Characteristics physicochemical of melanin from squid ink (loligo sp.) extracted by ethanol

F Abidin\textsuperscript{1}, L Sulmartiwi\textsuperscript{2*}, E Saputra\textsuperscript{2}

\textsuperscript{1}Program Study of Fisheries Product Technology, Faculty of Fisheries and Marine, Universitas Airlangga, Kampus C Jalan Mulyorejo, Surabaya 60115 East Java, Indonesia
\textsuperscript{2}Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Kampus C Jalan Mulyorejo, Surabaya 60115 East Java, Indonesia

Corresponding author: laksami-s@fpk.unair.ac.id

Abstract. Squid ink is liquid waste from the fishery product processing industry, it has the potential to pollute the environment because it contains a lot of organic material. Squid ink contains pigment such as melanin which is a biological material and has many benefits. Extraction using polar solvents namely Ethanol. The purpose of this study was to determine the effect of different concentrations of ethanol solvents on the extraction of melanin from squid ink (Loligo sp.) on physicochemical characteristics. The treatment used is the difference in the concentration of the solvent given, namely HCl 2 M (P0), Ethanol 2 M (P1), and Ethanol 3 M (P2) each treatment was repeated six times. The parameters of this study were protein content, fat content, and ash content, and pigment colour analysis (FT-IR) test. The results of the study showed that the difference in the concentration of the solvent used affected the yield, proximate values (protein content, fat content, and ash content), and did not eliminate the melanin content in it after the FT-IR test. The characteristics physicochemical of melanin has a different result on every parameter test. The concentration of ethanol for extracted melanin from squid ink affects the characteristics of physicochemical.

1. Introduction
Squid is one of Indonesia’s fishery resources which has economic value and is popular with the community [1]. According to data from the Ministry of Marine Affairs and Fisheries, it is stated that in 2010-2011 there was an increase in exports of squid by 34,925,401 kg to 48,803,318 kg. International trade class squid is marketed as frozen squid, dried salted squid and canned squid. Squid products produce large waste and are not utilized. One of the squid waste is squid ink [2].

Squid ink is the liquid waste of the fishery product processing industry which is found in almost all squid processing industries, so it has potential pollutes the environment because it contains a lot of organic matter. The waste product of the squid processing industry is in the form of liquid waste containing various kinds of organic materials such as protein, fat, pigments, and mineral salts so that it affects the characteristics of wastewater [3]. Melanin is a biological compound in the form of a pigment found in plants, animals and protists. The resulting pigment is a derivative of the amino acid tyrosine. The function of melanin in organisms is as protection from exposure to ultraviolet (UV) rays [4]. The
The melanin pigment contained in squid ink is naturally in the form of melanoprotein with melanin content of 90%, 5.8% protein, and 0.8% carbohydrate [1].

The melanin in squid ink isolation is done by using the extraction method [5]. The extraction process with the maceration method uses a solvent and each solvent has different abilities in taking bioactive compounds from a sample. Research on melanin from squid ink has been widely carried out, but the selection of the right solvent to extract melanin from squid ink is still limited, so this research is necessary to determine the effect differences in the concentration of ethanol solvent in melanin extraction from squid ink (Loligo sp.) on physicochemical characteristics (yield value, protein content, fat content, ash content, and pigment color analysis test).

2. Material and methods
2.1. Research material
The tools used in this study were autoclave, refrigerator, freezer, erlenmeyer 1000 ml, erlenmeyer 500 ml, erlenmeyer 50 ml, measuring cup 100 ml, FT-IR spectroscopy, trays, gloves, masks, volume pipettes, syringes, test tubes, freeze dry, refrigerated centrifuge, and magnetic stirrer. The material used is squid ink (Loligo sp.), aluminum foil, cotton, newsprint, alcohol, distilled water, acetone, 2 M HCl, 2 M and 3 M Ethanol.

2.2. Preparation of squid ink
The yield of squid ink produced was 0.40-0.60 grams/ind with an average weight of 69.97 grams of squid [6]. The ink for the squid is dark black and still mixed with water. The squid ink is then put into bottles that have been sterilized and stored in the freezer at 3°C to prevent damage to the squid ink. The squid ink that has been stored in the freezer is then dried using the freeze dry method to make it a dry powder to facilitate the extraction process.

