Effect of Melanin Free Ink Extracted From Squid (Loligo sp.) on Proximate and Sensory Characteristics of Soft-Bone Milkfish (Chanos chanos) During Storage

Tri Winarni Agustini1*, Hadiyanto2, Ima Wijayanti1, Ulfah Amalia1, Soottawat Benjakul3

1Department of Fish Products Technology, Faculty of Fisheries and Marine Science, Diponegoro University, Jalan Prof. Soedarto, SH Tembalang Semarang, 50275, Indonesia
2Department of Chemical Engineering, Faculty of Engineering, Diponegoro University, Jalan Prof. Soedarto, SH Tembalang Semarang, 50275, Indonesia
3Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112 Thailand

*Corresponding author Email: tagustini@yahoo.com

Abstract. Antioxidant could be extracted and isolated from squid inks. Squid ink in the form of melanin free ink (MFI) could be act as an electron donor which can stabilize free radicals in lipid oxidation. This study was carried out to assess the antioxidant activity of squid inks converted into MFI in different dilution and to optimize the extraction conditions for the application of MFI as an antioxidative agent on fish product. Three different types of MFI extracts i.e : pure squid ink, squid ink with 5 times dilution and squid ink with 10 times dilutions by using cooled ionized water (4ºC). The ink was then centrifuged at 18.000 x g for 30 minutes at cooled centrifuge (4ºC) followed by DPPH analysis. The results showed that the IC50 of MFI extracts were 2.84 ppm; 1.11 ppm and 0.34 ppm, respectively (p < 0.05). The results indicated that squid ink with 10 times dilution in extraction of MFI had the highest value in free radical inhibitory. Although the IC50 of three different dilutions are equally low, and are considered as very strong antioxidative agent, however, it showed that the MFI extracted from squid ink had the ability to prevent free radical

Keywords : antioxidant, squid ink, melanin free ink, extract, lipid oxidation

1. Introduction
Squid (Loligo sp.) production in Indonesia increase 30.40% starting from 2000-2010, within year 2009-2010 significant increase of 80.8% was occurred. Squid production in 2010 reached 10,860 tons [1]. Processing of squid has results in some wastes including viscera and ink which is potential as raw material for bioactive compound resources which can be utilized as antioxidant, antibacterial and so on. Squid ink can be used as antioxidant in the form of squid ink polysaccharides [2] and melanin free ink [3]. Application of squid ink on some fisheries products in Indonesia as preserving agent is still
lack in information. Therefore, it is necessary to conduct research on the use of squid ink and extract the melanin free ink to preserve the fish product.

Soft bone milkfish (*Chanos chanos*) is one of specific fish product produced and becoming typical food in Semarang city. Milkfish is one of fish species that is commonly cultured and developed in Central Java and its potential production reaches 6,975 Ha. Milkfish is one of prime commodity in Central Java and its production increase by year. In 2012 the production increase by 26% and this increase due to increasing demand of soft bone and boneless-milkfish [4]. This composition has brought about the fish for susceptible oxidation process. Therefore, it is necessary to involve antioxidant to preserve fish product based on milkfish during processing and storage. The use of synthetic antioxidant has been considered as non-safe because it is potential for cancer. Natural antioxidant has been developed and squid ink in the form of melanin free ink can be of potential antioxidant for milkfish and the like species.

Squid ink can be used as antioxidant agent as it contains L-dopa and dopamine which have hydroxyl group and capable to be oxygen donor. Squid ink in the form of melanin free ink could be electron donor which can stabilize free radicals. In addition this melanin-free ink capable to act as metal chelating agent so that lipid oxidation can be retarded especially in initiation phase [3].

Study on utilization of squid ink as antioxidant for fish product has been conducted by Vate and Benjakul [3]. Ink extract as melanin free ink has been applied into surimi of short-bodied mackerel. The results showed that concentration of melanin free ink of 100 and 200 mg/Kg (ppm) have significant in reducing lipid oxidation during 15 days of cold storage. Application of melanin free ink into different fish product has not been carried out. This study was aimed to observe the effect of melanin free ink in reducing lipid oxidation process of milkfish based product.

