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Chemical Composition of *Pellonula leonensis* Fish Oils from the Congo River Obtained by Soxhlet Extraction

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Abstract

The chemical composition of *Pellonula leonensis* fish oils from the Congo River (at Boko city) was carried out. The fatty acids were determined by gas chromatography. The Sn-2 position of fatty acids on glycerol was carried out by the ISO 6800 standard. The separation of the compounds (as free fatty acids, monoglycerides, diglycerides, triglycerides, sterols and methyl esters, etc.) was carried out by HPLC using gel permeation with refractometric detection. The phospholipid composition was by HPLC with an evaporative light scattering detector. We obtained oil contents of 32.10 (±0.46)%. The major fatty acids were Palmitic acid (27.41%) and oleic acid (24.23%). The SFA were of 44.50%. The MUFA represent 32.54% and PUFA 22.60%. Regarding the Sn-2 position on glycerol, 48.0% were by SFA and 51.3% were by unsaturated fatty acids. Among them 34.2% of fatty acids were 17.5% oleic acid molecules, 3.3% DHA molecules and 2.1% EPA molecules. Free fatty acids have contents of more than 62%, Diglycerides 16.63% and triglycerides 20.46%. Seven different phospholipids were identified, namely: Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylcholine (PC), Sphingomyelin (SM), Lyso Phosphatitdylcholine (LPC) and Lyso Phosphatidylethanolamine (LPE). According to the high levels in position 2 of the glycerol of palmitic acid, the consumption of *Pellonula leonensis* fish could be moderate.

Keywords

Pellonula leonensis, Fatty Acids, Sn-2 Position, Triacylglycerol, Phospholopids

1. Introduction

Food and food processing plays a vital role in human life. Very early in prehistory, man always had the need to subject the use of natural food materials to some sort of treatment before being consumed.

In sub-Saharan Africa, populations in riparian areas have fish as their main source of food [1]. In addition to their high protein value, fish constitute an important dietary source of lipids, vitamins, minerals and trace elements [2]. The lipids contained in fish are very important sources of energy and essential fatty acids, long-chain polyunsaturated fatty acids (LCFA) of the n-3 and n-6 omega series and fat-soluble vitamins. Which gives them an important nutritional role [3] [4].

Pellonula leonensis, a small fish called "Nsangui" in Congo-Brazzaville, is very popular with residents of the Congo River, Lake Nanga, the Sounda and Loémé rivers. It is regularly consumed by urban communities in the cities of Pointe-Noire and Brazzaville and rural communities. *Pellonula leonensis* is a freshwater clupeid; the most widespread in West and West Africa according to Gourène and Teugels [5]. The species is found in lagoons, lakes [6] as well as in the lower and upper watercourses of the Senegal River. *Pellonula leonensis* is also present in the lower and upper watercourses of the coastal basins from Cameroon to DR Congo [1].

The fish *Pellonula leonensis* consumed in Congo Brazzaville comes mainly from the Congo River and Lake Nanga. This species is of significant economic interest in the localities of Boko and Nanga where it is marketed fresh and dried in the sun on fine sand. In Brazzaville and particularly in Kombé (riparian area of the Congo River), *Pellonula leonensis* fished in the Congo River is marketed in fresh form and is widely consumed stewed by the populations.

However, its nutritional importance remains unknown. The nutritional value of fish is estimated by its composition of water, protein, lipids, carbohydrates, ash and vitamins. Water, proteins, lipids and ash (minerals) represent the four main constituents of fish muscle, the analysis of which is often called "proximal analysis". Proximal composition data are essential for many applications. However, several species of fish have not yet been studied; this is the case of the fish *Pellonula leonensis* from the Congo River and Lake Nanga in the Republic of Congo.

According to the interest of local populations in the consumption of "nsangi" *Pellonula leonensis*, it becomes important to study the chemical composition of this fish, to determine its nutritional value and to project its effects on local populations. In previous study we have tried to determine the proximal composition of fish [7].