2.3. Melanin extraction of squid ink (Loligo sp.)
Melanin extraction in squid ink was carried out according to the modified Magarelli et al [7] method and the modified Ikram et al [8] method. The extraction stage was carried out in 2 M ethanol solvent. The dried squid ink sample was weighed as much as 0.5 grams then mixed in 50 mL of solvent. Preparations are carried out in light-tight conditions. The same was done with the treatment of squid ink and 3M ethanol solvent. Then the solution was stirred using a magnetic stirrer for 30 minutes, then stored for 24 hours at 10°C. The precipitate was separated from the supernatant using a refrigerated centrifuge (10,000 rpm at 5°C for 15 minutes). The precipitate (solid) was washed or resuspended with 2 M ethanol and 3 M ethanol solutions according to the concentration of ethanol solvent at the beginning of extraction three times, followed by distilled water, acetone, and finally with distilled water again, then let stand for a while so the remaining solvent evaporates by itself. The control treatment in this study was to extract squid ink using 2 M HCl solvent. The dry powder of 0.5 grams of squid ink was mixed in 50 mL of 2 M HCl solvent. The next step, the samples are stored in a freezer before further testing.

2.4. Testing of the physicochemical characteristics of melanin
The melanin produced was then tested for physicochemical characteristics to determine the effect of using different solvents in the extraction process. Testing of melanin results includes the calculation of the final yield, protein content, fat content, ash content, and test of melanin pigment colour analysis using FT-IR spectroscopy.
2.5. Rendement
The yield is one of the important values in determining the level of effectiveness of a solvent in extracting a compound contained in a material. Yield is the ratio of the dry weight of the product to the initial weight of raw materials. The yield calculation was based on the ratio of the final weight to the initial weight multiplied by 100% [9] (formula 1.)

\[
\text{Yield (\%) } = \frac{\text{Total final weight} \times 100}{\text{Total initial weight}} \tag{1}
\]

2.6. Protein content
Protein content is the percentage of protein content contained in melanin in squid ink. The method that can be used for protein analysis is the spectrophotometric method. The spectrophotometric method is the determination of protein levels based on the absorbance of ultraviolet light by the amino acids tryptophan, tyrosine, and cysteine disulfide bonds which absorb strongly at wavelengths, especially at 280 nm [10].

2.7. Fat level
Fat content testing refers to AOAC) [11], which was carried out using the Soxhlet method. Fat content determination is carried out to determine the amount of fat contained in a solid component of the material [12]. Fat content can affect the quality and quality of an ingredient. Calculation of fat content was used formula 2, as follows:

\[
\text{Fat content (\%) } = \frac{(B - A)}{\text{Mass sample}} \times 100 \tag{2}
\]

Information:
A = weight of empty flask (g)
B = pumpkin weight + extracted fat (g)

2.8. Ash content
The purpose of the ash content analysis is to determine the mineral content contained in the experimental melanin. The water-evaporated sample was then put into a furnace with a temperature of 600°C where the dry plate weight and sample weight were known beforehand. The sample weighing is carried out after all the materials have turned gray [13]. The ash content calculte use formula 3.

\[
\text{Ash content (\%) } = \frac{B - A}{\text{Mass sample}} \times 100 \% \tag{3}
\]

Information:
A = weight of empty cup (g)
B = weight of cup with ash (g)

2.9. FT-IR test
Analysis of the melanin pigment content of squid ink (Loligo sp.) Using FT-IR Spectroscopy. Melanin is part of the protein contained in squid ink, so it can be seen by showing the OH and NH groups at a wavelength of 3236.33 cm\(^{-1}\) in the test results [14]. The working principle of FT-IR is that infrared light is absorbed by dry melanin samples and other rays through a gap which has a function as a control of the amount of energy, then transmitted through the surface of the sample, so that the infrared light passes through the detector and the measured signal is sent to the computer for data reading [15].

2.10. Data analyze
The data were processed using the one way ANOVA test then continued with Duncan's Mean Range Test (DMRT). Data analysis was performed using the SPSS program.
3. Results and discussion

Parameters physicochemical of melanin extraction is melanin yield, protein content, fat content, and moisture content. The results shown different values. The higher value of melanin yields is $83.66 \pm 6.34$ (P0), protein content with $11.48 \pm 0.10$ (P0), fat content with $0.45 \pm 0.03$ (P2), and moisture content with $1.77a \pm 0.02$ (P1) (Table 1.).