2. Materials and methods

2.1. Materials

Materials used in this study was squid ink extracted from squid (*Loligo* sp.). Squid was obtained from fish market in Semarang. Fish products intended to be observed include: soft-bone milkfish (year 1) and fish ball milkfish (year 2). The equipment used including for production of milkfish-based products (soft-bone milkfish and fish ball milkfish), extraction tools for MFI, cold centrifuge, apparatus for quality assessment of the products.

2.2. Methods

Study was started by extraction of squid ink into MFI (melanin free ink). Extract MFI is objected for antioxidant activity test. Year 1 focus on potential assessment of MFI as antioxidant agent and applying on soft-bone milkfish product. MFI was applied by spraying on the surface of product as edible coating. Concentration of MFI were 0.5%; 1% and 1.5% and then followed by storage at different temperatures of 0ºC, 10ºC and 30ºC and left for several days with interval assessment of 0, 5, 10, and 15 days. Parameters of the product evaluated include fat content, protein content and moisture content, PV, and sensory.

2.2.1. Preparation of MFI

Preparation of MFI accorded to Vate and Benjakul [3]. Squid was separated from its ink by cutting ink truck from the body and collect them inside the bowl. This squid ink was diluted 10 times by using cooled ionized water (4ºC). The ink was then centrifuged at 18,000 x g for 30 minutes at cooled centrifuge (4ºC).

2.2.2. Analysis of DPPH

DPPH test was carried out by using Bloish method (1958) in Vate and Benjakul [3]. Sample of approximately 1.5 ml was added with 0.15 mM DPPH in 95% ethanol solution. Sample
and DPPH were mixed by using vortex and left at room temperature and dark condition for 30 minutes. Absorbance was detected by using spectrophotometer at 517 nm. Blank sample was also prepared using the same procedure.

2.2.3. Proximate analysis
Proximate analysis was conducted for protein, lipid and moisture by using AOAC methods [5].

2.2.4. Analysis of Data
This study was designed for completely randomized design with Factorial of 4x4. First factor was MFI concentration (0%, 0.5%, 1% and 1.5%). Second factor was storage time (0, 5, 10 and 15 days). Parametric data include: lipid, protein, water content, PV were analysed by ANOVA [6], DPPH data was analyzed descriptively and compare with the references.

3. Results and discussion

3.1. DPPH Analysis of MFI
The results for DPPH of the MFI was summarized in Table 1 and it was confirmed that MFI contain bioactive compounds such as flavonoids and phenol. As stated by Vate and Benjakul [3] and Hakim [8], MFI contain approximately 0.004% and 0.021% for flavonoid and phenol, respectively. Flavonoids can act as antioxidant which can prevent lipid oxidation on food matrix. The flavonoid as antioxidant can take up free radicals by release hydrogen atom from hydroxyl group because flavonoid has very reactive hydroxyl. According to Formagio et al [8], most of flavonoids has antioxidant activity because the present of hydroxyl phenolic compounds in their molecule structure.

Table 1. The antioxidant activity of squid inks

| Antioxidant activity | A (pure squid ink) | B (squid ink with 5 x dilution) | C (squid ink with 10 x dilution) |
|----------------------|--------------------|---------------------------------|----------------------------------|
| IC₅₀ (%)             | 31.07              | 15.39                           | 9.46                             |

Explanation for IC₅₀ (the antioxidant activity):