What could be the lipid quality of this little fry, sometimes mistaken for a treat? Thus in the present work we study the glyceridic fraction of *Pellonula leonensis* oil by determining the position of fatty acids on glycerol, and the phospholipid composition in order to complete its nutritional quality and to determine the quality of its lipids.

2. Material and Methods

2.1. Biological Material

The biological material used in this study consists of *Pellonula leonensis* fish (Figure 1) fished in the Congo River in the south of Congo-Brazzaville in the localities of Boko (0˚58'17" South and 15˚32'19" East).

Pellonula leonensis is known by the vernacular names of "Nsangui" in southern Congo in the departments of Brazzaville and Pool. *Pellonula leonensis* is a freshwater clupeid present in the main rivers and bodies of water in the Congo, including the Congo River.

Fresh fish were dried in an oven at 70˚C for 48 hours and then ground using an electronic grinder. The resulting powder was wrapped and stored in aluminum foil.

(a) Fresh fish (b) Dried fish

Figure 1. *Pellonula leonensis* fish from the Congo River.

2.2. Methods

2.2.1. Soxhlet Oil Extraction

The Soxhlet (S) method was used to extract lipids from fish. A 30 g mass of fish mince was put into the extraction cartridge and placed in the Soxhlet apparatus. Using 200 mL of hexane contained in a 250 mL flask, the fat contained in the grind was depleted by refluxing the solvent (approx. 80˚C) for 6 hours. The extract was cooled and dried with anhydrous sodium sulfate. The oil was obtained after evaporation of the solvent in a rotary evaporator at 40˚C under vacuum at 200 mbar. The oil content was then determined relative to the mass of crushed material using Equation (1) below:

$$\%\ \text{Oil} = \frac{M_1 - M_0}{M_c} \times 100 \tag{1}$$

where: M_1 = mass of flask containing oil, M_0 = mass of empty flask, M_c = mass of crushed material, % oil = oil content.

2.2.2. Preparation of Methyl Esters and Fatty Acid (FA) Profile

The methyl esters of fatty acids were obtained by basic transesterification: 3 drops of oil were introduced into a flask using a Pasteur pipette. Then 3 pumice

stones were placed in the flask and 3 mL of sodium methoxide were added. After placing the saponification cane on the flask, we heated for 10 minutes at thermostat 1. Then we added 3 mL of acetyl chloride until the phenolphthalein discolored, heating for 10 minutes; and we turned off the heating. After cooling, 8 mL of hexane were added then 10 mL of water. A separation of the two phases (aqueous and hexanic) was observed. Then we collected 1 mL of the hexanic phase in a vial for GC analysis.

The analyzes were carried out on a Focus brand chromatograph equipped with an apolar column (50 m long, 0.25 mm internal diameter and 0.2 µm thick) and a flame ionization detector (FID) according to the following experimental conditions: carrier gas, helium at constant flow: 1 mL/mm; the oven temperature is programmed from 50˚C to 280˚C with a gradient of 5˚C/min; injector temperature: 250˚C; detector temperature: 280˚C. The quantity injected was 1 µL.

A mixture of known fatty acids in defined proportions was injected under the same conditions as the oil to be studied. The retention time and surface area of each control fatty acid were determined. The fatty acids of the oil studied were identified by comparison of retention times and were measured by their areas, referring to the area of an internal standard.

2.2.3. Determination of Fatty Acid Composition in Position 2 and 1 + 3 of glycerol

1) Analysis of Fatty Acids in the Sn-2 Position of Glycerol

The analysis of fatty acids in the Sn-2 position of glycerol was carried out according to ISO 6800. The standard pancreatic lipase hydrolysis procedure used to determine the FA composition in the β position of TAG was modified according to [8]. MAGs were rapidly isolated by preparative TLC on silicic acid previously impregnated in a solution of 5% boric acid in methanol (w/v) to prevent isomerization. Plates were developed with chloroform/acetone/acetic acid solution (85:15:1, by volume) to isolate sn-1(3) from sn-2-MAG. After development, the bands were revealed and visualized with the DCF solution (2′,7′-dichlorofluorescein in ethanol) under UV light (366 nm). The following bands were observed: α-MAG (Rf = 0.26); β-MAG (Rf = 0.38); α,β-DAG (Rf = 0.76); α,α'-DAG (Rf = 0.85); tertiary alcohols of deacylated AG and residual TAG (Rf = 0.95). Silica gel from the corresponding strips was scraped and transferred to Teflon-coated screw-cap tubes for methyl ester preparation. The methyl esters were prepared according to [9] after desorption in 5 mL of diethyl oxide, centrifugation, filtration and evaporation of the solvent.

