skin Enterprise, Malaysia and threadfin bream *(Nemipterus japonicus)* from fishermen at the jetty in Langkawi, Kedah. The scale and fin were removed from the fish, washed with distilled water, cut into small pieces and stored at -20°C until use. The mixture of scale and fin is referred as the 'waste'. All chemicals used were of analytical grade purchased from Sigma Aldrich, U.K.

*Extraction of collagen*

Collagen was extracted according to Minh Thuy *et al.*, (2014) with slight modification. All preparation procedures were carried out at temperature below 4°C. The waste (mixture of scale and fin) were dissolved in 0.1 M NaOH for 6 hrs at sample to NaOH ratio of 1:8 (w/v) to remove non-collageneous proteins followed by washing in cold distilled water until a neutral pH 7 was achieved. Sample was then soaked in calamansi juice for 12 hrs with occasional changes of the juice every 3 hrs. The supernatant was collected by centrifugation using a centrifuge (Model 320 R, Universal, Hettich, Malaysia) at 5, 000 rpm for 30 mins. This was followed by precipitation by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M Tris-HCl at pH 7.5. The resultant precipitates were collected by centrifugation using a centrifuge (Model 320 R, Universal, Hettich, Malaysia) at 5, 000 rpm for 30 min. The pellets were dissolved in calamansi juice, dialyzed against volumes of calamansi juice with ratio of 1:1 (v/v) and lyophilized using a freeze dryer (Model Alpha 1-4 Ld Plus, Martin, Hrist, Germany). Similar procedures were applied for extraction of collagen using calamansi juice for 24 hrs and extraction using 0.5 M citric acid for 12 and 24 hrs.

*Determination of collagen yield*

Yield was calculated based on the weight of lyophilised collagen and threadfin bream waste as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of lyophilised collagen (g)}}{\text{Weight of threadfin bream waste (g)}} \times 100$$

*Color measurement*

Collagen color was measured using Hunterlab Ultrascan Sphere Spectrocolorimeter (Model Minolta CR-400, Malaysia) and reported as the 'L' - 'lightness', 'a' - 'redness' and 'b' - 'yellowness' values. Blank used for calibration was white tile CM-A101.

*Sensory evaluation of collagen (odor)*

Lyophilized collagen was placed in amorphous bottle with four coded labels that were retrieved using permutation 9 digits. Panelists were asked to describe the odor of the collagen based on the simple odor recognition test (Rothe, 1988).

*Differential scanning calorimetry (DSC)*

DSC analysis was carried out to determine the maximum transition temperature ($T_{max}$) (Nalinanon *et al.*, 2007). Sample was rehydrated by adding 0.05 M acetic acid at the ratio of 1:20 (w/v) and left for 24 hrs at 4°C. Approximately 10 mg sample was weighed into an aluminium pan, sealed and then scanned at 1°C / min over the range of 20 to 50°C by using DSC (Model Pyris Diamond, Perkin Elmer, Malaysia). The maximum transition temperature ($T_{max}$) was estimated from the endothermic peak of the thermogram. Total denaturation enthalpy ($\Delta H$) was estimated by measuring the area of the DSC

thermogram.

*Viscosity measurement*

An amount of 3 g lyophilised collagen was dissolved in 100 mL distilled water. Viscosity was measured by inserting the solution into the Brookfield DV-I viscometer (U.S.A) with spindle No.1 at 60 rpm. The temperature was initially set at 55°C and then occasionally reduced every 1°C/min until 0°C.

*Amino acid analysis*

The amino acid composition was analyzed by method as described in Pranoto *et al.,* (2011) with slight modification. An amount of 0.1 g lyophilised collagen was hydrolyzed in 5 ml of 6 M HCl at 100°C for 22 hrs. The amino acid composition was determined by reverse phase HPLC AccQ Tag column (3.9 x 150 mm) at 36°C. An aliquot of 5 µL was injected into AccQ Tag HPLC (Model 1525, Binary HPLC pump) equipped with refractive index and multi fluorescence detectors (Waters, U.S.A). The mobile phases used were AccQ Tag Eluent A (concentrate) and 60 % acetonitrile as Eluent B.