- < 50 : very strong
- 50-100 : strong
- 101-150 : medium
- >150 : weak

Following DPPH analysis, the results showed that the IC₅₀ of MFI extracts were 31.07%, 15.39% and 9.46%, respectively (p < 0.05). These results were comparable with Nicy [9] in which maximum DPPH radical scavenging activities of MFI of cuttlefish were 8.83% (Sepia pharaonis); 20.12% (Sepia prabahari) and 11.81% (Sepia ramani). The results indicated that squid ink with 10 times dilution in extraction of MFI had the highest value in free radical inhibitory. The IC₅₀ of three different dilutions were low, meaning that all MFI have very strong antioxidant activity. It’s showed that the MFI extracted from squid ink had the ability to prevent free radical. MFI of the splendid squid (Loligo formosana) showed antioxidative activities and was effective in scavenging DPPH. It was observed that DPPH scavenging activities of such squid was 179.6±2.1 protein [3]. Furthermore Vate and Benjakul [3] stated that melanin free ink of the splendid squids (Loligo formosana) was able to prevent lipid oxidation in surimi gels during refrigerated storage. Vate et al [10] informed that MFI could prevent lipid oxidation in surimi gels during refrigerated storage. Sudhakar and Nazeer [11] reported that cuttlefish peptide could be used as natural antioxidant in preventing oxidation reactions in food processing. Susilo et al.[12], state that inhibitor concentration (IC₅₀) was effective concentration in
sample which could inhibit 50% of DPPH absorbance. The result shows that the dilution of squid inks increased the antioxidant activity of MFI to a significant extent. Luo and Liu [2] stated further that marine bioactive squids ink polysaccharides not only have strong scavenging activity on DPPH and hydroxyl radicals as well as total reducing power and are called antioxidant ability, but also protect DNA from oxidative damage induced by free radicals originated from combined action of UV and H$_2$O$_2$.

Derby [13] reported that cephalopod ink has anti-oxidant activity in both the melanin and melanin-free fraction of ink. Liu et al [14] showed squid ink has antioxidant function in growing broiler chickens; Fahmi and Soliman [15] informed that Sephia officinalis ink has invitro antioxidant activities; Zahara and Rabita [16] declared squid ink powder has antioxidant activities.

3.2. Proximate content
The different concentrations of MFI showed significantly different effect on proximate of water, protein, fat and ash content during storage at different temperatures (10ºC, 20ºC and 30ºC).

3.2.1. Water content
The different concentrations of MFI and storage temperature showed significant different effect on water content of soft bone milkfish during storage. As shown at Table 2, there is no effect of MFI to water content of soft bone milkfish during storage at 10ºC, whereas for temperature storage of 20ºC and 30ºC the effect were varied and for control showed that the water content decrease in the 9th days of storage at temperature of 30ºC. The decrease in water content at temperature 30ºC higher than 20ºC, with water content was 44.53%. This is due to higher storage temperatures resulting in higher water evaporation during storage. Soft bone milkfish with the addition of 0.75% and 1.5% MFI concentration showed that the water content changes especially for storage temperature of 20ºC and 30ºC. Temperature storage of 10ºC performed more stable water content on the samples, indicating that the suitable storage for soft bone milkfish is 10ºC. Interestingly, during storage at 20ºC, it was found that mold was growth on the samples causing some defect on the texture.

| Storage Temperature (ºC) | Storage Time (day) | Concentration of MFI |
|--------------------------|--------------------|----------------------|
|                          | 0                  | 0%                   |
|                          | 3                  | 0.75%                |
|                          | 9                  | 1.50%                |

Table 2. Water Content of Soft bone Milkfish with Different Concentration of MFI during Storage in 10ºC, 20ºC, 30ºC

At 20ºC and 30ºC showed the addition of 0.75% MFI concentration can increase the water content, it is due to the MFI that were added can form a layer of edible coating that can inhibit the release of water so that the evaporation process in soft bone milkfish did not occur. The increase levels of water content was due to the possibility of soft bone milkfish hydrolysis during the storage time and stuck in the flesh of fish because of the MFI coating.
3.2.2. Protein content
Protein content of the samples were analyzed during storage as shown on Table 3. By applying MFI as edible coating on the soft bone milkfish, it was expected that product deterioration can be retarded to some extend due to the role of MFI as antioxidant. Based on the data resulted, samples added MFI at all storage temperatures (10ºC, 20ºC and 30ºC) showed the same result in which protein content decreased at the end of the storage.