2) Analysis of fatty acids in position 1 + 3 of glycerol

The extract is purified in order to only work on triglycerides. The external positions 1 and 3 of the triglyceride are selectively transesterified with ethanol using a Candida Antartica enzyme to obtain 2-monoglycerides. A step by SPE makes it possible to separate the 2-monoglycerides from the other compounds produced; the fatty acid composition of these 2-monoglycerides will be determined by GC/FID after trans esterification with KOH/MEOH.

2.2.4. Determination of the Glyceride Composition of *Pellonula leonensis* Oil

The method used makes it possible to determine the percentage composition of a classic mixture ranging from free fatty acids to triglycerides. The separation of the compounds (free fatty acids, monoglycerides, diglycerides, triglycerides, sterols and methyl esters, etc.) was carried out by high-performance liquid chromatography by gel permeation with refractometric detection. The stationary phase consists of divinylbenzene styrene copolymer. The separation of molecules is done according to the size of the molecules and in descending order of molecular weight.

2.2.5. Determination of the Phospholipid Composition of *Pellonula leonensis* Oil

The method used makes it possible to determine the content and composition of phospholipids. The sample is dissolved in a mixture of chloroform/methanol solvent (2:1, by volume) then analyzed by high performance liquid chromatography with an evaporative light scattering detector.

3. Results and Discussion

3.1. Fatty acid composition

Fish oils are generally characterized by a fairly broad group of saturated and unsaturated fatty acids, usually associated with mixed triglycerides.

Data relating to the fatty acid composition and level of *Pellonula leonensis* fish oils from the Congo River are shown in Table 1.

Table 1. Fatty acid composition of *Pellonula leonensis* oil.

Common name	Fatty acids	Content (%)
Yield	% oil	32.10 (±0.46)
Lauric acid	C12:0	0.32
	C13:0	0.07
Myristic acid	C14:0	2.53
Myristoleic acid	C14:1	0.79
Pentadecanoic acid	C15:0	0.76
Palmitic acid	C16:0	27.41
Palmitoleic acid	C16:1	6.73
Hexadecadienoic acid	C16:2	1.17
Hiragonic acid	C16:3	0.34
Margaric acid	C17:0	2.81
Heptadecenoic acid	C17:1	0.53
Stearic acid	C18:0	9.45
Elaidic acid	C18:1 trans	0.37
Oleic acid	C18:1	24.23

Continued

Linoleic acid	C18:2	5.55
Gamma-linolenic acid	C18:3 (n-6)	0.31
Linolenic acid	C18:3 (n-3)	2.52
Stearidonic acid	C18:4 (n-3)	0.33
Arachidic acid	C20:0	0.52
Gondoic acid	C20:1	0.26
	C20:2 (n-6)	0.23
	C20:3 (n-6)	0.23
Arachidonic acid	C20:4 (n-6)	2.90
	C20:3 (n-3)	0.14
	C20:4 (n-3)	0.32
EPA	**C20:5 (n-3)**	3.64
behenic Acid	C22:0	0.42
	C22:4 (n-6)	0.26
	C21:5 (n-3)	0.10
	C22:5 (n-6)	0.37
DPA	C22:5 (n-3)	1.76
DHA	**C22:6 (n-3)**	2.43
Lignoceric acid	C24:0	0.20
Total	33 Fatty acids	100%

