# Penetration Of Solid Lipid Nanoparticle (Sln) Containing Extracts Collagen From Scales And Skin Of Red Snapper (Lutjanus Malabaricus)

**In Rahmi Fatria Fajar, Swasono R Tamat, Deni Rahmat**

**Abstract:** until now, needs of collagen are fulfilled mostly from beef or poultry. As there are more diseases found in poultry, the source of collagen from the skin and scales of fish becomes one promising alternative. The observed collagen parameters include proximate analysis, SDS-Page and heavy metals. To strengthen penetration of collagen, the application is made in form of solid lipid nanoparticles (SLN). SLN is made from Brij (5, 6, 7%) as the surfactant and Glycerol monostearate (GMS) (4, 5, 6%) as the cosurfactants and high-speed homogenization to produce a high penetration power. In-vitro penetration test is conducted by using Franz diffusion cells and mouse skin membrane. SDS-Page analysis shows various types of peptides contained in collagen with molecular weight of 100, 80, 70, 60, and 50 kDa. SLN compounds also meet the requirements which includes spherical morphology, particle size of 61.1 - 965.9 and zeta potential of (-3.16) – (12.37). The study concludes that collagen which is derived from the scales and skin of Red Snapper (Lutjanus malabaricus) can be applied in solid lipid nanoparticle (SLN) compound by using Brij (6%) and Glycerol monostearate (4%) as the surfactants and the cosurfactants, and having the highest penetration value of 897,125 μg/cm$^2$.

**Key words:** collagen, solid lipid nanoparticle (SLN), scales and skin of red snapper, penetration test

## 1 INTRODUCTION

Utilization of fish skin waste as collagen raw material is one alternative to increase the added value of fishery industrial waste and to reduce its negative effect (pollution) to the environment. Collagen is commonly isolated from skin tissues of terrestrial animals (cattle and pig). However, some consumers are found to be infected by bovine spongiform encephalopathy (BSE) and hoof-and-mouth disease due to the use of collagen produced from cattle skin tissue[1] and pigskin tissue, which are forbidden for Moslems. This condition opens an opportunity for utilizing fish skin as a source of collagen source alternative [1]. Collagen that is isolated from cattle or pig can not be absorbed by the skin perfectly due to its larger molecular structure. Collagen that is isolated from fish has smaller molecular structure so it can be better absorbed by the skin. Besides, the collagen structure isolated from fish is similar to human collagen structure. Collagen isolated from fish is able to function as a substrate at the in-vitro culture better (better splitting capabilities) than that from cattle or pig, making it have more benefits. From the above-mentioned problem, the writer will develop compound of solid lipid nanoparticle (SLN) that contains collagen from scales and skin of red snapper to make a high skin penetration power and higher therapeutic effects and at the same time, it will be easy and comfortable in use. The benefits of this research will provide an isolation method of collagen from red snapper scales, a method of making solid lipid nanoparticle (SLN) which contains active collagen substances collagen and an understanding of penetration speed of the compound [2].

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## 2 MATERIALS, TOOLS AND METHODS

### 2.1 Materials

The scales and skin of red snapper taken from Muara Angke, acetic acid (Brataco), sodium hydroxide (Brataco), sodium chloride (Brataco), Span 80 (Brataco) (Brataco), propilenglikol (Brataco), Brij (S2-PA-SG), BHT, methyl paraben (Brataco), propyl paraben (Brataco) , VCO (Optima), glycerin monostearate, L-dopa, enzyme tyrosinase (Sigma)

### 2.2 Tool

Centrifuge, pH meters, viscometer (Brookfield), Transmission Electron Microscope (TEM), Particle Analyzer (Delsa Nano C) Photon Correlation Spectroscopy (PSC), analytical scales (Mettler Toledo), homogenizer (IKA T25 digital ULTRA TURRAX), freezer dryer, UV-vis spectrophotometer (Shimadzu UV-1800), oven, microplate reader, Atomic Absorption Spectrophotometer (AAS), SDS-Franz

