Fatty Acid Composition, Extraction, Fractionation, and Stabilization of Bullfrog (*Rana catesbeiana*) Oil

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ABSTRACT: The oil extracted from the fat-storage organ (fat body) of the bullfrog (*Rana catesbeiana*) was characterized for its fatty acid composition. The main fatty acids were palmitic (18.1%), stearic (4.1%), myristic (2.7%), oleic (31.7%), and linoleic (12.9%) acids. Long-chain polyunsaturated fatty acids were also present in significant amounts, i.e., eicosapentaenoic (1.5%) and docosahexaenoic (4.7%), and were probably derived from the fish meal content of the diet. A partially fractionated oil was extracted from the homogenized and frozen fat body with an oleic acid content of 43.2%. The natural alkaloid boldine, added at 0.5 mg/g oil level, improved the oxidative stability by a factor ranging from 1.7 to 2.4, as assessed by the Oil Stability Index method between 90 and 110°C. The stabilization effect of boldine was higher than that of naringenin, morin, and quercitin and for the synthetic antioxidant butylated hydroxytoluene at the same concentration level.
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KEY WORDS: Antioxidants, boldine, bullfrog oil, extraction, fatty acid composition, flavonoids, fractionation, *Rana catesbeiana*, stabilization.

Frogs are vertebrate species widely distributed on the earth. During their early development stages, they live in an aquatic habitat until their lungs are developed and begin their final stage on land. The energy-storage organs in frogs are the liver and the fat body, in which glycogen and lipids are, respectively, deposited during the summer. The fat-body lipids are used during the prehibernating season and are mobilized to other body sites or transformed to other metabolites (1). Information on the fatty acid composition of frogs is rather scarce. Phospholipid fatty acid composition of *Rana esculenta* (2) livers and the fatty acid distribution pattern of the triacylglycerols of an unidentified frog species (3) have been reported. To our knowledge, no report on the fatty acid composition of the fat-body lipids has been reported.

Bullfrog (*R. catesbeiana*) rearing in Uruguay is focused to the production of frog legs for the food industry. From the remains of the slaughter procedure, the fat body can be easily removed. This organ represents *ca.* 2% of the frog weight, yielding approximately 3 g of oil per frog. The actual production of bullfrogs in Uruguay comprises about 1,200,000 frogs per year, which would represent approximately 3.6 tons of bullfrog oil. Although this amount would not be important in terms of oil production, its economic importance will depend on the possible uses of the oil.

In view of the possible commercial use of this new oil source, this work reports the fatty acid composition of the fat-body lipids of the bullfrog (*R. catesbeiana*) and the extraction, fractionation, and stabilization of bullfrog oil (BFO).

EXPERIMENTAL PROCEDURES

Samples. Bullfrogs were reared on a farm (Alesa; Canelones, Uruguay) up to their complete development (2 d as eggs, 90 d as tadpoles in water, and then 100 d as frogs on land). During the land-stage development, bullfrogs were fed with a diet comprised of 40% protein and 9% fish meal and soybean oil. After reproduction (summer), 300 adult specimens were slaughtered to obtain frog legs. Bullfrog fat bodies were manually removed and immediately frozen at −20°C. Within the next 24 h, frozen fat bodies were homogenized, placed in closed plastic tubes under nitrogen, and immersed in a water bath (98°C) for up to 1 h. The tubes were then centrifuged at $2{,}000 \times g$ for 30 min, and the supernatant oil was removed, dried over anhydrous sodium sulfate, and stored in glass vials under a nitrogen atmosphere at −20°C until analyzed.

Chemical and physical analysis. BFO was derivatized to fatty acid methyl esters by AOCS Method Ce 1b-89 (4). The methyl esters were analyzed in a fused-silica capillary column 30-m × 0.25-mm i.d. (0.25-µm film thickness), coated with OMEGAWAX-250 (Supelco, Bellefonte, PA) stationary phase, either isothermally (195°C) or temperature-programmed (194°C for 20 min, then 3°/min to 210°C, hold for 30 min, 20°/min to 240°C, hold for 30 min). A Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA) was used. Other operation parameters were: flame-ionization detector temperature 270°C, injection temperature 250°C, carrier gas hydrogen at a flow rate of 1 mL/min.

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Pure standards, semilogarithmic graphs of relative retention times (195°C) vs. carbon chainlengths, comparison of experimental equivalent chainlength (ECL) values to those reported in the literature (5), and fractional chainlength and separation factor type I, II, and III calculations were used for identification of the fatty acid methyl esters.

