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# Extraction of lipids and purification of linoleic acid from *Clarias macrocephalus* oil

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**Abstract**. Fatty acid composition of "keli" (*Clarias macrocephalus)* oil has been shown to contain 18.4% of linoleic acid (LA). Oil was obtained from three types of extraction methods namely Blight & Dryer method, Soxhlet method, and pressing method. Blight & Dryer method produced an acceptable total lipid content with good quality of oil. After saponification and acidification, purification of released LA by urea complex with urea to fatty acid ratio 5:1 and crystallization temperature of 20°C, had enhanced purity of LA to 47% with total fatty acid recovery of 25.2%. The LA obtained was converted to LA methyl ester prior to further purification by argentated silica gel chromatography. LA with a purity of 92% with total fatty acid recovery of 12.9% was obtained.
**Key Words**: *Clarias macrocephalus,* lipid extraction, purification linoleic acid, urea complexation, argentated silica gel chromatography.

**Abstrak**. Komposisi asid lemak minyak ikan keli (*Clarias macrocephalus)* menunjukkan ia mengandungi asid linoleik (LA) sebanyak 18.4%. Minyak diperoleh daripada tiga jenis kaedah pengekstrakan iaitu ; kaedah Blight & Dryer, kaedah Sohxlet, serta kaedah perahan. Kaedah Blight & Dryer memberikan jumlah lemak kasar yang memuaskan serta kualiti minyak yang baik. Setelah proses penyabunan dan pengasidan, pemurnian asid linoleik melalui kaedah pengkompleksan urea dengan nisbah urea : asid lemak 5:1 dan suhu pengkristalan pada 20 °C, memberikan ketulenan asid linoleik sebanyak 47% dan peratus dapatan semula asid lemak total sebanyak 25.2%. LA yang diperolehi ditukar kepada ester metil LA sebelum penulenan seterusnya dengan kromatografi turus gel silika berargentum dijalankan. LA dengan ketulenan 92% dan peratus dapatan semula asid lemak total sebanyak 12.9% diperolehi.
**Kata kunci**. *Clarias macrocephalus,* Pengekstrakan lemak, Penulenan Asid Linoleik, Pengkompleksan Urea, Kromatografi Turus Berargentum.

**Introduction**. Linoleic acid (C18:2) is an essential fatty acid (EFA) which the body cannot synthesized it and must be obtained through diet. When consumed, this omega-6 polyunsaturated fatty acid series can be metabolized to arachidonic acid (ARA) and docosapentaenoic acid (DPA) in the human body. It is an essential nutrient which is responsible for the development of a wide variety of abnormalities such as rheumatoid arthritis, autoimmune disorders, diabetic neuropathy and cardiovascular reproductive (Wu et al 2008).

Linoleic acid is normally obtained from seed oil. But in this study, oil from *C. macrocephalus* a local freshwater catfish was choosen as the source of linoleic acid. Freshwater fish oil has never gained attention as much as marine fish oil since it is not high in omega-3 fatty acid (DHA and EPA). However some of the fish for example *C. macrocephalus,* has its own nutritional value where its contain 17.9% of linoleic acid (LA) (Suriah et al 1994). Despite its nutritional value, catfish has a big market in Malaysia. According to Department of Fishery Malaysia (2009), in 2009 the retail value of catfish (*C. macrocephalus)* production from freshwater ponds is RM 393,276.27 million. The value is expected to grow from year to year. This fish also have the advantage of fast growth, easily to breed, locally originated and thus low in cost. Although the amount of LA in catfish is not as much as the amount of LA in seed oil, but its still can be considered as an alternative source of LA.

