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Effect of Fish Scale Hydrolysed Collagen on Stability of Water-in-Virgin Coconut Oil Emulsion for Potential Topical Application

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Abstract: Hydrolysed collagen intake has been shown to have a positive effect on flexibility and elasticity of the skin. However, topical use of hydrolysed collagen in emulsion form is under-explored. The aim of this study was to investigate the effect of hydrolysed collagen existence on the physical stability and droplet size of different formulations of water-in-virgin coconut oil emulsion. The obtained emulsion via. high shear homogenization, both before and after incorporated with hydrolysed collagen showed good physical stability with no separation layersand suitable to be administered on skin. Incorporating hydrolysed collagen caused a small additional decrease in emulsion droplet size compared to typical emulsion. The dispersion of small water-based hydrolysed collagen droplets in emulsion plays an important role for a better permeation of hydrolysed collagen into the skin, once it applied topically. The rheological properties of emulsion attribute to shear thinning behaviour which is appropriate for the needs in the topical application. The results recommend an emulsion system as an innovative approach for enhancing the transdermal penetration of hydrolysed collagen into skin for topical application.

Key words: Emulsion, high shear homogenization, hydrolysed collagen, stability, topical application, transdermal penetration

INTRODUCTION

The demands of collagen as useful biomaterials have been extensively increased in biomedical and cosmetic industries due to its potential in drug delivery, enhanced fibroblast cell proliferation and provided structural support to the skin which can appear youthful (Chak *et al*., 2013; Kalarikkal *et al*., 2016 and Borumand and Sibilla, 2014). Collagen is a natural protein in the body that provides a structure to the skin and works hand-in-hand with another protein, called elastinto increase the flexibility of skin as it needs to stretch and return to its original state as the body moves. As one ages, the fibroblasts activities start to slow down in producing new collagen fibres in the body. Therefore, the problem has gained attention among researcher to find alternative source of collagen. It is believed that taking collagen can facilitate the biosynthesis of natural collagen in the body and thus improved the structure of epidermal appearance (Schagen, 2017; Song and Li, 2017). The oral administration of collagen may have a slower effect due to slower release or may quickly pass the metabolism process without been digested thereby entering the rectum as an indigestible molecule waiting to be removed from the body (Alexander *et al*., 2012; Prausnitz and Langer, 2008). Topical administration allows the collagen targeting directly to the to the dermis layer where the fibroblast cells were placed. However, skin becomes a barrier to protect the body from varied external insults. Therefore, collagen preparation is very important to ensure it will penetrate into the targeted site, thus can provide the desired functionality.

Hydrolysed collagen from fish scales was prepared in this study as an alternative of bovine or porcine source is biocompatible, weak antigenicity and biodegradable (Tang and Saito, 2015; Yamada *et al*., 2014). The usage of fish scales to derive collagen will not only reduce pollution but may reclaim fish waste to high value added materials. However, low molecular weights (M_w<1, 500 Da) of prepared hydrolysed collagen tend to agglomerate

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due to strong Van der Waals interactions of fine particles, thus, reduce its penetration capability into skin (Baert *et al*., 2007; Dumitriu and Popa, 2013). Study on surface morphology of commercialized hydrolysed collagen was observed in the globular groups form proving the occurrence of particles agglomeration (Latip *et al*., 2015). Therefore, low transdermal penetration efficiency of hydrolysed collagen when administrated topically (Chai *et al*., 2010). Now a days, emulsion products are typically loaded with a lot of chemical substances to stabilise and preserve them (Juttulapa *et al*., 2017; Sasseville, 2014).

In this study, water-in-virgin coconut oil emulsion containing hydrolysed collagen was stabilised with small percentage of non-ionic surfactant that adsorb to the surface of droplet, forming a thin coating around the droplet that inhibit their agglomeration by generating repulsive forces between them (Chung and McClements, 2014). It was reported that the non-ionic surfactants was safe to use as ingredient in personal care products and cosmetics because they do not cause negative side effects to the user (Mahdi *et al*., 2011).

