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Characterization of Fish Viscera Oils Extracted by Slow Freezing

Caracterização de Óleos de Vísceras de Peixe Extraídos por Congelamento Lento

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Abstract

Fish viscera are often improperly discarded, polluting the environment. This material is rich in lipids, whose characterization allows determining their potential use in various applications, especially due to the presence of omega-3 fatty acids. The objective of this study was to characterize the physicochemical properties of oils extracted after slow freezing from the viscera of three species of freshwater fish: rainbow trout, pacu, and curimbatá, collected in three locations in the Southeast region of Brazil. The viscera were frozen at -20 º C and subsequently heated to 60 ºC, producing the separation of the lipid fraction into a supernatant layer. Oil yield was 42.53%, 27.58% and 13.75% ($p<0.05$) for pacu, rainbow trout and curimbatá respectively. Free fatty acids content, acid, peroxide, iodine and saponification value; density (40 ºC); dynamic and kynematic viscosity (40ºC); and refractive index (25 ºC and 40 ºC) were within the normal range for crude fish oils in the three species. The omega-3 fatty acid content was lower than that observed in marine oils used as EPA and DHA supplements, but higher than the levels of these fatty acids in fats from terrestrial animals. Thus, the oils from the viscera of rainbow trout, pacu, and curimbatá can be suitable for food purposes or biofuel production. The slow freezing extraction method proved effective for obtaining oil from fish viscera. This method is simple and does not require the use of solvents or sophisticated equipment, resulting in cost reduction, environmental and public health care.
Keywords: Fatty Acids. Feshwater Fish. Fish By-Products. Omega-3. Sustainability.

Resumo

As vísceras de peixe frequentemente são descartadas de forma inadequada, poluindo o ambiente. Este material é rico em lipídios cuja caracterização permite determinar seu potencial de uso em diversas aplicações, especialmente pela presença de ácidos graxos ômega-3. O objetivo deste trabalho foi caracterizar as propriedades físico-químicas de óleos extraídos após congelamento lento, de vísceras de três espécies de peixes de água doce: truta arco-íris, pacu e curimbatá, coletadas em três localidades da região Sudeste do Brasil. As vísceras foram congeladas a -20 ºC e posteriormente aquecidas a 60 ºC, separando a fração lipídica numa camada sobrenadante. O rendimento de óleo foi 42,53%; 27,58% e 13,75% ($p<0.05$) para pacu, truta arco-íris e curimbatá respectivamente. Os ácidos graxos livres, índices de acidez, peróxidos, iodo e saponificação; densidade (40 ºC), viscosidade dinâmica e cinemática (40 ºC); e índice de refração (25 ºC e 40 ºC) apresentaram valores normais para óleos brutos de peixe nas três espécies. O teor de ácidos graxos ômega-3 foi inferior ao observado nos óleos marinhos utilizados como suplemento de EPA e DHA, mas superior aos teores em gorduras provenientes de animais terrestres. Desta forma, os óleos de vísceras de truta arco-íris, pacu e curimbatá seriam adequados para fins alimentícios ou produção de biocombustíveis. O método de extração por congelamento lento resultou eficaz para obtenção de óleo de vísceras de peixe. Este método é simples e dispensa o uso de solventes ou equipamentos sofisticados, resultando na redução de custos, cuidado do meio ambiente e da saúde pública.
Palavras-chave: *Ácidos Graxos. Peixe de Água Doce. Resíduos de Peixe. Ômega-3. Sustentabilidade.*

1 Introduction

Global aquaculture production has increased from 10 million tons annually in 1990 to 90 million tons in 2020 (FAO, 2022). Considering a viscera-somatic index from 7% to 15% for fish, between 5.6 and 12 million tons would correspond to viscera, which constitutes a by-product that is not always properly utilized and is sometimes disposed of inadequately, polluting the environment.

The main use of fish viscera is in animal feed due to its protein and fat-rich composition. Characterizing the lipid fraction of this material is of interest in the expectation of obtaining oil rich in long-chain omega-3 fatty acids for human or animal consumption and supplementation, or for other potential uses such as biofuel production. However, fat extraction is usually performed using organic solvents (Brum; Arruda; Reginato-D'Arce, 2009), under appropriate conditions with the use of protective equipment to reduce health and/or environmental risks associated with handling and disposal, which also implies high costs.