Table 3. Protein Content of Soft bone Milkfish with Different Concentration of MFI during Storage at 10ºC, 20ºC, 30ºC

| Storage Temperature (ºC) | Storage Time (day) | Concentration of MFI |
|--------------------------|--------------------|----------------------|
|                          | 0                  | 0%                   |
|                          | 3                  | 0.75%                |
|                          | 9                  | 1.50%                |
| 10                       | 0                  | 29.27±0.27de         |
|                          | 2                  | 29.48±0.258d         |
|                          | 6                  | 28.14±0.11def        |
|                          | 9                  | 30.78±0.02bc         |
| 20                       | 0                  | 28.60±0.33ab         |
|                          | 3                  | 28.07±0.61bcd        |
|                          | 6                  | 28.85±1.35ab         |
|                          | 9                  | 30.63±0.37a          |
| 30                       | 0                  | 30.57±1.10ab         |
|                          | 3                  | 28.39±0.31bcd        |
|                          | 6                  | 21.06±0.21e          |
|                          | 9                  | 30.36±0.40a          |

At temperature storage of 10ºC soft bone milkfish with the addition of 0%, 0.75% and 1.5% MFI concentration showed that the protein content tend to decrease compared to the initial protein content. The decrease of soft bone milkfish’s protein content at 20ºC and 30ºC higher than 10ºC, soft bone milkfish with the addition of 0.75% MFI concentration showed that the protein content decrease 12.7% compared to the initial content. At 20ºC soft bone milkfish with the addition of 1.5% MFI concentration showed that the protein content decrease 20.5% and at 30ºC soft bone milkfish with the addition of 1.5% MFI concentration showed that the protein content decrease 11.8%.

3.2.3. Fat content
The use of MFI on soft bone milkfish was intended for preserving the product during storage. MFI which can act as antioxidant was expected for retarding lipid oxidation on the product so that it can prolong the shelf life. In the control treatment at 10ºC showed that the fat content decrease 32.8% compared to the initial content. At 20ºC showed that the fat content decrease 14.5% at the end of the storage and at 30ºC showed that the fat content decrease 17.5% at the end of the storage.

At the end of the storage, soft bone milkfish with the addition of 0.75% and 1.5% MFI concentration showed that the fat content higher than control and the fat content with the addition of 1.5% MFI higher than 0.75% MFI. This phenomenon was considered due to the possibility of edible coating of these MFI as antioxidants which can reduce the occurrence of fat degradation during storage. At 10 ºC at the end of the storage, MFI 1.5% was the best treatment to maintain the fat content, it is shown that the highest fat content was at 10ºC. It was due to the possibility of MFI acts as an antioxidant and supported by low storage temperature so that damage or fat oxidation can be inhibited to some extent. At 20ºC and 30ºC the fat content of soft bone milkfish control and with the
addition of MFI was lower than 10°C, but the addition of 1.5% of MFI in each storage temperature showed at the end of storage the fat content is higher than the control treatment and MFI 0.75%.

Table 4. Fat Content of Soft bond Milkfish with Different Concentration of MFI during Storage in 10°C, 20°C, 30°C

| Storage Temperature (ºC) | Storage Time (day) | Concentration of MFI | 0%       | 0.75%     | 1.50%     |
|-------------------------|--------------------|----------------------|----------|-----------|-----------|
|                         |                    |                      | 13.40±0.39<sup>a</sup> | 6.93±0.57<sup>de</sup> | 7.34±0.98<sup>de</sup> |
| 10                      | 0                  |                      | 8.06±0.21<sup>bcd</sup> | 8.88±0.28<sup>bc</sup> | 7.46±0.97<sup>bde</sup> |
|                         | 3                  |                      | 5.56±0.49<sup>de</sup> | 8.60±0.42<sup>bcd</sup> | 14.81±0.95<sup>a</sup> |
|                         | 6                  |                      | 9.00±0.36<sup>b</sup> | 9.37±0.01<sup>b</sup> | 13.50±0.37<sup>a</sup> |
|                         | 9                  |                      | 8.73±0.29<sup>e</sup> | 11.11±0.75<sup>cd</sup> | 9.55±0.95<sup>de</sup> |
| 20                      | 0                  |                      | 10.93±0.48<sup>cd</sup> | 16.39±0.47<sup>a</sup> | 10.83±0.69<sup>cd</sup> |
|                         | 3                  |                      | 13.19±0.06<sup>b</sup> | 12.25±0.92<sup>bc</sup> | 15.48±0.41<sup>a</sup> |
|                         | 6                  |                      | 9.55±0.05<sup>de</sup> | 11.83±0.30<sup>bc</sup> | 12.16±0.62<sup>bc</sup> |
|                         | 9                  |                      | 8.11±0.87<sup>fg</sup> | 12.10±0.75<sup>cd</sup> | 9.55±0.32<sup>ef</sup> |
| 30                      | 0                  |                      | 11.07±0.47<sup>de</sup> | 5.84±1.03<sup>b</sup> | 7.53±0.32<sup>gb</sup> |
|                         | 3                  |                      | 17.06±0.76<sup>a</sup> | 14.22±0.30<sup>bc</sup> | 13.18±0.56<sup>bc</sup> |
|                         | 6                  |                      | 9.15±0.06<sup>efg</sup> | 9.12±0.32<sup>efg</sup> | 10.68±0.26<sup>de</sup> |