Hydrolysed collagen and Virgin Coconut Oil (VCO) have gained more attention among researchers due to its unique characteristics in delivering lots of benefits to the health and cosmetic industries. It was found that VCO is traditionally used to enhance beauty appearance, moisturizes the skin and treat many skin disorders (Daud, 2008; Mansor *et al*., 2012). Malaysia remains as one of the top ten coconut producing countries in the world. Therefore, VCO usage is one of the great things that can increase the utilisation of coconut and income of farmers in our country. The stability of hydrolysed collagen was enhanced in water-in-VCO emulsion via. high shear homogenization technique. The physical stability of emulsion was observed and its suitability to be used in topical application was characterized. The collagen prepared along with the VCO should hold high commercial interests due to its double functionality in improvement of facial skin qualities (moisture contents and relative elasticity).

MATERIALS AND METHODS

Hydrolysed collagen type I was prepared from Tilapia fish scales using combination of hydrothermal extraction with enzymatic hydrolysis (produced in our polymer laboratory) (Izzati *et al*., 2017). VCO was purchased from Sanggul Biru Enterprise and Azzahra Sdn Bhd (Bangi, Malaysia), respectively. Protease enzyme 2.4L FG was purchased from Brentag specialities (USA) and both non-ionic surfactants of Span 60 (sorbitan monostearate) and Tween 60 (polysorbate 60) were purchased from Sigma-Aldrich, (USA). Deionized water was prepared using easy pure LF, barnstead (USA). All the materials and chemicals were used without further purification.

Table 1: Compositions of emulsion

	Compositions		
φ_w	Mixed surfactant (Span 60: Tween 60)	Deionized H_2O	VCO
0.75	5	74.10	20.90
0.83	5	80.75	14.25
0.94	5	90.25	4.75

Preparation of emulsion: The emulsion compositions of mixed span 60: Tween 60/water/VCO systems were selected from a previous study (Alwi, 2013; Jamil, 2012). The emulsion was preparedusing three compositions which are vary in water volume fraction, φ_w of 0.75, 0.83 and 0.94 but have afixed Hydrophilic-Lipophilic Balance (HLB) value (5.006) as summarized in Table 1. The water phase consist of water-based hydrolysed collagen that was prepared by dissolving the hydrolysed collagen in deionized water (0, 5, 10, 15, 20 and 25 wt.%) prior to mixing with a hydrophobic surfactant, Tween 60 whereas the oil phase consists of a mixture of VCO and span 60. Then the water phase was titrated into the VCO phase and homogenized using a digital Ultra-Turrax homogenizer (model T18 from IKA) at 19, 000 rpm for 15 min. Schematic illustration of the formation of water-based hydrolysed collagen droplets is shown in Fig. 1. The emulsion samples were kept at 27±1°C for 24 h and centrifuged for 5 min at 3000 rpm before any testing was performed. Emulsion that was prepared without hydrolysed collagen acts as a control sample.

Microscopic analysis: An Optical Polarising Microscope, OPM (Eclipse 50i POL, Nikon, USA) was used to observe the microstructure of resulting emulsion. The emulsion was mixed with a few drops of methylene blue, placed on a slide using a spatula and covered with a glass slide. Polarised Light Microscopy (PLM) modes were used to observe microstructure of an emulsion containing hydrolysed collagen (φ_w = 0.75).

Droplet size analysis: The droplet size of the freshly prepared emulsion was measured by a Zetasizer Nano ZS (Malvern, UK), based on Photon Correlation Spectroscopy (PCS) techniques. The samples were prepared by diluting approximately 0.005 g of emulsion in 5 mL of VCO with gentle stirring. The measurements were done at room temperature.

pH measurement: The pH of the prepared emulsion was measured by using a pH meter (Thermo Scientific Orion 2-star benchtop pH meter, Cole-Parmer Instruments,