The slow freezing oil extraction method involves exposing the viscera to a temperature of -18 °C until complete solidification, followed by heating to 60 °C to induce fat separation. This method can even be performed using conventional household equipment, allowing for the processing of raw materials in small and medium-scale analyses such as laboratories or small fish farms (Ortiz; Oña;

Guerra, 2008).

The objective of this study was to characterize the viscera oils of three freshwater fish species: rainbow trout (Oncorhynchus mykiss), pacu (Piaractus mesopotamicus), and curimbatá (Prochilodus lineatus), obtained through the slow freezing extraction method.

2 Material and Methods

A total of 40 kg of rainbow trout (O. mykiss) viscera from Santo Antônio do Pinhal, São Paulo, Brazil and 5 kg of pacu (P. mesopotamicus) viscera from Toledo, Paraná, Brazil, from intensive aquaculture, as well as 2.5 kg of Curimbatá (Prochilodus sp.) viscera from Pirassununga, São Paulo, Brazil from extensive aquaculture, were collected. The materials were transported under refrigeration to the Aquaculture Laboratory at the Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, and stored in freezers at -18 °C until complete solidification (around 48 hours).

The extraction of fish viscera oil by slow freezing method for rainbow trout, pacu, and Curimbatá was performed according to Ortiz, O**ña** and Guerra (2008): the still frozen viscera were cut into 3 cm cubes, placed in 600 mL beakers, and heated in a water bath at 60°C for 90 minutes, resulting in the separation of a supernatant lipid layer wich was collected using pipettes, vacuum-filtered, and stored at -20 °C for further analysis.

The content of free fatty acids (FFA), acid value (AV), peroxide value (PV), saponification value (SV), iodine value (IV), and refractive index at 25 °C and 40 °C (RI25 °C and RI40 °C, respectively) were determined according to Endo (2018). The density at 40 °C (d40 °C) was determined using an Anton Paar DMA 4500 density meter (Anton Paar GmbH, Ostfildern, Germany), and the dynamic and kinematic viscosity at 40°C (ηdyn40°C and ηkin40 °C, respectively) were measured using an Anton Paar Automated Micro Viscometer (AMVM) (Anton Paar GmbH, Ostfildern, Germany).

The fatty acid profile was determined by gas chromatography. A 25 mg aliquot of oil was transesterified according to Hartman and Lago (1973). Gas chromatography was performed using a Shimadzu gas chromatograph (Kyoto, Japan) equipped with a split injector (1:50) at 250 °C and a fused silica capillary column (CP-SIL 88, Chromopack, Middleburg, Netherlands) with dimensions of 100 m x 0.25 mm x 0.20 µm. The injected sample volume was 2 µL. The flame ionization detector temperature was set at 260 °C. The initial temperature of the chromatograph was set at 120 °C for 8 minutes, followed by an increase to 160 °C for 4 minutes (rate of 20 °C/min), then to 195 °C for 10 minutes (rate of 3 °C/min), further increased to 220 °C for 3 minutes (rate of 35 °C/min), and finally held at 240 °C for 5 minutes (rate of 20 °C/min), totaling 46 minutes (Sancho et al., 2011). Hydrogen was used as the carrier gas at a linear velocity of 34 cm/s, and nitrogen was used as the makeup gas with a flow rate of 30 ml/min. The identification of methyl esters was performed by comparing the retention times of peaks in the sample with those of the commercial FAME Mix C4-C24 standard (Supelco, Bellefonte, Pennsylvania, USA).

A completely randomized design was employed, and analysis of variance (ANOVA) was performed using the mixed procedure in SAS (9.22). Assumptions of error normality and variance heterogeneity were verified using the residual option in the above-mentioned procedure, and differences between means were determined using Tukey's range test at a significance level of 5%.