### 2.3 The isolation of collagen from scales and skin of Red Snapper (a modified Ogawa-M method) [3]

The collagen isolation process of black Nile Tilapia's skin samples using Ogawa method et al. (2004) with modifications. The stages are as follows:

a. For main raw-material preparation, skin samples are cut into 1-cm-x-1-cm pieces.
b. To remove the non-collagen protein, the samples are soaked in a 0.1-M NaOH solution by 1:10 comparison. The blend of skins and scales with NaOH is then kept for 24 hours at the temperature of 28° c.
c. The blend is then extracted by acetic acid ($CH_3COOH$) with 0.5-M concentration. The soaking process accompanied by the stirring is completed in 2 days.
d. Next, the samples are filtered by using 1000-mesh Dacron fabric/gauze fabric; separate the macerate from the scales and skin. The collagen is then purified in salting-out method by using NaCl salt



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until reaching a 0.9-M concentration in 24 hours at the temperature of 25° C until the wet collagen is obtained.

e. The last stage is drying/lyophilization in freeze-drying method.

## 2.4 The test of collagen from scales and skin of Red Snapper

Collagen from scales and skin of red snapper is characterized by conducting proximate test (proteins, moisture, ash, and fat content), SDS-PAGE test and heavy-metal test.

**a. Proximate Test (moisture, ash, proteins and fat content)**

**1) Moisture content [4]**

Determining moisture content is conducted by taking 1-2 gram sample and placing it in an empty vial, together which its lid is previously weighed, dried in the oven and cooled in the dessicator. Vial containing the sample is then closed and put in the oven for 3 hours with a temperature of 105° C. The vial is then cooled in the dessicator and after cold, the vial is weighed. Moisture content is calculated with the formula:

Moisture content: $\frac{W}{W_1} x\ 100\%$

Description:

W = sample & vial weight in grams before being dried

W1 = sample & vial weight in grams after being dried

**2) Ash content [4]**

Determining ash content is conducted by taking 1-2 gram sample and put in a crucible that is previously weighed and burned in the muffle furnace and then cooled in a desiccator. The crucible containing samples is then put into the furnace and burned perfectly. The burning is done at 550°-C temperature. Finally, the crucible containing ashes is cooled in a desiccator and weighed. The ash content is calculated by the following formula:

Ash content = $\frac{W1 - W2}{W}$ x 100%

Description:

W = sample & crucible weight before burnt in grams

W1 = sample & crucible weight after burnt in grams

W2 = empty crucible weight in grams

**3) Protein content [4]**

Determination of protein content is conducted by micro-Kjeldahl method. 0.51-gram sample is prepared and put into 100-mL Kjeldahl flask. Then it is added by 2.5 grams of SeO powder, 2,100 grams of $K_2SO_4$, and 30 grams of $CuSO_4.5H_2O$ and 25 mL of $H_2SO_4$ (p). The sample is destructed for 2 hours until the liquid becomes clear green and is then cooled and added by 100-mL distilled water. By using 5-mL pipette, 5-ml 30% NaOH is inserted into the flask, together with PP indicator and then the flask is distilled. Distilled liquid is placed in a 125-mL Erlenmeyer which contains 10-mL $H_3BO_3$ and PP indicator. Finally, it is titrated by using 0.01-N HCl until the color becomes pink.

**4) Fat content [4]**

2-gram sample is weighed and wrapped with filter paper and is then covered with fat-free cotton and put into fat flask. After that, it is put into a soxhlet extractor with the position of condenser be on top and the fat flask be below. Petroleum benzene is added into the fat flask and it is extracted for about 6 hours at 40°-C temperature until the solvent which falls back into the fat flask becomes clear. The solvent inside the fat flask is distilled in order to evaporate all fat solvent. After that, the extracted flask is dried in an oven at 105°-C temperature. Next, the flask is cooled in a desiccator and then weighed. Determination of fat content uses the following formula:

Fat content = $\frac{W1 - W2}{W}$ x 100%

Description:

W = flask weight in grams

W1 = flask & fat weight before the extraction in grams

W2 = flask & fat weight after extraction in grams

**b. SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electroforesis) [5]**

The sample is dissolved in 5% SDS and the mixture is incubated at the temperature of 85° C for 1 hour in a temperature-controlled water bath. The mixture is centrifuged at 8,500 rpm in 5 minutes at room temperature. The resulted supernatant is mixed with the buffer (HCL Tris of 60 mM and 6.8 pH; containing 2% SDS and 25% glycerol) with 1:10 ratio (v/v) and containing 10% β-merkaptoetanol (β-ME). The mixture is heated in boiling water for 2 minutes. 15-μL sample is then inserted into polyacrylamide gels, consisting of 8% running gel and 4% stacking gel and electrophoresis is applied at 50-mA/gel constant current for 2 hours. After electrophoresis finishes, gels are marked with 0.05% (b/v) R-250 blue Coomassie in 15% (v/v) methanol and 5% (v/v) acetic acid for 1 hour, then the samples are marked by a mixture of 30% (v/v) methanol and 10% (v/v) acetic acid for 1 hour. The weight of protein molecular sample is estimated based on molecular marker weight.

**c. Analysis of heavy metals [6]**

Heavy metal analysis begins with the making of calibration curve from standard solutions of Hg, Pb and As. The resulted absorbance will have a linear relationship with the measured analyte concentration, following the law of Lambert-Beer. The making of calibration curve begins with the creation of lead, tin and cadmium standard solution dilution series. To get the desired concentration, primary solution of each metal is diluted. The contents of heavy metals (Hg, Pb and As) are analyzed by using Atomic Absorption Spectrophotometer (AAS).

### 2.5 Formulations of Solid Lipid Nanoparticle (SLN) [7]

Preliminary tests are carried out to determine the best conditions and the best composition of surfactants and cosurfaktan materials to produce stable and homogeneous solid lipid nanoparticle (SLN) compounds. The formation of solid lipid nanoparticles (SLN) compounds uses high-speed homogenization technique. The composition of solid lipid nanoparticle (SLN) materials includes VCO with 40% concentration, 0.02% BHT, optimization of 5% - 6% - 7% Brij concentrations, optimization 4% - 5% - 6% gliseril monostearat concentration, 20% propilenglikol, 0.02% propyl paraben, 0.1% methyl paraben, 80% span, and 2.5% - 3% - 3.5% collagen concentrations as active substances.

### 2.6 Characterization of Solid Lipid Nanoparticles (SLN) [8]

Characterization of solid lipid nanoparticle (SLN) is applied on the three formulations, where the nature and characteristics are expected similar to those when created.

a. Morphology test for solid lipid nanoparticle (SLN)
   Shape and morphology of solid lipid nanoparticles (SLN) are observed by using Transmission Electron Microscopy (TEM).
b. Determination of solid lipid nanoparticle (SLN) particle size
   Solid lipid nanoparticles (SLN) particle size is observed by using Particle Analyzer with Photon Correlation Spectroscopy (PSC) technique. The results obtained from the Particle Analyzer are particle size data from the formula [9].
c. Determination of solid lipid nanoparticle (SLN) zeta potential
   Zeta potential value of solid lipid nanoparticles (SLN) is determined by using Delsa Nano C Particle Analyzer. Electrophoretic light scattering (ELS) method is applied in this analysis so the electrophoretic mobility of the particles can be measured [9].

### 2.7 Evaluation of Solid Lipid Nanoparticle (SLN) Physical Compound

Organoleptic observation is applied to color, odor, pH and viscosity of the compound produced.