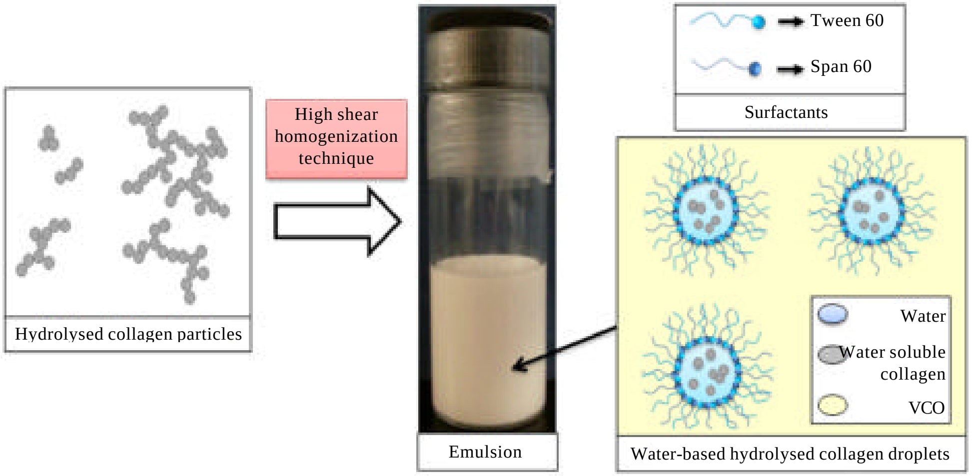

Fig. 1: Schematic illustration of the formation of water-based hydrolysed collagen droplets

USA). The measurement of one sample was repeated three times at 25°C. The average measurements for each sample were calculated and recorded.

Stability of emulsion: All freshly prepared emulsions were transferred into glass vials and then centrifuged at 3000 rpm for 10 min. After centrifuged, the creaming indexes of all emulsions were measured using the following Eq. 1:

$$\text{Creaming index}\ (\%) = \frac{\text{Height of emulsion layer (cm)}}{\text{Total height of emulsion sample (cm)}} \times 100\% \quad (1)$$

Rheological properties: Rheological properties of emulsion were measured by arheometer (Physica MCR 301, Anton Paar, USA) and data were collected in Rheoplus/32 V3.21 Software. A cone and plate system (CP25/1°, 25 mm) was used. A fresh sample was loaded for each run. The thickness of the sample at the centre of the sensor was 0.05 mm. The sample was allowed to rest and adapt for 5 min before each measurement was performed. All the tests were performed at constant temperature (25°C). The strain sweep test was measured in the strain ranging from 0.01-100% at a constant frequency of 1 Hz. Oscillation measurements enable us to obtain the Linear Viscoelastic Region (LVR) and provides the structural properties of each system. The flow characterization of the emulsion samples was obtained by recording shear stress and apparent viscosity values with increasing shearrates ranging from 0.0001-1000 sec^{-1} (logarithmic scale).

RESULTS AND DISCUSSION

Formation of emulsion: The emulsion does not flow even when the vialis upside down, showing concentrated emulsion produced. Hydrolysed collagen has been successfully loaded up to 20 wt.% into the water phase of the emulsion system without undergoing any phase change, even after being allowed to stand for 24 h. As was reported by previous study, the greater volume fraction of dispersed phase compared to continued phase with a maximal packing volume has resulted in the formation of a closed-packed droplets structure (Pal, 1999). Concentrated emulsion could retard the free motion of the droplet thereby delaying flocculation and coalescence of the droplets (Ng *et al*., 2014).

Microscopic study: The microscopic images of the typical emulsion and emulsion containing 5 wt.% of hydrolysed collagen at various φ_w of 0.75, 0.83 and 0.94 are shown in Fig. 2. The droplets dispersed widely in emulsion were light blue in color and showed spherical in shape. Since, methylene blue used in preparation of sample analysis is a water-soluble compound, the dispersion can be attributed as a type of water-in-oil (w/o) emulsion. In general, the size of the emulsion droplet containing 5 wt.% of hydrolysed collagen is much smaller than typical emulsion, irrespective of the water volume fraction studied. The microscopic results also showed that the droplet sizes of emulsions were decreased with increasing φ_w of 0.75-0.94. Similar results have been reported by Anisa and Nour (2010) that the 20-80% w/o which lower water volume fraction exhibit fine emulsion droplets compared to the 50-50% w/o. They also found that the