The attempt to purify linoleic acid from *C. macrocephalus* includes two steps which are urea complex fractionation and argentated silica gel column chromatography. Urea complex fractionation was established by Bengen in 1940 for separation of straight-chain

compounds from branched or cyclic compounds (Guil-Guerrero & Belarbi 2001). Today, this method is well-known as a technique for elimination of saturated (SFA) and monounsaturated fatty acids (MUFA). SFA and MUFA is easily complex with urea and crystallize out on cooling to form urea complexed fraction (UCF), and may subsequently be removed by filtration. The filtrate or non-urea complexed fraction (NUCF) is enriched in polyunsaturated fatty acid (PUFA). Urea complex fractionation method has the advantage where complexed crystals form are extremely stable, and filtration does not necessarily need to be carried out at very low temperatures. Previous study have also reported the other advantage of this method which includes a technically simple method which are energy efficient, involve mild operating conditions, and utilize inexpensive renewable materials (Wu et al 2008)

Second step of purification involves adsorption on silver nitrate-impregnated silica gel. This method has been successfully employed to purify specific fatty acid such as γ-linolenic acid (GLA) from *Spirulina platensis* with purity >96% (Sajilata et al 2008), and GLA from seed oils with purity of 86% (Guil-Guerrero et al 2000). Since urea fractionation can only separate LA from SFA and MUFA, this method was aimed to separate LA from the other PUFA present by solid phase extraction in a column chromatography. Isolation of a desired fatty acid is achieve by elution of solvent to stationary phase where separation process occur is base on number, position, and geometric configuration of double bonds (Guil-Guerrero & Belarbi 2001; Cert & Moreda 1998).

**Material and Method**. Catfish (*C. macrocephalus*) was obtained from the local wet market. The fish was gutted, washed, and flesh was separated from other parts. The flesh was stored in the freezer prior to the oil extraction. All chemicals used were an analytical grade.

The fish oil were extracted by "Bligh and Dyer" method with slight modifications from Kinsella et al (1977). Fish filets (30 g) were ground for 2 min before it is transferred into 500 mL beaker. Mixture of methanol (60 mL) and chloroform (30 mL) was then added to the blended fish and stirred by glass rod. One volume of chloroform (30 mL) was added to the mixture and after stirring for an additional 30 sec, distilled water (30 mL) was added. The homogenate was left for 3 hours before filtered under vacuum. The filtrate was transferred to a separating funnel to separate the organic and the water soluble layer. The oil layer is then added with sodium sulphate anhydrous before once again filtered, and concentrated with a rotary evaporator at 60 °C.

Soxhlet method was carried out by the method of Shamsudin & Salimon (2006), with slight modifications. Fish fillet was first ground, dried by using freeze dryer for 24 hours. The dried fish was placed in a white cotton sack. The extraction using Soxhlet method was carried out using 150 mL mixture of solvent (chloroform : methanol, 1:1) for 8 hours. The heating power was set at 80-90°C. The crude extract solutions obtained were concentrated and dried using vacuum rotary evaporator at temperature 60 °C or less to remove the solvents.

Extraction by pressing method was carried out according to Sathivel et al (2003). The 100 g portion of sample was finely ground for 2 min. Water was added (water/ ground sample, 5:1 v/wt) and the mixture was heated at 70°C for 15 min. The solid particles were separated from the liquid phase by filtering through cheesecloth, and the particles were pressed to remove most of the liquid. The crude oil was separated from the water phase and sample particles by centrifuging at 4,000 rpm for 30 min.

The Acid Value and Peroxide value was conducted based on official standard method AOCS 5a-40 (1998).

Saponification of oil was done according to methods by Hai-bo et al (2009). Crude catfish oil (60 g) were saponified using a mixture of NaOH (1.6 g) and 95% (v/v) aqueous ethanol (300 mL), by refluxing at 70°C for 1 hour under the protection of nitrogen. Distilled water (100 mL) was added to dissolve the saponified mixture, and the aqueous layer containing saponified matter was acidified with 6N HCl. The mixture solution was shaked, and hold on for 15 min before water layer was drain off. 100 mL hexane was added to dissolve oil layer. Distilled water (100 mL × 3) was added to wash

the oil layer until neutral. The hexane layer containing free fatty acids was then dried over anhydrous sodium sulphate and solvent was removed at 60°C by rotary evaporator.