3 Results and Discussion

A significant difference ($p<0.05$) was observed in the yields of fish viscera oil extracted by slow freezing method among the three evaluated species. The highest yield was observed in pacu viscera oil (42.53 ± 2.73), followed by trout viscera oil (27.58 ± 2.42), and Curimbatá viscera oil (13.75 ± 1.34).

Pacu has high capacity to accumulate fat reserves and has omnivorous feeding habit, which facilitates the use of diets with high or even exclusive incorporation of plant-based ingredients (Segura et al., 2017) at a low cost (Oliveira et al., 2020). However, an imbalance in the protein-to-energy ratio can increase visceral fat accumulation, reducing meat yield without significantly affecting weight gain (Signor et al., 2010; Araujo et al., 2020). The fat content in rainbow trout viscera was like that reported by Dumas et al. (2007) at 31.2% and lower than the value observed by Weber et al. (2015) at 42% in 14-month-old females, indicating good nutritional management during their rearing. Curimbatá viscera had a lower fat content, likely due to lower nutrient availability in the extensive system.

Freezing the viscera at -18 °C (slow freezing) probably causes the formation of intracellular ice crystals that damage the cell membranes, like what is observed in plant tissues subjected to the same process (M**élo**; De Lima; Do Nascimento, 2000). These histological lesions allow the release of lipids upon thawing the material, and their displacement to the superficial layer due to their lower density compared to the predominantly aqueous fraction in tissues. The freezing extraction process was applied by Ortiz, Oña and Guerra (2008), using viscera from a commercial rainbow trout stock, highlighting the advantage of using conventional freezers and stoves. Jean, Lee and Wu (1999) applied the same principles for separating oil from oily sludge from a petroleum refinery, which was frozen at -20 °C and then thawed at room temperature for 12 hours after solidification, resulting in a dark sediment, an intermediate aqueous layer, and a superficial layer containing 51.5% of the total lipids. This result could not be achieved by ultra-freezing (-198 °C), emphasizing the need for slow material solidification

to achieve a high fat recovery. Thus, the extraction of fish viscera oil by slow freezing method from the three fish species was effective in obtaining good yields of crude oil.

Arias, Gómez and Zapata (2017) evaluated a method for oil extraction from tilapia viscera using "heating followed by freezing", where the viscera, after being extracted from the fish, were heated (60 to 80 °C) for 20 to 40 minutes and subsequently frozen at -18°C for 24 hours, resulting in a superficial layer of fat that was analyzed. According to their description, the process should avoid freezing the viscera before heating to prevent the material emulsification, as this would hinder separation phase. Conversely, in the present study, the applied process sequence was 'freezing followed by heating,' and no indications of raw material emulsification were observed. It is recommended to compare the methods to identify potential improvements.

The results of the physicochemical characterization (except for fatty acid profile) of oils extracted from rainbow trout, pacu, and Curimbatá by slow freezing are presented in Table 1.

Table 1 - Physicochemical characterization of crude fish viscera oil extracted by slow freezing method from rainbow trout, pacu and Curimbatá

Variable	Unit	Fish viscera oil		
		Rainbow trout	Pacu	Curimbatá
FFA	%	6.06 ± 0.13^{A}	4.92 ± 0.39^{B}	4.12 ± 0.11^{C}
AV	mg g^{-1}	12.05 ± 0.27^{A}	9.79 ± 0.78^{B}	8.19 ± 0.22^{C}
PV	Meq Kg^{-1}	7.26 ± 0.29^{B}	6.81 ± 0.21^{B}	27.27 ± 0.94^{A}
IV	cg g^{-1}	91.02 ± 9.22^{AB}	73.00 ± 4.69^{B}	112.25 ± 8.64^{A}
SV	mg g^{-1}	226.49 ± 8.15	237.80 ± 2.24	234.23 ± 7.71
d40ºC†	g cm^{-3}	0.9016^{B}	0.8999^{C}	0.9038^{A}
ηdyn40ºC	mPa s	32.2097 ± 0.1816^{B}	33.0987 ± 0.2590^{A}	29.5691 ± 0.0957^{C}
ηkin40ºC	mm^2 s^{-1}	35.7230 ± 0.2014^{B}	36.7796 ± 0.2879^{A}	32.7175 ± 0.1060^{C}
RI25ºC		1.4703 ± 0.0002^{B}	1.4691 ± 0.0003^{C}	1.4715 ± 0.0000^{A}
RI40ºC		1.4652 ± 0.0003^{A}	1.4620 ± 0.0000^{B}	1.4655 ± 0.0000^{A}