3.2.4. Ash content
The ash content of soft bone milkfish with the addition of MFI were higher than control treatment at all temperatures at the beginning of storage. The addition of MFI gave the additional of mineral so that the ash content increased. The ash content changed during storage. In all treatments showed a decrease in ash content during the storage time.

Table 5. Ash Content of Soft bond Milkfish with Different Concentration of MFI during Storage in 10°C, 20°C, 30°C

| Storage Temperature (ºC) | Storage Time (day) | Concentration of MFI | 4.45±0.16<sup>de</sup> | 9.15±0.68<sup>a</sup> | 5.76±0.40<sup>de</sup> |
|-------------------------|--------------------|----------------------|-----------------------|-----------------------|-----------------------|
|                         |                    |                      | 1.96±0.31<sup>es</sup> | 1.29±0.21<sup>es</sup> | 3.88±0.74<sup>f</sup> |
|                         |                    |                      | 6.43±0.59<sup>bc</sup> | 3.57±0.38<sup>f</sup> | 4.17±0.24<sup>f</sup> |
|                         |                    |                      | 8.17±0.53<sup>ab</sup> | 5.91±0.57<sup>cd</sup> | 4.94±0.39<sup>def</sup> |
| 20                      | 0                  |                      | 3.95±0.03<sup>bc</sup> | 5.33±0.58<sup>ab</sup> | 7.02±0.76<sup>a</sup> |
|                         | 3                  |                      | 3.60±0.15<sup>c</sup> | 3.70±0.81<sup>bc</sup> | 4.21±0.43<sup>bc</sup> |
|                         | 6                  |                      | 4.48±0.11<sup>bc</sup> | 4.58±0.52<sup>bc</sup> | 3.66±0.50<sup>bc</sup> |
|                         | 9                  |                      | 3.41±0.26<sup>c</sup> | 6.71±1.21<sup>a</sup> | 3.33±0.41<sup>c</sup> |
| 30                      | 0                  |                      | 3.23±0.37<sup>de</sup> | 3.59±0.70<sup>cd</sup> | 3.63±0.19<sup>bc</sup> |
|                         | 3                  |                      | 2.59±0.62<sup>de</sup> | 4.48±0.51<sup>ab</sup> | 1.97±0.42<sup>e</sup> |
|                         | 6                  |                      | 3.62±0.29<sup>bcd</sup> | 5.58±0.67<sup>a</sup> | 4.71±0.76<sup>ab</sup> |
|                         | 9                  |                      | 2.86±0.68<sup>de</sup> | 3.59±0.317<sup>bcd</sup> | 4.75±0.17<sup>ab</sup> |

3.3. Sensory analysis
At 10°C MFI concentration gave significant effect on sensory value. At the beginning of the storage, sensory value of control treatment were higher than that of MFI treatments, but during the storage,
control treatment showed decreased on sensory value. At the end of the storage, MFI 1.5% has the highest sensory value compared to that of 0% and 0.75% MFI. At 10ºC all treatments are acceptable. This occurrence profound that using MFI as edible coating on soft bone milkfish can significantly retard deterioration of the product.