The water content of the fat body was determined by heating the homogenized sample in a vacuum oven at 40°C and reduced pressure to constant weight. The free fatty acid content was determined by IUPAC method 2.201 (6). Iodine and saponification values were determined according to AOCS procedures Cd 1c-85 and Cd 3-25, respectively (4). The cold test was conducted according to AOCS method Cc 11-53 (4).

Antioxidant solutions. The flavonoids quercitin (3,3′,4′,5,7-pentahydroxyflavone), morin (2′,3,4′,5,7-pentahydroxyflavone) and naringenin (4′,5,7-trihydroxyflavanone), the alkaloid boldine [(*S*)-2,9-dihydroxy-1,10-dimethoxyaporphine], and the synthetic antioxidant butylated hydroxytoluene (BHT) were used at a level of 0.5 mg/g oil each. Quercitin, morin, naringenin, and BHT were purchased from Aldrich (Milwaukee, WI). Boldine was extracted from the bark of *Peumus boldus* Mol. and crystallized from chloroform (7). Stock alcoholic solutions (5 mg/mL) of each antioxidant were stored at 4°C under nitrogen.

Oil stability index (OSI) analysis. A Rancimat model 679 (Metrohm, Switzerland) was used at 90, 100, and 110°C. BFO (2.5 ± 0.05 g) was placed in each tube, and 50 μL of the alcoholic solution of the antioxidant was added. To the control, 50 μL of ethyl alcohol was added.

The tubes were tested at the selected temperature at an air flow of 20 L/h. The induction periods (IP) were automatically evaluated by the apparatus. The starting conductivity values of the distilled water used was 2–4 $\mu S \cdot cm^{-1}$. After each run, the glassware was rigorously cleaned, according to the procedure described elsewhere (8). Results for each temperature are means of duplicate runs.

Data treatment and statistical analysis. The efficiency of each antioxidant was calculated as the ratio between the IP of the stabilized and pure BFO.

Correlations were performed by means of least square regression analysis. Linearity of the fitted lines was assessed by the *F*-test, and the validity of the coefficients was determined by the *T*-test (9). A significant level of 5% was considered in all data analyses.

RESULTS

A total of 62 fatty acids was identified; those above 0.5% are listed in Table 1. After 60 min heating, the saturated fatty acid content in the BFO was 27.4%, dominated by palmitic (16:0) acid with 18.1% and followed by stearic (18:0) and myristic (14:0) acids, with 4.1 and 2.7%, respectively. The monoethylenic fatty acids in the BFO were dominated largely by the ubiquitous oleic acid (18:1n-9) with 31.7%, followed by 8.0% palmitoleic acid (16:1n-7). Among the polyunsaturated fatty acids, linoleic (18:2n-6) acid was dominant with 12.9%.

TABLE 1
Fatty Acid Compositions of Bullfrog Oil and the Oil Extracted after Different Heating Times (percentage by weight)

	Composition of BFO extracted after different times of heating (min) at 98°C				
Fatty acids	0	5	15	30	60
14:0	1.9	2.8	2.7	2.7	2.7
15:0	0.5	0.6	0.5	0.5	0.5
16:0	17.5	17.6	18.1	18.0	18.1
18:0	5.1	3.9	4.1	4.1	4.1
16:1n-7	7.8	8.5	8.0	8.2	8.0
18:1n-9	43.2	35.9	34.7	34.3	31.7
20:1n-9	1.3	1.2	1.3	1.2	1.3
18:2n-6	8.1	12.6	12.9	12.7	12.9
18:3n-3	0.9	1.3	1.4	1.4	1.4
20:5n-3	1.0	1.3	1.5	1.5	1.5
22:5n-3	0.6	0.9	1.0	1.0	1.0
22:6n-3	3.6	3.7	4.7	4.5	4.7
ΣSAT[a]	25.0	24.9	25.4	25.3	27.4
ΣMUFA[a]	52.3	45.6	44.0	43.7	46.7
ΣPUFA[a]	14.2	19.8	21.5	21.1	24.3
Total reported[b]	91.5	90.3	90.9	90.1	87.9

[a]SAT, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]Includes fatty acids in percentages greater than 0.5%; BFO, bullfrog oil.

The more common n-6 and n-3 polyunsaturated fatty acids were also present. Generally, the n-3 isomer exceeded the n-6 isomer for a given fatty acid, as for 18:3, 18:4, and 22:5. For other minor polyunsaturated fatty acids, both isomers were present in equal amounts. The presence of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids was significant and accounted for 6.1% of the total fatty acids. The ratio n-6/n-3 fatty acids was 1.76, indicating the predominance of the n-6 isomers, mainly owing to the 18:2n-6 content.