Mean ± standard deviation of three replicates; different letters in the same row indicate significant difference ($p<0.05$) by Tukey's range test; FFA = free fatty acids expressed as a percentage of oleic acid; AV = acid value, grams of KOH required to neutralize the acids present in 1g of sample; PV = peroxide value, milliequivalents of active oxygen per kilogram of fat; IV = iodine value, centigrams of iodine absorbed per gram of sample; SV = saponification value, milligrams of potassium hydroxide required to saponify one gram of oil; d40ºC = density at 40ºC; ηdyn40ºC = dynamic viscosity at 40ºC; ηkin40ºC = kinematic viscosity at 40 ºC; RI25ºC and RI40ºC = refractive index at 25ºC and 40ºC respectively; † the standard deviation of the density at 40ºC in all cases was 0.0000.
Source: research data.

Several indicators can be used to determine the quality of oils. FFA, AV, IV, and SV are chemical characteristics; d40 ºC, ηdyn40 ºC, ηkin40 ºC, RI25 ºC, and RI40 ºC are physical characteristics, and PV is a characteristic of deterioration (ENDO, 2018). According to Bimbo (1998), crude marine fish oils for food grade have FFA content ranging from 1 to 7%; PV from 3 to 20 Meq.Kg^{-1}; IV in the lower limit from 95 to 180 cg.g^{-1} and in the upper limit from160 to 200 cg.g^{-1}; density at 30 ºC and 45 °C of 0.90 and 0.91 g.cm^3, respectively, and viscosity at 50 °C from 20 to 30 mPa.s. According to Scrimgeour (2005) the SV of fish oil varies from 180 to 192, and for RI, Rahman et al. (2023) observed values ranging from 1.455 to 1.46 in pangasius fish oil (Pangasius pangasius).

The oils of the three species evaluated in this study have characteristics that fall within the reference values mentioned, except for Curimbatá viscera oil, whose PV was the highest among the three species and exceeded 20 Meq.Kg^{-1}. The Curimbatá viscera were collected as these specimens were caught over a period of 5 hours, which possibly increased peroxidation because of temperature variation when opening and closing the cooling box. The IV in rainbow trout and Pacu viscera oil was lower than the values observed in the references, influenced by the lower unsaturation of these oils compared to highly unsaturated marine oils, and the SV was higher due to the higher presence of fatty acids with a lower average chain length (Hass, 2005). The higher IV of Curimbatá viscera oil would have a direct relationship with density, considering that fatty acids with a longer carbon chain have a higher molecular weight and the capacity to accommodate a greater number of double bonds. However, in general, the density tended to be lower than that of marine fish oil. The viscosity at 40 °C (dynamic and kinematic) showed an inverse relationship with IV because the melting point of fatty acids is higher when the number of double bonds is lower. The refractive index decreased as the temperature increased, and it was observed that the RI40ºC values were lower than the RI25ºC values, understanding that the viscosity of oils exposed to higher temperatures decreases, facilitating the passage of light (the physical principle of RI). The variations in this physicochemical characteristics of the fish viscera oil obtained by slow freezing method are consistent with their fatty acid composition, which has a lower degree of unsaturation than marine oils.

The viability of fish viscera oil extraction through slow freezing from an industrial perspective would depend on a cost analysis of facilities, equipment, and energy. However, on a small and medium scale, the applicability of this method is suitable allowing for the rapid, economical, and safe

production, while eliminating health and environmental risks due to the absence of solvents.

Table 2 shows the characterization of the fatty acid profile of oils from pacu, rainbow trout, and curimbatá viscera extracted by slow freezing. The most abundant fatty acids in the three oils evaluated were 16:0, 16:1n-7, 18:0, 18:1n-9, and 18:2n-6.