The fatty acids identified in *Pellonula leonensis* fish oil from the Congo River (Boko) sample consist of ten (10) saturated fatty acids which include C12:0, C13:0, C14:0, C15: 0, C16:0, C17:0, C18:0, C20:0, C22:0 and C24:0; six (6) monounsaturated fatty acids which are C14:1, C16:1 (n-9), C17:1, C18:1 (n-9), C18:1 trans and C20:1 (n-9); and sixteen (16) polyunsaturated acids which are C16:2, C16:3, C18:2 (n-6), C18:3 (n-6), C18:3 (n-3), C20:2 (n-6), C20:3 (n-6), C20:4 (n-6), C20:3 (n-3), C20:4 (n-3), C20:5 (n-3), C22:4 (n-6), C21:5 (n-3), C22:5 (n-6), C22:5 (n-3) and C22:6 (n-3). The profile of major (with contents greater than 10%) and minor (with contents less than 10%) fatty acids is summarized as follows:

C16:0 > C18:1 > C18:0 > C16:1 > C18:2 > C20:5 (n-3) > C20:4 (n-6) > C17:0 > C14:0 > C18:3 (n-3) > C22:6 (n-3) > C22:5 (n-3) > C16:2

Palmitic acid (27.41%) is the most abundant fatty acid in *Pellonula leonensis* fish oil. It is followed by oleic acid (24.23%). Table 2 shows the different groups of fatty acids constituting the oil of the fish *Pellonula leonensis*. It appears from this table that almost half of the fatty acids in this oil (44.50%) are saturated fatty acids. Monounsaturated fatty acids represent 32.54% of the fatty acids in this oil, while polyunsaturated fatty acids represent 22.60% of the overall fatty acid content. It should therefore be noted that *Pellonula leonensis* fish oil is mostly unsaturated (55.14%).

We also observe the presence of traces of trans fatty acids (C18:1 trans). It is well known that this trans fatty acid isomer is produced from its cis-unsaturated counterpart (C18:1 cis) during hot Soxhlet extraction of oils.

Many researchers consider that a diet is healthy when it contains a lipid fraction rich in polyunsaturated fatty acids, particularly omega 3 [10]. Thus, a lower ratio between $\omega 6/\omega 3$ fatty acids would be desirable to reduce the risk of many chronic diseases whose prevalence is high in developing countries. The exact value of the $\omega 6/\omega 3$ ratio is given in numerous documents, but some studies indicate that the optimal ratio may vary depending on the disease considered [11]. For the fish oil *Pellonula leonensis* studied, the ratio $\omega 6/\omega 3$ is equal to 0.88.

Table 2. Groups of fatty acids in *Pellonula leonensis* oil.

Types of fatty acids	Content (%)
Saturated fatty acids	44.50 ± 2.18
Monounsaturated fatty acids	32.54 ± 2.83
Polyunsaturated fatty acids	22.60 ± 2.47
Of which (n-3)	11.23
Of which (n-6)	9.87
Trans fatty acids	0.37

3.2. Composition of Fatty Acids in Position 2 and 1 + 3 of Glycerol

Table 3 shows the sn-2 fatty acid composition of glycerol from *Pellonula leonensis* fish oil.

Regarding the Sn-2 position of glycerol, which is important for the stability and bioavailability of fatty acids [12]-[15], the results show that the *Pellonula leonensis* fish oil has high levels of saturated fatty acids, including palmitic acid. 30.1% of the triacylglycerol molecules in *Pellonula leonensis* oil contain palmitic acid and 21.4% of the triacylglycerol molecules contain oleic acid. However, 34.2% of the fatty acids in the Sn-2 position of glycerol are palmitic acids, 17.5% are oleic acid molecules, 3.3% DHA molecules and 2.1% EPA molecules. According to the average distribution of fatty acids, 65.7% and 26.0% of DHA and EPA molecules respectively are in position 2 of glycerol.

It should be noted that a predominance of saturated fatty acids in this position induces a higher and more pronounced circulating concentration of chylomicrons in the blood after digestion, which could contribute to cardiovascular risk [16]-[18].

However, for *Pellonula leonensis* oil, 48.0% of fatty acids in the sn-2 position of glycerol are saturated acids and 51.3% are unsaturated fatty acids (Table 3).

Table 3. Composition of fatty acids in position 2 and 1 + 3 of glycerol.