![Figure 1](image1.png)

**Figure 1.** The Spectrum of Fourier Transform Infrared (FT-IR) Results on 2 M HCl (A), 2 M Ethanol (B), 3 M Ethanol (C)
Table 1. Physicochemical Melanin Extraction (Average (%) ± SD)

| Treatment | Melanin Yield | Protein       | Fat           | Moisture      |
|-----------|---------------|---------------|---------------|---------------|
| P0        | 83.66a ± 6.34 | 11.48a ± 0.10 | 0.23c ± 0.03  | 1.23c ± 0.02  |
| P1        | 63.00c ± 3.00 | 10.12c ± 0.10 | 0.33b ± 0.05  | 1.77a ± 0.02  |
| P2        | 67.66b ± 3.66 | 10.37b ± 0.13 | 0.45a ± 0.03  | 1.53b ± 0.02  |

Description: Superscript notation shows the comparison between treatments. Different letter notations indicated significant differences (P <0.05). Note; P0 = HCl 2 M; P1 = Ethanol 2 M; P2 = Ethanol 3 M.

Melanin is a pigment contained in *Loligo* sp. Squid ink. Natural melanin in squid ink is melanoprotein which contains 10% - 15% protein in it. Squid ink melanin is an alkaloid which has many benefits in the health sector. The alkaloid properties of squid ink have disadvantages, namely that it is easily damaged in storage at high temperatures [1].

The yield value shows the amount of melanin content of squid ink that remains after the extraction process. Based on the yield table, the melanin of squid ink produced in each treatment decreased from the initial yield of raw materials. The results of the calculation of the final yield of melanin in squid ink have an average of 83.66%; 63.005%; and 67.66%. The extraction process affects the final weight of a material. This is because in the extraction process the content contained in squid ink melanin such as protein, fat, minerals, and many organic and inorganic compounds are dissolved in solvents. The hydrolysis process uses the extraction method using a solvent which has the same properties [16].

The protein content test results showed the amount of protein contained in the squid ink melanin after the extraction process. Based on the results of protein content testing, the melanin content of squid ink had an average level of 11.48%; 10.12%; and 10.37%. These results indicate that the extraction process does not reduce the melanin protein content of squid ink in large quantities. Squid ink contains melanin in the form of melanoproteins with protein content of 10% - 15% [17]. The three treatments have an effect on the results of the protein content obtained.

Factors that can affect protein levels are the type of solution used during the extraction process [18]. The control treatment with 2 M HCl (P0) solvent had the highest protein content based on the test results, which was 11.48%. HCl is a solvent that is able to fully dissociate in the melanin of squid ink, so that it is able to attract more mineral compounds and other impurities in a material [19]. The results of the protein content test for the lowest main treatment were Ethanol 2 M (P1) of 10.12%. The results of the main treatment have differences due to the smaller concentration of the ethanol solvent used.

Fat content determination aims to show how much fat content is in the melanin of squid ink after the extraction process. Squid ink has a fat content of 0.2% - 1.4% [20]. The results of the fat content test showed an average of 0.23% for the 2 M HCl treatment (P0), 0.33% for the 2 M Ethanol (P1) treatment, and 0.45% for the 3 M Ethanol (P2) treatment. The control treatment was the best treatment with a fat content value of 0.23%.

This is because HCl is an acidic solvent, so some of the fat breaks down into other compounds, so that the detected fat is lower [9]. The difference in the yield of fat content was caused by sample preparation, extraction time, solvent quantity, solvent temperature, and solvent type. The difference in the fat content test results between 2 M Ethanol (P1) and 3 M Ethanol (P2) was caused by the difference in the concentration used. The higher of the solvent concentration, the higher of the level of polarity of a solvent, so that the greater the solubility of fat in the solvent and decomposes into other compounds [21]. Based on the available literature, an error occurred in our study which can be predicted because the time used in extraction using 3 M Ethanol (P2) is slightly faster than 2 M Ethanol (P1).

The next physicochemical test is the ash content test. Ash content analysis shows the amount of minerals contained in a material. The ash content is a parameter of the effectiveness of a solvent in the extraction process to remove the mineral content contained in melanin in squid ink. The mean yields of melanin ash content of squid ink were 1.23% (P0), 1.77% (P1), and 1.53% (P2), respectively.