Fractionation with urea was refered to (Guil-Guerrero & Belarbi 2001) with modification. 100g urea was dissolved in 500 mL 95% ethanol by heating. After a clear, homogeneous solution had formed, 20g fatty acid was added into the solution for 15 min with continuous stirring. The mixture was allowed to crystallize at 20°C for 20 hour with continous stirring. The solution was filtered under vacuum, and the filtrate was kept at 7°C for 5 h and then filtered again. The filtrate was mixed with HCl (10% vol/vol) and hexane to thoroughly stirred for 1 h. The hexane layer was separated and washed by distilled water (100 mL x 3). Anhydrous sodium sulphate was used to dry the hexane layer before solvent was removed by rotary evaporator at 60°C.

Esterification of PUFA from urea crystallization was carried out by mixing FFA (1.5 mL) with 15 ml hexane and 15 mL sodium methoxide. Mixture were vortex for 25 sec and left to separate. The FAME layer (upper layer) was collected and subjected to GC analysis or Ag-silica gel chromatography.

The Ag-silica gel chromatography was prepared as follows method by (Guil-Guerrero & Belarbi 2001; Ghebreyessus et al 2006). 30 g of silica gel 60 (0.06–0.2 mm, 70–230 mesh ASTM) for column chromatography was added with a solution of silver nitrate (12 g) in disttiled water (24 mL). The silver-impregnated silica gel was activated by overnight heating at 120°C. This material was cooled and kept in the dark in a desiccator until needed. The packed height of glass chromatography columns was varied from 15-17 cm in a 1cm diameter column. The exit of the chromatography column was plugged with a sintered glass disc to retain solids. Chromatography columns were packed as follows: A slurry of Ag-silica gel (30 g) in hexane (60 mL) was poured into a column. A slight flow of hexane was allowed to occur while the packing process. The hexane level was lowered until it was 1 cm above the stationary phase. An aliquot of the FAME solution was applied to the chromatography column with loadings 20% of extract-to-stationary phase. The column was eluted with (50-60mL) hexane for one time and followed by a 5 times of toluene as the elution solvents. Each eluates were collected as fractions and analysed by gas chromatography (GC).

Fatty acid profile was analysed with Shimadzu GC-17A with a BPX70 column (30 m × 0.25 mm × 0.25 µm). Injection and detection (FID) temperatures were set at 250°C and 280°C, respectively and nitrogen was used as the carrier gas with column flow rate of 0.4 mL/min. FAMEs were identified by comparing the retention time of the integrated peak of the individual FAME with those of authentic standard FAME from Supelco analytical (USA). The purity of linoleic acid was determined by injection of standard linoleic acid together with sample to ensure both peak of linoleic acid in sample and standard overlap.

**Results and Discussion**. Fractionation of lipid was carried out by three extraction method namely Soxhlet, Blight & Dryer, and pressing method. From gas chromatography (GC) analysis, *C. macrocephalus* oil were found to be comprised of palmitic acid, C16:0 (22.61%), palmitoleic acid C16:1 (4.47%), stearic acid C18:0 (5.39%), oleic acid C18:1 (41.09%), linoleic acid C18:2 (18.40%), arachidonic acid C20:0 (1.31%), and octadecatetraenoic acid C18:4 (0.74%). The percentage of LA in this study was slightly higher than reported by Suriah et al (1994). Table 1 shows comparison of physicochemical properties for three investigated extraction methods. From the result, Soxhlet method showed the highest yield of lipid with 36.71% of total lipid content, followed by "Blight and Dryer" method with 26%, and lastly pressing method with 17%. However, Soxhlet method yield low quality of oil by high AV (3.53 ± 0.29) and PV (6.27 ± 1.53). AV and PV resulted from "Bligh and Dryer" (1.39 ± 0.01, 1.32 ± 0.57) shows no significant different with AV and PV from pressing (1.40 ± 0.02, 1.65 ± 0.58).

The total lipid content from organic solvent extraction (Soxhlet and "Bligh and Dryer") was higher than non organic solvent extraction (pressing). This is due to the fact that lipids are soluble in organic solvent, but insoluble in water. Soxhlet method has advantages over the other two methods because during extraction, it allows sample to have permanent contact with hot and fresh solvent (Manirakiza et al 2001).