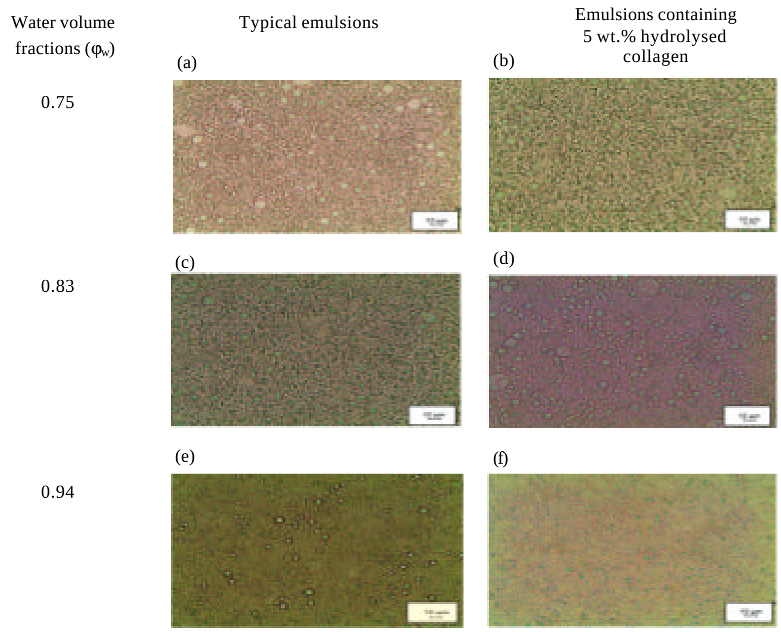

Fig. 2: a-f) Microscopic images of typical emulsion and emulsion containing 5 wt.% hydrolysed collagen at different water volume fraction, φ_w of 0.75, 0.83 and 0.94

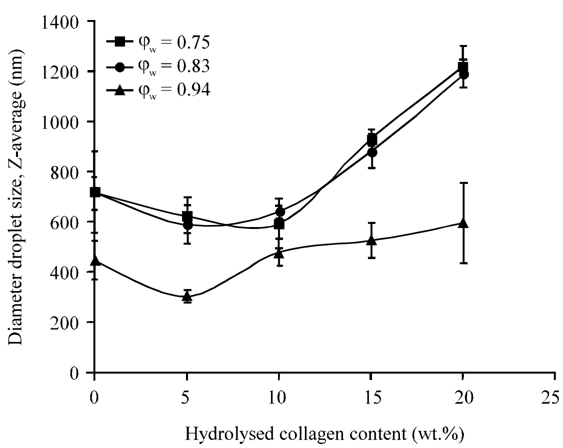

Fig. 3: Droplet size of emulsions at different water volume fractions, φ_w of 0.75, 0.83 and 0.94 as a function of hydrolysed collagen content

viscosity increased linearly with increasing of φ_w have supported the result of the concentrated emulsion prepared in this study. High water volume fraction of the emulsion system leading to decrease in the particle distances as well as an increase of resistance to flow, due to the increase of hydrogen bonds.

Droplet size measurement: The average droplet size (Z-average) of the emulsions at different φ_w of 0.75, 0.83 and 0.94 as a function of hydrolysed collagen content is shown in Fig. 3. The average droplet size of typical emulsion was found decreased with increasing φ_w. All samples at different φ_w exhibit similar trends in terms of droplet size changes against collagen content. The droplet size of the emulsion prepared at higher φ_w of 0.83 and 0.94 reduced to a minimum with the addition of hydrolysed collagen as low as 5 wt.%. Meanwhile the minimum droplet size of the emulsion prepared at φ_w of 0.75 was obtained after adding 10 wt.% hydrolysed collagen. A strong interaction between adjacent collagen particles caused by hydrogen bonding leads to a decrease in the size of the emulsion droplets, especially, for this closed-packed emulsion structure. The small size of the emulsion droplets allows for easy diffusion through the epidermal barrier when considering application to the skin In this study, the preparation of small and monodisperse droplets of emulsion is the main goal to ensure that the hydrolysed collagen can be absorbed through the skin easily when it was topically applied.