*Hydroxyproline content*

Hydroxyproline content was determined as described in Nalinanon *et al*., (2007). Sample was hydrolysed with 6 M HCl at 110°C for 24 hrs and then clarified with activated carbon followed by filtration. The filtrate was neutralized with NaOH to obtain pH 6.0-6.5. Neutralized sample of 0.1 ml was transferred into a test tube where 0.2 ml isopropanol was added and vortexed. An amount of 0.1 ml oxidant solution (mixture of 7% (w/v) chloramine T and citrate buffer, pH 6) was added. Then, 1.3 ml Ehrlich's reagent solution and isopropanol at the ratio of 3:13 (v/v) were added. The mixture was agitated and heated at 60°C for 25 mins in water bath and then cooled under running water. The solution was diluted to 5 ml with isopropanol. Absorbance was measured against water at 558 nm. The hydroxyproline standard solutions with concentration ranging from 10 to 60 mg/kg were used for the standard curve preparation.

*Molecular weight distribution by SDS-PAGE*

SDS-PAGE was performed to determine the molecular weight distribution of the protein according to Normah *et al*., (2013). An amount of 0.25 g collagen was dissolved in 0.5 mL distilled water and shaken until dissolved. Solubilised samples were mixed at 1:1 (v/v) ratio with sample buffer. An amount of 10 µL sample was then loaded into NU-PAGE (U.S.A) gel comprising of 12% resolving and 4% stacking gel. Electrophoresis was performed using electrophoresis cell (Model EV 231, Consort, Germany), run for 30 min at 100-125 mA/gel. The gel was stained in blue staining Biorad solution and destained in ultrapure water.

*Statistical analysis*

Analysis was conducted in triplicate and data was averaged. The data was analyzed by utilizing the analysis of variance (ANOVA) and Duncan's multiple range tests to determine the significant difference between the means. Data was analyzed using Statistical Analysis System (SAS) Version 9.2 for Windows (SAS Institute Inc., 2009).

## Results and Discussion

*Yield, color and odor of threadfin bream collagen*

Collagen from threadfin bream waste was extracted using calamansi juice and 0.5 M citric acid. The yield was 22 % (12 hrs) and 20.37% (24 hrs) for collagen extracted using calamansi juice and 8.3 % (12 hrs) and 6.9 % (24 hrs) for collagen extracted using citric acid. Shorter extraction time (12 hrs) for collagen extraction of threadfin bream resulted in higher yield regardless of the extraction solutions used. Low yield at longer extraction time was probably due to collagen degradation after very long exposure under the acidic environment. Extraction using calamansi juice resulted in higher yield than those using citric acid most probably due to the natural citric acid present in calamansi juice which generally led to a greater yield. The pH of the calamansi juice was 3.37 which is slightly higher than those of citric acid (pH 2.41). In another study, the yield from deep-sea redfish was 6.80 % when the collagen was extracted for 24 hrs in 0.5 M acetic acid (Wang *et al*., 2008). Extraction of collagen usually requires long time generally 48 hrs (Nalinanon *et al*., 2008). However, by increasing the concentrations of acid for extraction such as from 0.1 to 0.5 M, yield could be increased (Kiew and Mashitah, 2013).

The collagen produced using calamansi juice were light yellow (L = 93.70, a = -1.84, b = 13.44) while those produced using 0.5 M citric acid were white (L = 94.82, a = 0.31, b = 0.20). Color of barramundi acid soluble collagen (Lates calcarifer) produced using acetic acid at 4°C was white (L = 0.14, a = 3.16, b = 0) (Jamilah *et al.,* 2013). The natural color of calamansi juice may have influenced the color of the extracted collagen.