Fatty acids	Tricylglycérols	Average composition		Average distribution	
		Position 2	Position 1 + 3	Position 2	Position 1 + 3
C12:0	0.3	0.3	0.3	36.6	63.4

Continued

C13:0	0.2	0.1	0.2	18.2	81.8
C14:0	2.6	3.2	2.4	40.1	59.9
C15:0	1.7	2.3	1.5	43.5	56.5
C16:0	**30.1**	**34.2**	**28.0**	**37.9**	**62.1**
C16:1	7.9	10.1	6.8	42.6	57.4
C16:2	1.4	1.7	1.2	41.8	58.2
C16:3	0.3	0.3	0.3	34.0	66.0
C17:0	3.3	1.8	2.6	25.9	74.1
C17:1	0.3	0.2	0.3	23.8	76.2
C18:0	11.1	5.5	13.9	16.6	83.4
C18:1 trans	0.7	0.6	0.7	30.9	69.1
C18:1 cis	**21.4**	**17.5**	**23.3**	**27.3**	**72.7**
C18:2 cis	4.8	6.0	4.2	41.8	58.2
C18:3 (n-6)	0.3	0.3	0.3	33.7	66.3
C18:3 (n-3)	2.3	2.9	2.0	41.6	58.4
C18:4 (n-3)	0.3	0.3	0.3	38.6	61.4
C20:0	0.8	0.3	1.0	12.0	88.0
C20:1	0.7	0.4	0.9	19.3	80.7
C20:2 (n-6)	0.3	0.1	0.4	13.8	86.2
C20:3 (n-6)	0.3	0.2	0.3	26.0	74.0
C20:4 (n-6)	2.3	1.8	2.6	26.4	73.6
C20:4 (n-3)	0.3	0.3	0.3	34.9	65.1
C20:5 (n-3)	**2.7**	**2.1**	**3.0**	**26.0**	**74.0**
C22:0	0.5	0.2	0.7	12.2	87.8
C22:1 (n-9)	0.2	0.1	0.2	14.4	85.6
C22:4 (n-6)	0.2	0.3	0.2	47.2	52.8
C22:5 (n-6)	0.4	0.6	0.3	55.0	45.0
C22:5 (n-3)	1.6	2.8	1.0	58.9	41.1
C22:6 (n-3)	**1.7**	**3.3**	**0.9**	**65.7**	**34.3**
C24:0	0.2	0.1	0.2	19.4	80.6

3.3. Glyceridic Composition

The polar compounds of minor glycerides include several families of compounds of very different molecular sizes, which explains why they can be easily separated and quantified by high-performance gel permeation liquid chromatography with refractometric detection.

Triacylglycerols, Diacylglycerols, Monoacylglycerols and free fatty acids are eluted from the chromatographic column in reverse order of their molecular size.

The results of the glyceride composition from the chromatographic analysis

are given in the following Table 4:

Table 4. Glyceridic composition of *Pellonula leonensis* oil.

Parameters determined	Contents (%)
Polymers (oligomers approx. 1500 g/mol)	0.85
Triglycerides	20.46
Diglycerides	16.63
Monoglycerides	<0.1
Free fatty acids and others (EMAG, sterols, etc.)	62.06

The compounds identified correspond to three main alterations that oils undergo when exposed to high temperature, atmospheric oxygen and humidity. The corresponding alterations for this study are thermal, oxidative and hydrolytic and can take place even during oil extraction processes. These compounds were quantified by HPSEC with a refractive index detector using pure TGs as external standards and assuming the same response factor for each of them. According to the results obtained, free fatty acids have contents of more than 62%. The Diglyceride content is 16.63% and triglycerides only present 20.46%. The oil studied, extracted with a hot Soxhlet, shows hydrolytic alteration. The extraction method used therefore favored the hydrolysis of triglycerides given the high level of Diglycerides and free fatty acids present in the oil [19].

3.4. Phospholipid Composition

Phospholipids are fundamental constituents of natural membranes; their amphiphilic properties come from the presence of both a hydrophobic tail and a hydrophilic head. This characteristic affects their role, behavior and function. They belong to the class of polar lipids and are literally defined as "phosphorus-containing lipids" [20].