### 2.8 In vitro penetration test for Solid Lipid Nanoparticle (SLN) Compounds by Using Franz Diffusion Cell [10]

Gel compound penetration test for mouse abdominal skin is performed by using Franz diffusion cells (1.77-cm$^2$ area diffusion size, 13.0-mL compartment volume, pH-7.4 phosphate buffer solution at the receptor compartment and 37+0.5° C temperature). The shaven mouse abdomen skin is placed between the donor compartment and the receptor compartment with facing-up stratum-corneum position. Solid lipid nanoparticle compound is weighed each 1 g, then is applied on the skin surface of the donor compartment. Next, 2-mL sample is taken periodically in 3 hours from the receptor compartment by using a syringe and is replaced by a similar amount of pH-7.4 phosphate buffer solution. The sample is then diluted by using pH-7.4 phosphate buffer solution in a 10-mL measuring flask and the absorption is measured by using UV-Vis spectrophotometer.

### 2.9 Stability test of Solid Lipid Nanoparticle (SLN) [11]

Stability test of gel compounds includse color, odor, pH and viscosity. Compounds are evaluated at low temperature (4±2° C), room temperature (27±2° C) and high temperature (40±2° C) in 12 weeks with observations conducted every 2 weeks, and cycling test conducted in 6 cycles.

### 2.10 Solid Lipid Nanoparticle (SLN) Irritation Test [12]

Primary skin irritation is measured by a technique of patch test on bruised and normal skins from clean-shaven albino rabbit. There are three subjects used for each glass slide tested. The method is conducted by placing 0.5 ml (for liquid) or 0.5 g (for solid and semi solid) under one-inch patch. Then, the entire animal body is wrapped with rubber-coated cloth for 24 exposing hours. This procedure helps in maintaining the tested patch on its position, besides preventing the evaporation of volatile substances. After 24 hours of exposure, the patches are removed and the coming reactions are evaluated based on score. The test-result reading is conducted 72 hours afterwards, and the final scores should represent the 24-and-72-hour readings. Finally, the reactions are observed subjectively and objectively in accordance with Table 1.

***Table 1.*** *Scoring Category of Skin Condition*

| Erythema | | Edema | |
|---|---|---|---|
| Type | Value | Type | Value |
| No erythema | 0 | No edema | 0 |
| Little erythema (almost invisible) | 1 | Extremely slight edema | 1 |
| Obviously visible erythema | 2 | Slight edema (obvious edge and magnification) | 2 |
| Mild-to-strong erythema | 3 | Mild edema (about 1 mm thick) | 3 |
| Severe erythema (wound visible) | 4 | Severe edema (more than 1 mm thick) | 4 |

## 3. RESULTS AND DISCUSSION

### 3.1 Proximate Test

Proximate test results of collagen derived from Red Snapper scales and skin are 7.28% moisture, 0.06% ash, 3.36% fat and 59.83% protein. 59.83%-protein level shows that the fish skin can serve as raw material for collagen products. According to SNI [13], in general the average content of skin collagen skin is 21.50% for wet weight and 71.20% for dry weight.

### 3.2 SDS-PAGE

SDS-PAGE analysis result shows that 10%-SDS solution and Commasie blue dye are used, based on the reference of the previous research [5]. The analysis results are presented at Figure 1.



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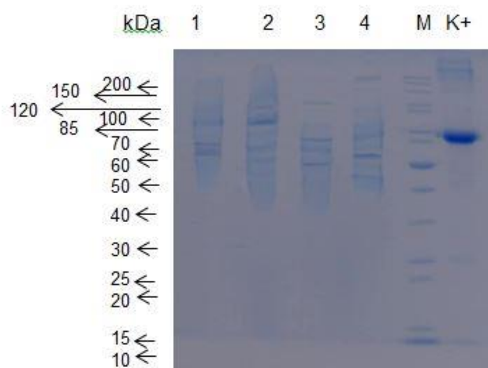



***Figure 1.** SDS-PAGE electrophoresis pattern*

Description:
(1) fish scales with ethyl acetate solvent; (2) fish scales with water solvent; (3) of fish skin with ethyl acetate solvent; (4) fish skin water solvent; (M) marker; (K +) Positive control.