Odor recognition test was conducted where panelists described the odor of each collagen. Generally, both calamansi juice collagens were described as sweet citrus odor by more than 85%

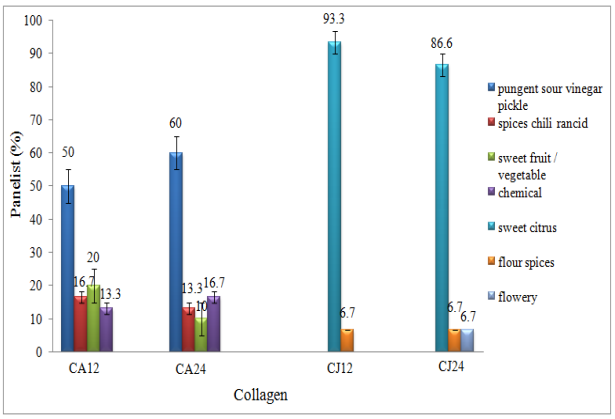


Figure 1. Odor of threadfin bream collagen extracted using 0.5 M citric acid (CA) and calamansi juice (CJ) for 12 and 24 hrs based on the percentage of panelist description on each identified odor

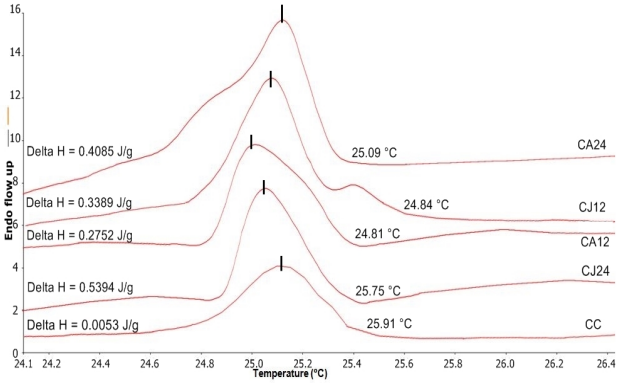


Figure 2. DSC thermal transition curve of commercial collagen (CC) and threadfin bream collagens extracted using calamansi juice (CJ) and 0.5M citric acid (CA) for 12 and 24 hrs

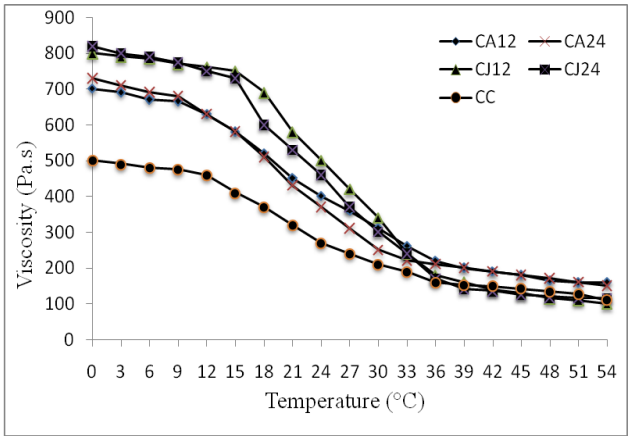


Figure 3. Viscosity of commercial collagen (CC) and threadfin bream collagens extracted using calamansi juice (CJ) and 0.5M citric acid (CA) for 12 and 24 hrs at different temperatures

panelists whereas citric acid collagens showed mixed description of odor with pungent sour odor which was recognized by as many as 60% panelists (Figure 1). However, there was no fishy odor detected in the collagens. One of the defects of fish collagen is fishy off-odor (Nagai *et al*., 2001; Sadowska *et al*., 2003). Therefore, collagen extracted using calamansi juice has the potential to be used in commercial products such as beverages and soup mixes due to its pleasant odor.