Table 6. Organoleptic value Soft bone Milkfish with Different Concentration of MFI during Storage at 10ºC, 20ºC, 30ºC

| Storage Temperature (ºC) | Storage Time (day) | Concentration of MFI |
|-------------------------|-------------------|----------------------|
|                         | 0                 | 0%                   |
|                         | 3                 | 0.75%                |
|                         | 6                 | 1.50%                |
| 10                      | 0                 | 8.24±0.544           |
|                         | 3                 | 8.027±0.620          |
|                         | 6                 | 7.827±0.486          |
|                         | 9                 | 7.727±0.595          |
| 20                      | 0                 | 8.213±0.378          |
|                         | 3                 | 6.3±0.787            |
|                         | 6                 | 4.467±1.091          |
|                         | 9                 | 4.707±0.486          |
| 30                      | 0                 | 7.92±0.511           |
|                         | 3                 | 8.12±0.629           |
|                         | 6                 | 7.447±0.545          |
|                         | 9                 | 4.98±0.539           |

At 20ºC organoleptic value on all treatments are not acceptable in the 3rd day. At 20ºC the control and MFI treatment was overgrown with molds in the 3rd day. At 20ºC MFI cannot able to inhibit the growth of mold, it was due to the possibility of 20ºC is the optimum temperature for molds growth.

At 30ºC MFI concentration did not give significantly different effect in organoleptic value but the storage time gave significantly different effect. At 30ºC organoleptic value of soft bone milkfish was better than 20ºC. Organoleptic value on all treatments decreased and still suitable for consumption in the 6th day, but not acceptable in the 9th day.

4. Conclusion
Result of this tentative study concluded that the squid ink potentially used as an antioxidant agent after processing into Melanin-Free Ink.

5. Acknowledgement
Further thanks to Diponegoro University, which has funded this research through the International Scientific Publication (RPI) 2016 budget.

References
[1] Ministry of Marine and Fisheries Affairs 2011 Marine and Fisheries in Figures (Ministry of Marine and Fisheries Affairs, Indonesia)
[2] Luo P and Liu H 2013 African J. of Pharmacy and Pharmacology 7(21) : 1382-1388.
[3] Vate NK and Benjakul S 2013 International Aquatic Research 5(9): 1-12
[4] Department of Marine and Fisheries of Central Java 2013 Profile of Aquaculture Field 2013. http://diskanlut-jateng.go.id.
[5] [AOAC] Association of Official Analytical Chemist 2005 Official Methods of Analysis of The Association of Official Analytical Chemist (Arlington, Virginia USA : AOAC Inc)
[6] Steel dan Torrie. 1991. *Principle and Procedure of Statistic* (PT Gramedia Pustaka Utama, Jakarta)

[7] Hakim 2017 *Effect of Melanin Free Ink as Antioxidant on Milkfish Fishbal during Refrigerated Storage* Research Report of Bachelor Degree Faculty of Fisheries and Marine Science, Diponegoro University Indonesia

[8] Formagio ASN, Carla Roberta Ferreira Volobuff, Matheus Santiago, Claudia Andrea Lima Cardoso, Maria do Carmo Vieira and Zefa Valdevina Pereira 2014 *Antioxidants* 3, 745-757

[9] Nicy Brita 2016 *Antioxidant And Antibacterial Properties Of Cuttlefish Ink Collected From Selected Cuttlefish Landed At Thoothukudi Coast* Thesis of Master Degree Fisheries College And Research Institute Tamil Nadu Fisheries University Thoothukudi

[10] Vate NK, Benjakul S, Agustini TW 2015 *J. Sci. Food Agric.* 95(11): 2201-7

[11] Sudhakar and Nazeer 2015 *Lebensmittel-Wissenschaft und-Technologie* 64(2):593-601

[12] Susilo J, Istianatus S, Syamsul R 2013 Uji Aktivitas Antioksidan Ekstrak Etanol Daun Poslen (*Talinum triangulare* (jacq.) Wild) Dengan Metode DPPH (2,2 – Diphenyl – 1-picrylhydrazyl) Farmasi STIKES Ngudi Waluyo Jawa Tengah

[13] Derby CD 2014 *Marine Drugs* 12: 2700-2730.

[14] Liu H, Ping Luo1, Shaohong Chen and Jianghua Shang 2011 *Asian-Australian Journal Animal Science* 24 (12) :1752 – 1756

[15] Fahmy SR, Soliman AM 2013 *Afr. J. Pharm. Pharmacol.* 7, 1512–1522.

[16] Zaharah F MY and Rabeta 2018 *Food Research* 2-7