Table 2 - Fatty acid profile (% of total fatty acids) of crude fish viscera oil (VO) extracted by slow freezing method from rainbow trout, pacu and Curimbatá

Fatty acid	Rainbow trout VO	Pacu VO	Curimbatá VO
11:0	0.02 ± 0.00^{B}	0.02 ± 0.00^{B}	0.07 ± 0.01^{A}
12:0	0.03 ± 0.00^{B}	0.05 ± 0.00^{B}	0.10 ± 0.01^{A}
14:0	1.25 ± 0.03^{B}	3.41 ± 0.40^{A}	3.60 ± 0.34^{A}
14:1n-9	0.07 ± 0.00^{B}	0.32 ± 0.03^{A}	0.07 ± 0.01^{B}
15:0	0.09 ± 0.01^{B}	0.19 ± 0.03^{B}	0.52 ± 0.04^{A}
16:0	20.00 ± 0.07^{B}	25.84 ± 0.46^{A}	27.57 ± 1.15^{A}
16:1n-7	6.98 ± 0.07^{B}	6.62 ± 0.29^{B}	13.49 ± 0.99^{A}
17:0	0.12 ± 0.01^{C}	0.33 ± 0.03^{B}	0.85 ± 0.02^{A}
18:0	5.62 ± 0.10^{B}	10.01 ± 0.71^{A}	6.80 ± 0.19^{B}
18:1n-9 (t)	nd	0.12 ± 0.01^{B}	1.18 ± 0.06^{A}
18:1n-9	38.94 ± 0.26^{A}	35.80 ± 2.53^{A}	21.57 ± 0.30^{B}
18:2n-6	19.41 ± 0.13^{A}	13.43 ± 1.38^{B}	10.02 ± 0.48^{C}
18:3n-6	0.73 ± 0.02^{A}	0.22 ± 0.01^{C}	0.35 ± 0.01^{B}
20:1n-9	nd	0.73 ± 0.08^{B}	3.46 ± 0.18^{A}
18:3n-3	0.76 ± 0.00^{B}	0.88 ± 0.05^{B}	3.03 ± 0.11^{A}
21:0	0.02 ± 0.00^{B}	0.04 ± 0.01^{B}	0.07 ± 0.01^{A}
20:2n-6	1.70 ± 0.05^{A}	0.50 ± 0.04^{C}	0.89 ± 0.05^{B}
20:3n-6	1.38 ± 0.03^{A}	0.50 ± 0.03^{C}	1.15 ± 0.02^{B}
22:1n-9	0.03 ± 0.00^{B}	nd	0.51 ± 0.03^{A}
20:4n-6	1.10 ± 0.04^{B}	0.57 ± 0.03^{C}	1.35 ± 0.03^{A}
20:5n-3	0.09 ± 0.03^{B}	0.12 ± 0.02^{B}	2.11 ± 0.10^{A}
22:6n-3	0.66 ± 0.20^{B}	0.10 ± 0.00^{C}	1.17 ± 0.03^{A}
ΣSFA	28.15 ± 0.09^{B}	39.91 ± 1.37^{A}	39.63 ± 1.42^{A}
ΣMUFA	46.02 ± 0.29^{A}	43.66 ± 2.59^{AB}	39.10 ± 0.76^{B}
ΣPUFA	25.82 ± 0.21^{A}	16.32 ± 1.39^{C}	20.07 ± 0.61^{B}
ΣHUFA	3.22 ± 0.29^{B}	1.29 ± 0.03^{C}	5.77 ± 0.09^{A}
ΣTRANS	0.02 ± 0.00^{B}	0.12 ± 0.01^{B}	1.20 ± 0.06^{A}
Σn-3	1.50 ± 0.23^{B}	1.10 ± 0.05^{B}	6.30 ± 0.21^{A}
Σn-6	24.31 ± 0.07^{A}	15.22 ± 1.39^{B}	13.76 ± 0.41^{B}
Σn-6/Σn-3	16.41 ± 2.37^{A}	13.87 ± 1.44^{A}	2.18 ± 0.02^{B}

Fatty acids as means ± standard deviation of three replicates; t = trans; nd = not detected; different uppercase letters in the same row indicate significant difference ($p<0.05$ - Tukey's range test); ΣSFA, ΣMUFA, ΣPUFA, ΣHUFA, Σn-3, Σn-6 = sum of saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, highly unsaturated fatty acids, n-3 fatty acids, and n-6 fatty acids, respectively.
Source: research data.