Table 1

Comparison of physicochemical properties for three investigated extraction methods

| Extraction method | Total lipid content (%) | Acid Value (AV) | Peroxide Value (PV) |
|---|---|---|---|
| Blight and Dryer | 26.00 ± 0.61[a] | 1.39 ± 0.01[b] | 1.32 ± 0.57[b] |
| Soxhlet | 36.71 ± 5.45[b] | 3.53 ± 0.29[a] | 6.27 ± 1.53[a] |
| Pressing | 17.00 ± 3.28[c] | 1.40 ± 0.02[b] | 1.65 ± 0.58[b] |

[a-c]Means of % in the same column with different superscript letter differ significantly ($p < 0.05$)
Results are means ±SD of three determinations

This allowed maximum total lipid content obtained. However, since fish oil is rich in polyunsaturated fatty acid, and easily exposed to hydrolytic spoilage and oxidative deterioration, the effect of extraction method towards quality of oil should also be the major concern. AV and PV analysis shows that Blight & Dryer method gives a higher quality oil compared to Sohxlet method. Hydrolysis of lipid is measured by AV, and primary oxidized product of fats and oil is measured by PV. High value of PV and AV could be attributed to the fact that Soxhlet extraction was carried out for 8 hours, and oil is subjected to oxygen and light much longer than the other two methods. Heat, light, oxygen, and the absence of antioxidants are factor that will effect on accelerating the rate of oxidative and hydrolytic rancidity (Stansby 1990; Ketaren 1986). Although pressing and Blight & Dryer method gives nearly the same value of AV and PV, Blight & Dryer method was choosen as oil extraction method in this study since it recover more oil than pressing method.

After extraction, oil undergo saponification and acidification in order to separate fatty acids from glycerol. Isolation of LA begins with urea complex fractionation where the formation of urea complex is an exothermic process. Urea complex formation is initiated by allowing the solution of FFA, urea, and ethanol to cool at 20 °C for 20 hours. Cooling over a long period of time is carried out in order to maximize the complex formation. After filtration, non-urea complex fraction (NUCF) was washed by water to eliminated traces of urea in the solution before the concentrated fatty acid was subjected to GC analysis. Table 2 shows the fatty acid composition before and after urea complexation.

Table 2

Fatty acid composition before and after urea complexation[a]

| | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | C20:0 | C18:4 | C20:4 |
|---|---|---|---|---|---|---|---|---|
| Before urea complexation | 23.42 ±0.03 | 3.97 ±0.01 | 6.74 ±0.07 | 44.14 ±0.14 | 16.59 ±0.04 | 0.85 ±0.04 | 0.47 ±0.03 | 0.53 ±0.04 |
| After urea complexation | 1.81 ±1.05 | 0.68 ±0.96 | 0.85 ±1.2 | 6.23 ±5.93 | 43.09 ±2.64 | 21.32 ±1.92 | 9.81 ±0.74 | 0.56 ±0.79 |

[a]Results are means ±SD of two determinations

Urea trap the straight chain organic compound such as saturated (SFA) and monounsaturated fatty acid (MUFA) as urea complex fraction (UCF) and leaves the more unsaturated acids including LA in solution or NUCF. Urea molecules bond together via hydrogen bonding, while it bond to fatty acids via strong van der Waals attraction (Hayes et al 1998). Previous studies (Wu et al 2008; Guil-Guerrero & Belarbi 2001; Wanasundara & Shahidi 1999) have shown that it is possible to separate mixture of fatty acids according to degree of unsaturation and chain length. The tendency of fatty acids to combine with urea decreases with increasing unsaturation and decreasing chain length.

After urea fractionation, fatty acid composition shows expected trends with reduction in saturated and monounsaturated fatty acid content (C16:0, C16:1, C18:0, and C18:1), and increase in long chain fatty acid and PUFA content (C20:0, C18:2, C18:4, and C20:4). The purity of LA was 47% with recovery of 25%. Small amount of SFA and MUFA is still exist in the NUCF since some of the saturated fatty acids do not bind with urea during crystallization (Wu et al 2008). Urea fractionation has shown unable to separate LA from the other fatty acid with higher polarity such as C20:0, C18:4 and C18:4. This fact had lead to second purification step by Ag-silica column chromatography.