Identification of phospholipids in the oil of *Pellonula leonenis* from the Congo River fished in the Boko area shows at least the presence of seven different phospholipids.

Lipids form a particularly complex family due to the possible isomers of fatty acids, and the large number and variety of other constituent elements. Their physicochemical properties and their biological roles arise from this structural complexity.

Table 5 gives the contents of the different phospholipids identified in *Pellonula leonensis* fish oil.

Table 5. Phospholipid composition of *Pellonula leonensis* oil.

Parameters determined	Contents (g/100 g of oil)
Phosphatidylglycerol (PG)	<0.02
Phosphatidylethanolamine (PE)	<0.02

Continued

Phosphatidylinositol (PI)	<0.02
Phosphatidylcholine (PC)	<0.02
Sphingomyeline (SM)	<0.02
Lyso Phosphatitdylcholine (LPC)	<0.02
Lyso Phosphatidylethanolamine (LPE) + Phosphatidylserine (PS) + Acide Phosphatidic (AP)	<0.1
Total phospholipids	**<0.1**

Phosphatidylglycerols play a role in the activity of respiratory chain enzymes involved in oxidative phosphorylation [21]. The phosphatidylglycerol content found in *Pellonula leonenisis* fish oil is less than 0.02%.

Phosphatidylethanolamines are among the most abundant phospholipids in both the animal and plant kingdoms. In animals, they are found in abundance in the liver and brain, which is why they were formerly called "cephalins". They play a role in membrane constitution, particularly in myelin sheaths, and in the formation of very low density lipoproteins. However, they are at levels less than 0.02% in the oil studied.

Phosphatidylinositols are lipids that are rapidly metabolized in the cell to provide diacylglycerols and inositol phosphate used in regulatory processes. Their content in *Pellonula leonensis* fish oil from the Congo River is less than 0.02%

Phosphatidylcholines are the most abundant lipids in cell membranes of animal tissues. These lipids, also called lecithins, have emulsifying properties which are widely used in the food industry. They are found in *Pellonula leonenis* fish oil at levels less than 0.02%.

Phosphatidylserines, weakly acidic lipids, are present in all cell membranes. They are also involved in platelet activation and in the activation of protein kinase C.

Pellonula leonensis oil provides total phospholipids at levels less than 0.1%.

4. Conclusions

Palmitic acid (27.41%) is the most abundant fatty acid in *Pellonula leonensis* fish oil. It is followed by oleic acid (24.23%). It appears from this that almost half of the fatty acids in this oil (44.50%) are saturated fatty acids. Monounsaturated fatty acids represent 32.54% of the fatty acids in this oil, while polyunsaturated fatty acids represent 22.60% of the overall fatty acid content. It should therefore be noted that *Pellonula leonensis* fish oil is mostly unsaturated (55.14%).

Regarding the Sn-2 position of glycerol, 48.0% of fatty acids in the sn-2 position of glycerol are saturated acids and 51.3% are unsaturated fatty acids. 34.2% of fatty acids in the Sn-2 position of glycerol are palmitic acids, 17.5% oleic acid molecules, 3.3% DHA molecules and 2.1% EPA molecules.

According to the results obtained, free fatty acids have contents of more than 62%. The Diglyceride content is 16.63% and triglycerides only present 20.46%.

The oil studied, extracted with a hot Soxhlet, shows hydrolytic alteration. The extraction method used therefore favored the hydrolysis of triglycerides.

The Identification of phospholipids in the oil of *Pellonula leonenis* from the Congo River fished in the Boko area showed the presence of at least seven different phospholipids, namely: Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylcholine (PC), Sphingomyelin (SM), LysoPhosphatidylcholine (LPC) and Lyso Phosphatidylethanolamine (LPE). Therefore, the presence of these phospholipids in the oil of *Pellonula leonensis* fish leads to low cadiovacular diseases and improves the saltatory transmission of nerve impulses.

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Conflicts of Interest

No conflicts of interest for this article.

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