Squid ink has an ash content of 2.74% [22]. The smaller the mineral content in a material, the higher the purity level of the material. Another objective of determining the value of ash content is to provide an overview of the mineral content from the initial process until the extract is formed. The best results
were obtained by the control treatment (P0) using 2 M HCl solvent, while for the main treatment the best results were shown in the 3 M ethanol (P2) treatment. The denser the ethanol concentration used, the smaller the value of the ash content, because it is able to dissolve the components of the ash material properly [19].

The final test is the analysis of the color of the melanin pigment in squid ink using Fourier Transform Infrared (FT-IR) Spectroscopy. The squid ink melanin tested using FT-IR has gone through an extraction process with various solvents. FT-IR test was conducted to determine the availability of melanin in squid ink after extraction. FT-IR is used to analyze organic and inorganic compounds, as well as qualitative and quantitative analysis by looking at the absorption strength of compounds at certain wavelengths [23].

The OH bond is in the 2500 cm$^{-1}$ - 3650 cm$^{-1}$ group and the NH bond is in the 3300 cm$^{-1}$ - 3500 cm$^{-1}$ group [24]. The results of each treatment that have been carried out contain melanin in it, because melanin is part of the protein contained in squid ink, this is confirmed in the research [20] by showing melanin in OH and NH groups at the peak of wavelength widening vibrations. 3236.33 cm$^{-1}$ in the test results.

4. Conclusion
It is concluded that based on molecular identification of parasite Haliotrema susanae in barramundi (Lates calcarifer) in Lampung Waters were stranded at 748 bp and the prevalence level and intensity respectively are 80% and 29.86 (parasites/fish).

5. References
[1] Susiana and Rochmady 2018 Indo J of Water Manag. 1(1): 14-30.
[2] Hulalata A, Makapedua D M, and Paparang R S 2013 J of Fish ProdTech Media, 1(2).
[3] Oktavia, D A 2012 AgroinTek 6(2): 65-71.
[4] Suryaningsih B E and Soebono H 2016 Bio of Melanocy 43 (2): 78-82.
[5] Tetti M 2014 J. of Health 7(2): 361-367.
[6] Vioni N, E Liviawaty, I Rostini, and N Kurniawati 2017 JPHP. 21 (1): 78- 85.
[7] Magarelli M, Passamonti P, Renieri C 2010 Veterin Zootecnia 5(2): PP 18-28.
[8] Ikram E H K, Eng K H, Jalil, A M M, Ismail, A, Idris S, Azlan A and Mokhtar R A M 2009 J of Food Com and Anal. 22(5), 388-393.
[9] Chamidah A, Marsono Y, Harmayani E, and Haryadi 2013 Agritech. 33(3): 251-257.
[10] Dewatisari F, Rumiyanti L, and Rakhmawati I 2017 J of App Agri Res. 17(3): 197-202.
[11] Association of Official Analytical Chemist (AOAC). 2007. Arlington, Published by The Association of Analytical Chemist, inc. Virginia. USA.
[12] Nimah N 2017 Effect of HCl Concentration on Demineralization Process on Gelatin Production from Broiler Chicken Bone (Gallus domesticus). ESSAY. Maulana Malik Ibrahim State Islamic University of Malang. 79 p.
[13] Anwar C 2013 Analysis of the Gel Strength of Jelly Candy Products from Cone Fish Skin Gelatin (Chiloscyllium punctatum) with the Addition of Carrageenan and Seaweed (Euchema spinosum), Bogor, Bogor Agricultural Institute. 21 p.
[14] Ilhamdi H and Yahya M F 2017 Engineering Litkayasa Bulletin.15 (1).
[15] Thermo, Nicolet 2001 Introduction to FTIR Spectrometry. Thermo Nicolet Inc., Madison. USA.
[16] Wiyarsi A and E Priyambodo 2009 The Effect of Chitosan Concentration from Shrimp Shells on the Efficiency of Heavy Metal Trapping. Essay. (Yogyakarta. Faculty of Mathematics and Natural Sciences, Yogyakarta State University). p 110
[17] Ringgenies D, Sasongko A S, and Sedjati S 2013 Characterization of Squid Ink (Sepiothus lessoniana) and its toxicity. (Semarang, Marine. Diponegoro University).p 88
[18] Adhibuana M J, Hintono A, and Pramono Y B 2018 J of Food Tech 2 (1): 17-20.
[19] Subagjo A 2006. Food Chemistry 95(1): 65-70.
6. Acknowledgements

Thank you also for the help of the technician of Mari-culture Center, Lampung.