The obtaining of a new n-3 long chain PUFA (LC-PUFA) supplement from fish viscera was one of the main commercial expectations for this byproduct. Unfortunately, the levels of these fatty acids observed in the evaluated fish viscera oils are much lower than those of marine oils marketed as nutritional supplements of n-3 LC-PUFA, which typically contain 21% to 30% EPA+DHA (Karsli, 2021). The highest content of EPA+DHA in the present study was 3.28% in curimbatá viscera oil. However, modest levels of EPA+DHA, such as those observed in the present study, are higher than those frequently found in oils and fats derived from terrestrial animals such as pigs, poultry, and cattle (Alm, 2013), which are commonly used in feed manufacturing. It should be noted that oils and fats of plant origin only contain linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) as the sole omega-6 and omega-3 fatty acids respectively. New primary sources of n-3 LC-PUFA such as microalgae or genetically modified plant oils, among others (Tocher et al., 2019), are not yet fully available in the Brazilian market. The fatty acid profile of fish is mostly a reflection of the fatty acid profile of their diet, and low levels of EPA and DHA in the feed do not affect the weight gain of rainbow trout, despite being a cold-water carnivorous fish (Rinchard; Czesny; Dabrowsky, 2007), nor do they affect the majority of tropical fish.

The production of biofuels constitutes another alternative for the utilization of fish viscera oil extracted by slow freezing method, as it does not compete with the use of land, as it is the case with edible vegetable oils which are costly compared to oils and fats unsuitable for food purposes (Morone; Cottoni; Giudice, 2023). Biodiesel is a biofuel that can be used directly in diesel engines or blended in various proportions with conventional fossil fuel oil. It is a mixture of fatty acid esters obtained by transesterification of oils or fats of animal or vegetable origin with low molecular weight alcohols (methanol or ethanol), generating glycerol as the main byproduct (Mata et al., 2010). The efficient production of biodiesel is only achieved with refined or high-purity oils and fats, with a maximum FFA content of 0.5% and no moisture (Di bitonto; Pastore, 2019). Oils of the required quality are expensive, representing 85% of the production cost of biodiesel. The alternative of using high-acidity crude oils obtained from various types of waste, such as the evaluated fish viscera oils in the present study, implies the development of economical and efficient methods, such as calcium oxide (CaO) catalyzed oil transesterification (Gebremariam; Marchetti, 2018). Oils from the viscera of marine fish (sardine, mackerel, and pink perch), which have a higher degree of unsaturation (IV of 135.8 $cg.g^{-1}$) than rainbow trout, pacu and Curimbatá viscera oils (Table 1), are suitable for producing biodiesel with physicochemical characteristics in accordance with ASTM standards (Karkal; Kudre, 2022), signaling the suitability of fish viscera oil extracted by slow freezing method for biodiesel production.

4 Conclusion

The method of extracting the lipid fraction from freshwater fish viscera through slow freezing followed by heating allows for the obtention of a product with similar physicochemical characteristics to other crude fish oils. It is an alternative extraction method that results simple and practical, particularly on a small and medium scale, and eliminates the need for solvents, making it a cost-effective and environmentally friendly approach.

The oils from the viscera of the evaluated freshwater fish species can be considered alternative sources of important fatty acids, as although their concentration of n-3 LC PUFA is lower than that of traditional marine oils, it is higher than that of fats from terrestrial animals (poultry, pigs, cattle) and vegetable oils, which are widely used in animal feed.

The slow freezing extraction method allows for obtaining fish viscera oil with suitable physicochemical characteristics for biodiesel production, but pre-treatments are recommended, especially to reduce the acidity index.

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