Test results have a molecular weight of 100, 80, 70, 60 and 50 kDa. These results indicate that there are different types of peptides contained in Red Snapper scales collagen [14]. Based on the pattern and migration distance, peptide ribbon (protein) for extracted collagen is assumed to be type-I collagen which consists of two twin peptide chains, namely α1 and α2 chains. Type-I collagen is the most known collagen which is found in all connective tissues, including skin and bones. This collagen contains 1-3 glycine, contains neither tryptophan nor cysteine, and contains little thyroxine and histidine.

### 3.3 The Analysis Results of Heavy Metals

The heavy-metal-analysis results of the collagen derived from the scales and skin of Red Snapper (Lutjanus malabaricus) include metals of Hg, As and Pb with the content as follows: Hg is 0.0694 mg/mL, As is 0.0864 mg/mL and Pb is 0.0705 mg/mL. Based on the test results, Pb, Hg, and As heavy-metal levels of Red Snapper scales and skin collagen are still under heavy-metal thresholds for fish and its processed products specified by SNI 7387-2009 that is 0.3 mg/Kg for Pb; 0.5 mg/Kg for Hg and 0.1 mg/Kg for As. This shows that Red Snapper (Lutjanus malabaricus) scales and skin are safe to use as the raw material for collagen.

### 3.4 The Formulation of Solid Lipid Nanoparticle (SLN)

The best optimization result of the surfactant and cosurfaktan is made into the next formula where the best surfactant (brij) is at 6% concentrations and cosurfaktan (GMS) is at 4%. The formula contains 2.5%, 3% and 3.5% active substances.

### 3.5 Evaluation of Solid Lipid Nanoparticle (SLN) Characteristics

#### Particle Size

The small size of the vesicles is crucial to topical delivery, therefore it is important to know the particle average size and size distribution. The diameter size data of the three formulas (1, 2 and 3) after the extrusion process respectively gives the value of 364.1; 965.9 and 61.1 nm (Table 2). The diameter values are used to compare the vesicle size of the three formulas since the values can reflect the size of the particles in the bulk sample volume. The other parameter obtained from the test using PSA is the value of PDI (Polydispersity Index) which illustrates the value of its homogeneity vesicles. Compounds revealed homogeneous have PDI value until 0.6. PDI of Formulas 1, 2 and 3 after consecutive extrusion is 0.54; 0.08 and 0.18. This shows that Formula 3 is the most homogeneous.

***Table 2***
*The test results of SLN particle size*

| Formula | Diameter (nm) | Pd Index | % Intensity |
|---|---|---|---|
| Formula I | 364,1 | 0,54 | 99,8 |
| Formula II | 965,9 | 0,18 | 97,1 |
| Formula III | 61,1 | 0,08 | 100 |

#### Zeta Potential

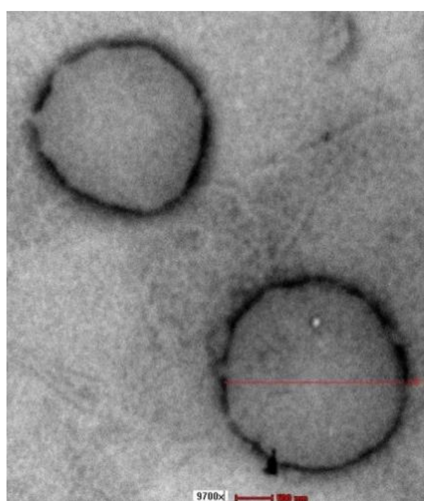
Zeta potential has an application in system stability containing dispersed particles, since this potential regulates the degree of repulsion between dispersed particles of equal charge and adjacent each other. Zeta potential is a good predictor of gelation phenomena [8]. The zeta potential test results of the solid lipid nanoparticles are as follow: Formula 1 is -3.16 mV, Formula 2 is -14.77 mV and Formula 3 is -12.37 mV. Zeta potential reflects the potential charge of the particle and is affected by the composition of the particles and the medium where the nanoparticles are dispersed[8]. The particle charge shows a negative zeta potential because it tends to absorb hydroxyl ions. The negative charge is also affected by Brij which is an anionic surfactant. The results of the zeta potential test of solid lipid nanoparticles are presented in Table 3.