Regardless of the water volume fraction (φ_w), all the emulsion droplet sizes increased after further addition of hydrolysed collagen up to 20 wt.%. The results showed that the smallest droplet size of emulsion produced at φ_w of 0.94 even with the presence of 20 wt.% hydrolysed collagen. The distribution of smaller droplet size can be clearly seen in the microscopic image of emulsion in

Fig. 2. The minimum emulsion droplet size achieved with the addition of 5 wt.% (φ_w = 0.83 and 0.94) and 10 wt.% (φ_w = 0.83) of hydrolysed collagen may be caused by disturbances in the balance of HLB. After a certain amount, the emulsion droplets were not able to accommodate the presence of collagen which is a polar compound that will attract the polar surfactant. This causes a disruption to the interaction between the surfactant molecules at the interface membrane. The phase membrane becomes weaker and the presence of high quantities of collagen particles leads to the enlargement of the emulsion droplet. Previous study was reported that the solute interactions are greater with an increasing hydrophilic chain which leads to an increase in the aqueous solubility of the particles and then adecrease to the driving force of the surfactant to form micelles (Gasic *et al*., 2002).

pH values: The pH of a product for skin application is required to be the pH of skin in order to ensure the product will be well tolerated (Hekimoglu, 2004). It was reported that a pH range of 5-7 is the level buffered by the skin (Yorgancioglu and Bayramoglu, 2013). The pH values of the emulsion prepared in this study are in the range of 6.40-7.64. The pH values of the emulsion containing hydrolysed collagenare relatively low compared to typical emulsion. It is due to the presence of an amino acid chain in the hydrolysed collagen structure. Nevertheless, pH values around 6.0 are still non-irritating pH value for the skin (Bernardi *et al*., 2011).

Phase stability: Even after 12 weeks of storage, the creaming indexes of all samples were maintained at 100%, indicates no phase separation observed during the storage. Both typical emulsion and emulsion with hydrolysed collagen appeared to be stable, irrespective of the water volume fraction (φ_w). According to the results obtained, the formulation used is successful to keep an emulsion stable even with the presence of the hydrolysed collagen. It was indicated that the ratio of surfactant as low as five used in this study is sufficient and able to produce a stable emulsion containing hydrolysed collagen up to 20 wt.%. It was reported that the emulsifying properties of collagen also play an important role in stability of oil-in-water emulsion by reducing oil droplet size (De Castro Santana *et al*., 2012).

Rheological properties

Strain sweep test: Strain sweep tests have been performed on both samples, typical emulsion and emulsion containing 5 wt.% of hydrolysed collagenas shown in Fig. 4a, b, respectively. All emulsion sowned a higher value of “G than G” within the Linear Viscoelastic Region (LVR) which is the typical behaviour of viscoelastic samples. Although, the range of LVR for both emulsions is almost the same but the downward trend of G’ for the emulsion containing hydrolysed collagen is quite slow compared to the typical emulsion. This indicates that the emulsion containing collagen is more easily disrupted. In addition, the decreasing trend of G’ for that emulsion is observed after the LVR value compared to the typical emulsion. This shows that the disruption that occurs in the structure of the water-based hydrolysed collagen droplets caused the decrease in viscosity at high strain amplitude.