*Thermal stability of the collagen*

Thermal transition curve of threadfin bream collagen is shown in Figure 2. All collagens showed single maximum transition temperature ($T_{max}$) within an approximate temperature range of 24.81 to 25.91°C. Mammalian collagen denatured at 37 to 45°C (Cheng *et al.*, 2009). This suggested that threadfin bream scale and fin collagen denatured at lower temperature than mammalian collagen. Low denaturation temperature indicated a low degree of proline hydroxylation in fish collagen (Hwang *et al.*, 2007). Thermostability of collagen depends on several factors such as species, environment, subunit compositions ie the α chains in addition to imino acid composition (Kittiphattanabawon *et al*., 2005; Pati *et al.,* 2010; Palpandi *et al*., 2010; Duan *et al*., 2012). The sea water temperature of threadfin bream habitat in the Straits of Malacca is on the average 28°C (Water temperature Langkawi, 2014). Extraction of collagen from spotted golden goatfish scale in 0.5 M acetic acid for 48 hrs achieved collagen with $T_{max}$ of 41.58°C while bighead carp scale extracted in 0.5 M acetic acid containing 0.1% (w/v) pepsin for three days showed $T_{max}$ of 35.2°C (Matmaroh *et al*., 2011; Liu *et al*., 2012).

*Viscosity*

Collagen was subjected to heat treatment at different temperature as shown in Figure 3. Collagens extracted using calamansi juice were more viscous than others. Viscosity of calamansi juice collagen decreased gradually beginning from 15°C while citric acid and commercial collagen beginning from 9°C. Almost complete melting was achieved beginning from 39°C for all the collagens. In comparison to other reported marine species collagen; bigeye snapper showed that viscosity decreased continuously up to 30°C and the rate of decreased was retarded in the range of 35 to 50°C (Kittiphatanabawon *et al*., 2005). Collagen denatures at temperature above 40°C which causes break down of hydrogen bonds due to the nature of mixture random-coil single, double and triple strands (Wong, 1997).

*Amino acid composition*

The amino acid composition is shown in Table 1. Proline was relatively high in collagen extracted from threadfin bream waste. Proline content in the scales of different fish were 95/1000 residues from deep-sea redfish extracted with 0.5 M acetic acid

Table 1. Amino acid composition of commercial collagen (CC), threadfin bream collagens extracted using calamansi juice (CJ) and 0.5M citric acid (CA) for 12 and 24 hrs

| Amino acids (/1000 residues) | CA12 | CA24 | CJ12 | CJ24 | CC |
|---|---|---|---|---|---|
| Alanine | 39.623 | 57.165 | 20.849 | 245.981 | 11.185 |
| Arginine | 74.146 | 52.085 | 0.214 | 0.558 | 41.413 |
| Asparagine | 2.548 | 6.785 | 1.805 | 1.948 | 1.208 |
| Cysteine | 10.318 | 26.834 | 19.275 | 51.487 | 15.477 |
| Glutamine | 2.519 | 17.056 | 2.396 | 2.364 | 4.616 |
| Glycine | 3.894 | 15.454 | 1.989 | 2.173 | 7.318 |
| Histidine | 22.013 | 88.742 | 1.683 | 3.346 | 2.064 |
| Isoleucine | 9.652 | 9.865 | 3.120 | 2.556 | 0.988 |
| Leucine | 9.497 | 11.976 | 5.183 | 16.015 | 1.312 |
| Lysine | 10.371 | 1.283 | 0.269 | 0.631 | 0.593 |
| Methionine | 3.952 | 12.589 | 8.193 | 7.094 | 20.304 |
| Phenylalanine | 265.928 | 201.706 | 0.142 | 1.092 | 1.996 |
| Hydroxyproline | 18.960 | 5.500 | 13.500 | 7.560 | 5.160 |
| Proline | 230.797 | 200.701 | 275.499 | 254.718 | 21.254 |
| Serine | 1.732 | 7.848 | 0.227 | 0.225 | 0.071 |
| Threonine | 0.000 | 98.111 | 0.000 | 94.438 | 13.361 |
| Tyrosine | 157.887 | 128.071 | 13.137 | 6.513 | 4.076 |