***Table 3***
*Zeta potential test results*

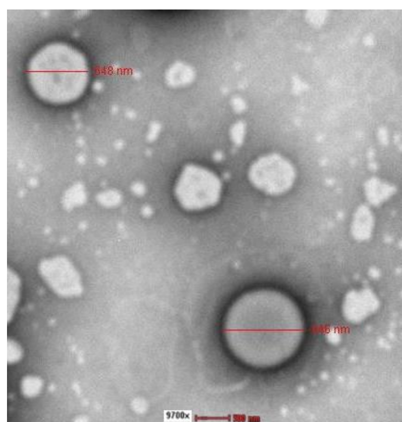
| | Formula I | Formula II | Formula III |
|---|---|---|---|
| | Zeta Potential (mV) | | |
| Sample 1 | -3.73 | -11.89 | -12.63 |
| Sample 2 | -2.82 | -30.89 | -11.68 |
| Sample 3 | -3.42 | -12.5 | -12.75 |
| Sample 4 | -3.6 | -15.08 | -12.7 |
| Sample 5 | -2.78 | -14 | -11.87 |
| Sample 6 | -2.53 | -13.69 | -11.95 |
| Sample 7 | -3.26 | -13.63 | -11.41 |
| Sample 8 | -3.17 | -11.83 | -13.47 |
| Sample 9 | -2.77 | -10.76 | -12.43 |
| Sample 10 | -3.47 | -13.44 | -12.76 |
| Average | -3.16 | -14.77 | -12.37 |

#### Shape and Morfology Observation

Transmission Electron Microscopy (TEM) test results are shown at Figure 2.



(a)



(b)

***Figure 2.***
*Morphology test result of solid lipid nanoparticle (SLN) - Formula I (a), Formula II(b)*

The TEM result shows that the compound morphology has a uniformly rounded shape. This indicates that the active substance is stable and protected by the emulsifier.

### 3.6 Physical Evaluation of Solid Lipid Nanoparticle (SLN) Compounds

Organoleptic observation, pH

Solid Lipid Nanoparticle (SLN) compounds have a milky white color, a distinctive odor (VCO), and thick. They have a pH value of respectively Formulas 1, 2 and 3 for 6.09; 6.89 and 7.12.

### 3.7 Viscosity and Fluidity Test

Based on the observation, the three formulas experience decreasing viscosity when stirring with high RPM. It is because the higher the RPM is, the lower the compound viscosity will be (melting), making the compounds be easier to pour. Solid lipid nanoparticle (SLN) compounds have plastic fluidity. Plastic is a non-newton fluidity where with the friction force, the compounds will flow in ideal viscosity. In general, the compounds have a plastic fluidity where the higher friction force is applied, the viscosity will decrease, making the compounds flow easily from the tube and more easily spread on the skin surface.

### 3.8 Evaluation of Solid-Lipid-Nanoparticles Penetration by Using Franz Diffusion Cells

In vitro penetration tests are conducted by using membrane as a skin model. The membrane can be a biological membrane from animals or artificial membranes like cellophane. The purpose of penetration test is to know the comparative amount of solid lipid nanoparticles collagen penetrated through skin in a specific time interval. Mouse skin is used as a membrane because it is easily obtainable and has permeability similar to humans', regardless its bigger permeability level than humans'. The solution used as the liquid on the receptor compartment is 7.4-pH phosphate buffer which is similar to human-body biological fluids. The temperature during the test is maintained at 37+0,5$^{o}$ C by using water jacket. This temperature is similar to normal human body temperature. The temperature should be kept constant since the temperature change will affect the penetration of active substances of the compounds. The test is conducted in 3 hours and sampling is applied at 6 points, which are in 30; 60; 90; 120; 150 and 180 minutes. In vitro percutaneous penetration test has two main parameters, namely the cumulative amount of the penetrating active substance and the penetration rate or flux. The cumulative amount of penetrating collagen and its 3-hour flux is shown in Figures 3 and 4.