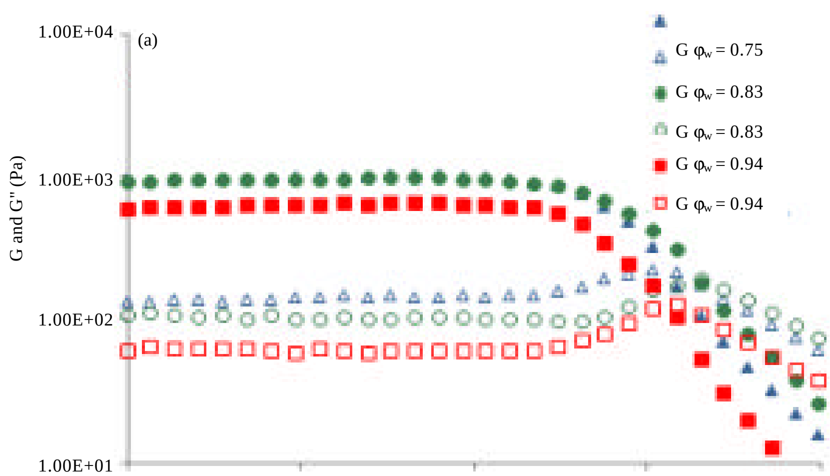

Fig. 4: Continue

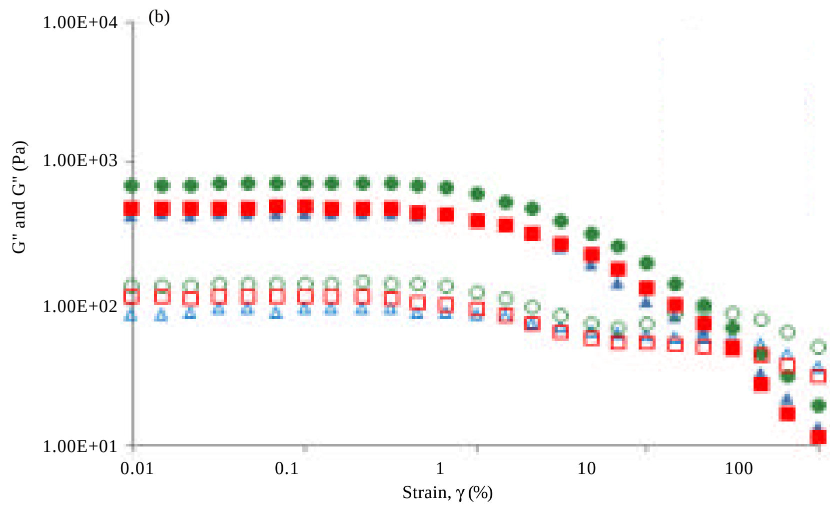

Fig. 4: “G & G” comparison of; a) Typical emulsion and b) Emulsion containing 5 wt.% hydrolysed collagen

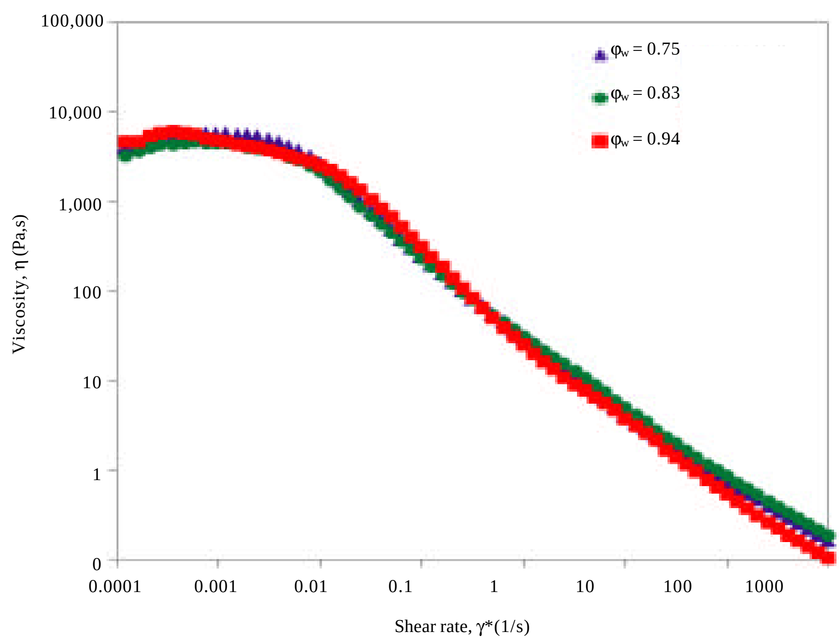

Fig. 5: Flow curve of the emulsion containing 5 wt.% of hydrolysed collagen at φ_w of 0.75, 0.83 and 0.94