at a sample/solution ratio of 1:100 (w/v) for 24 hr; 115/1000 residues from carp extracted with 0.5 M acetic acid at sample/acid ratio of 1:2.5 (w/v) for 4 days and 108/1000 residues from spotted golden goatfish extracted with 0.5 M acetic acid for 48 hr (Wang *et al*., 2008; Duan *et al.*, 2009; Matmaroh *et al.*, 2011). Hydroxyproline content for 12 hrs of extraction was higher than 24 hrs regardless of the extraction solution used (Table 1). Centrifugation after extraction could lead to the removal of non-collagenous substances and as a result, higher hydroxyproline and collagen contents were observed in the extracted collagen than in the skin Nalinanon *et al.*, (2007). Tyrosine content in citric acid collagens is higher than those from calamansi juice collagens. It has been shown that the skin of ornate threadfin bream collagen was rich in glycine, alanine and proline but relatively low in tyrosine and histidine (Nalinanon *et al.*, 2011). Generally, type I collagen consists of low amount of cysteine and methionine (Owusu-Apenten, 2002). Therefore amino acids composition suggested that threadfin bream waste collagen were of type I consistent with the reported collagens (Duan *et al*., 2009; Pati *et al*., 2010). No tryptophan could be detected in all the collagens.

*Molecular weight distribution*

The electrophoresis pattern of threadfin bream collagen is shown in Figure 4. Threadfin bream waste showed wide molecular weight bands ranging from 10 to 220 kDa. Calamansi juice collagens had bands from <10 to 220 kDa, whereas in citric acid collagens, only two bands were evident which are at 10 and 120 kDa. Smear band is present in commercial collagen at 30 kDa. Collagen extracted with calamansi juice exhibited molecular weight that is similar with raw calamansi juice at <10 to 220 kDa. The bands for collagen treated with calamansi juice for 24 hrs were more intense than calamansi juice extracted collagen at 12 hrs. Type I collagen consists of α-chains at 100 to 120 kDa (Minh Thuy *et al*., 2014). Citric acid collagens and waste showed very faint bands for α1 and α2 chains. Such bands were not observed in calamansi juice collagens which most probably have been degraded during the extraction process. Other marine species collagens reported to be of type I collagen include deep-sea redfish with bands at 116 to 120 kDa and spotted golden goatfish at 114 to 106 kDa (Wang *et al*., 2008; Matmaroh *et al*., 2011).

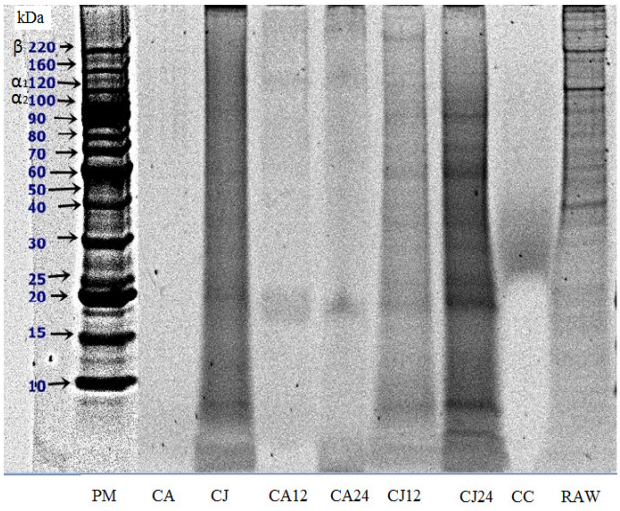


Figure 4. SDS-PAGE pattern of threadfin bream collagens, commercial collagen and raw threadfin bream. From left protein marker (PM), citric acid (CA), calamansi juice (CJ), citric acid 12 hrs collagen (CA12), citric acid 24 hrs collagen (CA24), calamansi juice 12 hrs collagen (CJ12), calamansi juice 24 hrs collagen (CJ24), commercial collagen (CC) and threadfin bream waste (RAW)

## Conclusion

The extraction of threadfin bream waste collagen for 12 hrs using calamansi juice led to higher yield, more viscous collagen with reasonably good $T_{max}$ value and characterised by a pleasant odor compared to citric acid extraction. Collagen extracted using calamansi juice were more viscous than citric acid extracted collagens but the melting point for both extractions were within an approximate range between 24.81 to 25.91°C. This indicated that extraction using calamansi juice produced better collagen quality in terms of viscosity. Therefore, calamansi juice could be an alternative medium for collagen extraction.

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