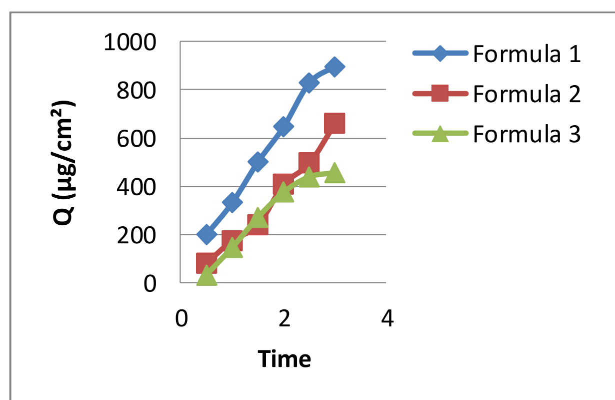



***Figure 3.*** *Profile graphic of cumulative amount of penetrating collagen*

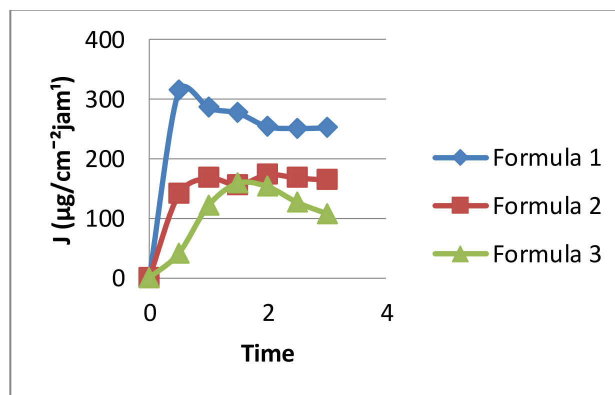



***Figure 4.*** *Profile graphic of collagen flux*

Based on the test, the cumulative amount of penetrated formula I (897,125) is higher than of formula II (660,425) and formula III (456,695). It is because the consistency of formula I is lower than of formula II and formula III, so it is easier to penetrate the skin membranes, resulting more collagen able to penetrate into the skin. In addition, the formation of solid lipid nanoparticle can protect collagen which is usually easy to be oxidized into the compounds. One of the other factors that influences the penetration through membranes is a hydrated corneum stratum. This hydration effect will increase the moisture content of the skin, where the water will open a compact corneum layer structure and the keratin threads in epidermal layer will expand, making skin permeable. Water in the compounds can also increase the amount of penetrated collagen. Based on collagen flux profile, formulas I, II and III have ascending and descending curves. The ascending curve indicates that the concentrated gradient between donor and receptor compartments is high, while the descending curve indicates that the collagen concentration at donor receptors begins to decrease.

### 3.9 Stability Test

Solid lipid nanoparticle (SLN) compound shows better physical stability by observing the change in color, odor, pH and viscosity and so the formula is declared stable for 12 weeks.

### 3.10 Irritation test

Irritation test on rabbit skin is intended to detect the irritation effect caused by the compounds to make it safe when applied into human's skins. Irritation test result is shown at Table V. 30. Irritation test result based on primary irritation index is 0 and is classified as not affected. This suggests that the three formulas do not show the irritation effect into the skin, so it is safe to use.

## 4 CONCLUSION

1. Collagen can be isolated from the scales and skin of Red Snapper (Lutjanus malabaricus) with 1.06% yield.
2. The above-mentioned 2.5% collagen with 6% brij used as the surfactant and 4% glycerol monostearat used as the co-surfactants can be formulated into good solid lipid nanoparticle (SLN) compound, with the size of 364.1, nm and in accordance with physicochemical requirement.
3. Collagen extract has inhibiting activity in the tyrosinase enzyme with 244.92-ppm $IC_{50}$.
4. Solid-lipid-nanoparticles compound can penetrate into the skin, has a good stability for 3 months and does not cause irritation to the skin.

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