The oil extracted from the homogenized and frozen fat bodies, heated for 30 min, had a iodine value of 98 and a saponification value of 192. The yield of extracted oil vs. time of heating in the water bath is represented in Figure 1 for the dry extraction procedure. The yields varied from 40.1% (no heating) to 61.7% (30-min heating). Data adjusted by nonlinear regression analysis gave a maximum stationary value (time = ∞) of 62.9%. The water content of the starting material was 19.7% (w/w), so this value corresponded to 78.3% yield in a dry basis. No change in the peroxide values nor in the anisidine values was found for the oils obtained with different heating times, although the acid values showed an increase of *ca*. 7% between the nonheated and the 30-min-heated samples. The residue after centrifugation was constituted of the remains of the tissue and a white layer over them, which decreased in thickness with the duration of heating.

In Table 2, the temperature of the heated mass prior to centrifugation and the results of the cold test of the extracted oils by the dry procedure are shown. None of the oils extracted at different heating times passed the cold test. The time needed to form a visible cloud under the cold-test conditions was recorded and is summarized in Table 2 as "cloud time." The results indicate that, when the homogenized and frozen fat

TABLE 2
Temperature of the Homogenized Bullfrog Fat Bodies After Heating in a Water Bath (98°C) for Different Times, and the Correspondent Cold Tests and "Cloud Times"[a] of the Extracted Oils[b]

Time of heating (min)	Temperature (°C)	Cold test	"Cloud time"
0	7 ± 2	Not pass	34 ± 1
5	67 ± 2	Not pass	5.0 ± 0.5
15	88 ± 1	Not pass	2.0 ± 0.5
30	91 ± 2	Not pass	2.0 ± 0.5

[a]"Cloud time" is defined as the time (min) needed for the oil to show a visible cloud under cold test conditions.
[b]Results are the means ± standard deviation (n = 4).

bodies were not heated, the "cloud time" increased more than 15 times compared to the heated fat bodies.

The effect of heating on the fatty acid composition of the oil extracted from the homogenized fat bodies was reflected in a decrease of monounsaturated fatty acids and an increase of polyunsaturated fatty acids; the saturated fatty acids remained constant (Table 1). Among the monounsaturated fatty acids, oleic acid showed a marked increase in the oil obtained without heating with respect to the other oils. In general, the main polyunsaturated fatty acids showed a decrease in the nonheated sample. The fatty acid compositions of the oils extracted after 15 and 30 min of heating were practically the same, indicating that a steady composition was reached in accordance with the yields represented in Figure 1.

The IP for the five antioxidants tested on BFO oxidation were compared to pure BFO at 90, 100, and 110°C (Table 3).

Among the flavonoids tested, naringenin did not show any stabilization effect on BFO oxidation at any of the three temperatures ($P < 0.05$). The stabilization order observed was quercitin > morin > naringenin, independently of the temperature tested.

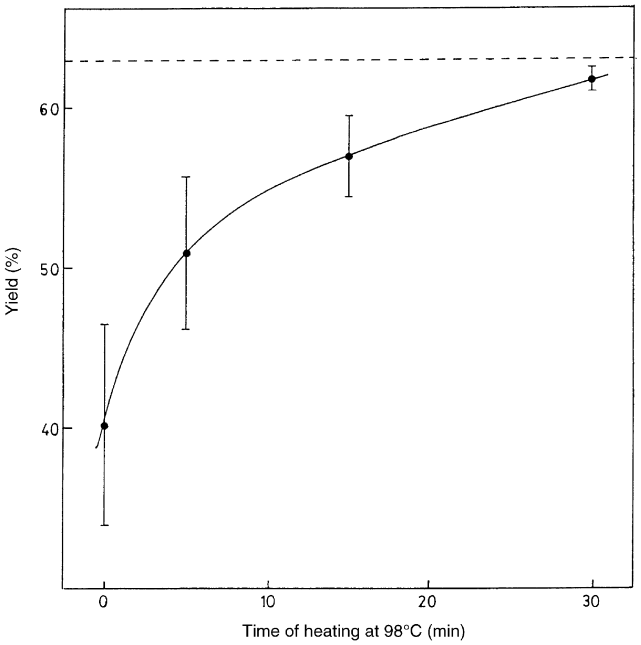

FIG. 1. Yield of extracted oil vs. time of heating in a water bath (98°C). The dashed line represents the theoretical maximum yield attainable by this procedure, obtained by nonlinear regression analysis (n = 4).