Before applied to column chromatography, mixture of fatty acid obtained from urea fractionation was esterified to form fatty acid methyl ester (FAME) by sodium methoxide. FAME was left in the column for 30 min before elution start, to allow FAME form polar complexes with silver ion Ag+. The column was eluted with the following sequence of Solvents: 1 time with hexane, and another 5 times with toluene. The fraction collected from each elution was subjected to gas chromatography analysis. Table 3 shows the fractionation of fatty acid by Ag-silica gel column chromatography.

Table 3

Fractionation of fatty acid by Ag-silica gel column chromatography[a]

| FAME | Solvent eluent | | | | |
|---|---|---|---|---|---|
| | 1st elution | 2nd elution | 3rd elution | 4th elution | 5th elution |
| C12 : 0 | 72.91±5.6 | 0.53±0.74 | 0 | 0 | 0 |
| C16 : 0 | 26±4.53 | 2.52±1.39 | 1.11±0.55 | 0 | 0 |
| C16 : 1 | 0 | 16.54±0.33 | 1.06±0.33 | 3.74±0 | 5.37±1.19 |
| C18 : 0 | 0 | 7.94±0.30 | 0.93±0.95 | 0 | 0 |
| C18 : 1 | 0 | 25.97±3.02 | 1.41±0.43 | 4.13±0.61 | 5.43±0.59 |
| C18 : 2 | 0 | 44.25±6.45 | 92.69±0.81 | 91.35±0.49 | 78.37±2.04 |
| C20 : 0 | 0 | 0 | 0 | 0.78±1.10 | 9.17±5.39 |
| C18 : 4 | 0 | 0 | 0 | 0 | 2.13±0.9 |

[a]Results are means ±SD of two determinations

The resolving power of Ag-silica gel is cause by reversible charge-transfer complexation of Ag+ with carbon–carbon double bonds (Sajilata et al 2008). The number and geometric configuration of double bonds determine the order of elution of fatty acid (Sajilata et al 2008). Analysis of fatty acid shows elution by hexane was found to be free from any fatty acid because hexane was only responsible to bring sample through out the column. From Table 3, toluene elutes fatty acid according to its polarity where SFA elute first, followed by the MUFA and PUFA. The first wash of toluene have shown to only elute SFA (C16:0 and C18:0) since this ester associate least strongly with the stationary phase. The second wash of toluene start to elute MUFA (C16:1 and C18:1) 42.5%, PUFA (C18:2) 44%, and SFA (C12:0, C16:0, and C18:0) 11%. As washing by solvent continue in third wash, 92% of LA was successfully concentrated accompanied by 2% of MUFA (C16:1 and C16:2) and also 2% of SFA (C16:0 and C18:0). The forth wash of toluene yield 91% of LA with the present of 7% MUFA (C16:1 and C18:1) and 1.56% of arachidonic acid (C20:0). The fifth wash of toluene shows the decrease in percentage of LA with increasing in arachidonic and the present of C18:4 fatty acid. If washing with solvent is continue, higher polar fatty acid is expected to be collected. Since the third and forth wash contain more than 90% of LA, both fraction were pooled together to obtain LA methyl ester recovery of 12.74%

Figure 1 shows comparison between chromatogram of fatty acid after saponification and acidification of oil, fatty acid after urea fractionation and fatty acid after Ag-silica column chromatography. It is clearly shown that after urea crystallization, most of fatty

acid with lower polarity than LA such as C16:0, C16:1, C18:0, and C18:1, had formed a complex with urea thus eliminated from PUFA concentrate. The second purification method by Ag-silica gel chromatography continue to separate LA from the higher polarity fatty acid such as C20:0 and C18:4.