However, the strength of the internal structure in an emulsion can be measured by tan δ = “G/G” due to its dependent on the differences between “G and G”. A clear comparison can be seen from the summary of the G’ and Tan δ values of all emulsions in Table 2. Although, the presence of hydrolysed collagen increases the Tan δ value of emulsion, reconfirming the occurrence of a small disruption in the internal structure but there was no significant difference in the tan δ value of all emulsions studied against the content of hydrolysed collagen. The value of Tan δ for all samples prepared can still be considered small as it is >0.3 Table 2. Liu *et al.* (2007) was stated that the Tan δ value of emulsion lower than one exhibited solid-like behavior. This implies that the emulsion is constructed of strong internal interactions (Fig. 5).

Shear flow test: The continuous shear flow test provides important information regarding the ways in which the structure changes to comply with the applied shear

Table 2: Storage modulus (G) and tan δ values of emulsion at 0.1% strain

δ_w	Hydrolysed collagen in water phase (wt.%)	Storage modulus, G (Pa)	Tan δ
0.75	0	969	0.15
	5	434	0.20
	10	1080	0.25
	15	1970	0.27
	20	2540	0.23
0.83	0	962	0.11
	5	724	0.20
	10	1940	0.25
	15	3870	0.29
	20	1310	0.27
0.94	0	631	0.09
	5	479	0.23
	10	867	0.28
	15	664	0.27
	20	1640	0.27

flow in different conditions such as storage, processing and application. The flow curve in Fig. 5 shows emulsion containing 5 wt.% hydrolysed collagen exhibited non-Newtonian characteristics, since, the emulsion underwent deformation when subjected to the shearing tension test and did not exhibit constant viscosity. The emulsion behaved as a pseudoplastic or with shear-thinning as the emulsion viscosity decreases with shear. The shear rate remains at zero until a threshold shear stress is reached and the pseudoplastic sample flow begins. Such, behaviour is due to the existence of an intermolecular network which initially acts like a solid and becomes a fluid when the applied stress exceeds the strength of the network. Shear thinning behaviour is the most relevant criteria for an application in the cosmetic field (Kwak *et al*., 2015).

The rheological behaviour of the emulsion in shear flow test also depends on the concentration of the dispersed phase (Foudazi *et al*., 2012). It was clearly shown that the flow properties of the emulsion changed drastically with increasing shear. This has proved that the emulsion droplets are densely packed and easy to deform. The emulsion prepared at the highest water volume fraction ($\varphi_w = 0.94$) has the highest viscosity at low shear. This could be confirmed by the droplet size measurement, in which the emulsion droplets prepared at φ_w of 0.94 shows the smallest size than the others. Similar results have been reported by Malkin *et al*. (2004) that the emulsion with small droplet size exhibited high viscosity at a low shear rate.

CONCLUSION

A stable water-in-oil emulsion containing fish scale hydrolysed collagen was successfully prepared via. high shear homogenization technique. The hydrolysed collagen can be uploaded up to 20 wt.% into an emulsion system of span 60/VCO-Tween 60/H_2O at φ_w of 0.75, 0.83 and 0.94 without phase separation. A high shear homogenization weakens the intermolecular interaction of hydrolysed collagen particles and forms the high dispersion of water-based hydrolysed collagen droplets in virgin coconut oil phase. Aminimum droplet size (Z-average) of 305 nm was obtained after loaded with 5 wt.% hydrolysed collagen at φ_w of 0.94. All results obtained show that the emulsion system is an ideal medium for the dispersion of hydrolysed collagen particles. The small size of water-based hydrolysed collagen droplets will allow the easily diffusion of hydrolysed collagen particles through the skin. This finding also showed that the pH of the emulsionis acceptable for topical application. In addition, the emulsion prepared has pseudoplastic and shear-thinning characteristics that are suitable for cosmetic application such as creams and moisturizers. Emulsion containing hydrolysed collagen has a potential to bedirectly applied to the skin. The addition of hydrolysed collagen as an active compound in the emulsion system can give value added to the VCO-based emulsion. As this is a preliminary study, further studies will include *in vitro* and *in vivo* evaluation for topical delivery.

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