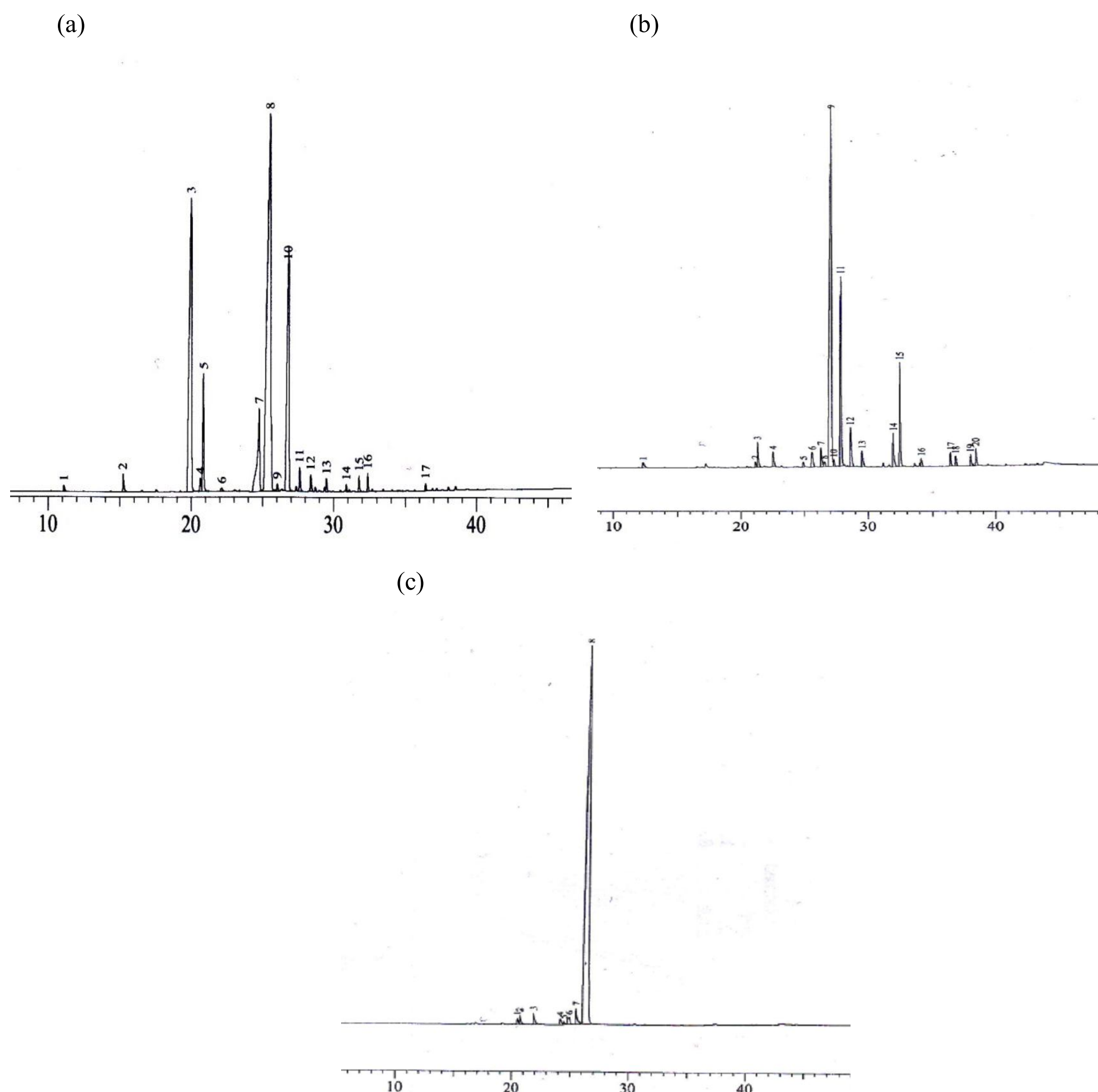


Figure 1. GC chromatogram of (a) Free fatty acid after saponification and acidification of oil (b) NUCF (c) Purified LA after Ag-silica gel chromatography.

**Conclusions**. The use of different approach during extraction results in different lipid recovery. Blight & Dryer method gives acceptable total lipid content with good quality of oil. Combination of urea complex fractionation and Ag-silica gel chromatography have shown to obtained 92.7% purity and recover 12.9% of LA. Urea fractionation concentrate LA by forming crystal complex with SFA and MUFA. LA and the other PUFA was left in non urea complex fraction. The power of Ag-silica gel chromatography to selectively isolate specific desired fatty acid according to its polarity have successfully concentrate LA with a high purity by using toluene as its elution solvent.

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