TABLE 3
Induction Periods (mean ± standard deviation) for Bullfrog Oil and Stabilized Bullfrog Oil with Several Antioxidants (0.5 mg/g oil) at 90, 100, and 110°C

	Induction period[a]		
Sample	90°C	100°C	110°C
BFO	7.0 ± 0.5[b]	3.4 ± 0.4[d]	1.7 ± 0.0[g]
BFO + naringenin	6.7 ± 0.1[b]	3.5 ± 0.2[d]	1.8 ± 0.1[g]
BFO + morin	8.9 ± 0.5[c]	4.4 ± 0.7[e]	2.3 ± 0.1[h]
BFO + quercitin	10.6 ± 0.1	5.3 ± 0.4[f]	2.9 ± 0.5[i]
BFO + BHT	9.4 ± 0.5[c]	4.3 ± 0.7[e]	2.4 ± 0.1[h]
BFO + boldine	16.6 ± 0.7	5.7 ± 0.8[f]	3.3 ± 0.5[i]

[a]Values with the same superscript in the same column did not differ significantly at $P < 0.05$. BHT, butylated hydroxytoluene. See Table 1 for other abbreviation.

BHT showed the same stabilization effect as morin at the three temperatures tested ($P < 0.05$). On the other hand, boldine showed the same antioxidant effect as quercitin at 100 and 110°C, but it was significantly higher at 90°C ($P < 0.05$).

A linear relationship was obtained between the logarithms of IP and the temperature for pure BFO and all stabilized oils, except for boldine ($P < 0.05$), with a mean temperature coefficient of 0.03 per °C (Table 4).

DISCUSSION

Fatty acid composition. Eleven of the 62 fatty acids identified were present in proportions greater than 1%, namely 14:0, 16:0, 18:0, 16:1n-7, 18:1n-9, 18:1n-7, 20:1n-9, 18:2n-6, 18:3n-3, 22:5n-3 and 22:6n-3, and accounted for 89% of the total fatty acids. These fatty acids may give an adequate picture of BFO, and they are enough to account for its physical properties.

The saturated fatty acid composition of BFO is typical of that found in other animal fats, in which even-chainlength fatty acids predominate. The monoethylenic fatty acids are dominated by 18:1n-9 and 16:1n-7, both derived from the Δ^9 unsaturation of the parent saturated compounds. Among the eicosenoids, 20:1n-9 was the isomer present in the highest amount.

Linoleic (18:2n-6) is the main diethylenic fatty acid and one of the main fatty acids in BFO, accounting for 12.9% of the total fatty acids. Its origin is from dietary sources because vertebrate

TABLE 4
Parameters of Linear Regression Analysis for the Dependence of the Logarithm of the Induction Periods on Temperature (range 90–110°C)

Sample	Slope (°C^{-1})	Intercept	T^a	F^b	r^c
BFO	–0.031 (0.000)	3.6	–106*	11163*	>0.9999
BFO + naringenin	–0.029 (0.000)	3.4	–99*	9747*	>0.9999
BFO + morin	–0.029 (0.001)	3.6	–25*	631*	0.9992
BFO + quercitin	–0.031 (0.000)	3.8	–1076*	1200000*	>0.9999
BFO + BHT	–0.030 (0.002)	3.7	–13*	169*	0.9971
BFO + boldine	–0.036 (0.007)	4.4	–5.3**	29**	0.9830

[a]Results are the average of two independent runs, and the standard deviations are in brackets. T-test ($P < 0.05$). See Tables 1 and 3 for abbreviations.
[b]F-test ($P < 0.05$).
[c]Correlation coefficient. *Significant at $P < 0.05$. **Not significant at $P < 0.05$.

animals are incapable of synthesizing it. This essential fatty acid is the precursor of the n-6 series, but the following fatty acids of this series are in proportions less than 0.5% each. Linolenic acid (18:3n-3), another essential fatty acid, also comes from dietary sources and accounts for 1.4% of the total fatty acids. This acid is the precursor of the n-3 series, and the following fatty acids of this series are also present in only minor proportions. The ratio n-6/n-3 was 1.76, a figure similar to that found in other land animals. The presence of 20:5n-3 and 22:6n-3 can be explained by the presence of fish meal in the frog diet (10).

In comparison to other animal fats and oils, BFO iodine and saponification values are higher than those of lard, pork, beef and mutton fats, and lower than those reported for freshwater and marine fish oils (11). The iodine and saponification values of BFO are closer to land animals than to marine animals.

Extraction and dry fractionation of BFO. Oils from fat-storage organs can easily be obtained by centrifugation, as reported for livers of the Southwest Atlantic hake (12). The different yields obtained with different heating times, together with the increase in the "cloud time" in the nonheated oil, suggest a partial fractionation due to temperature. Although the increase in the cloud point unequivocally indicates a thermal fractionation, the fatty acid compositions of the extracted oils (Table 1) are difficult to interpret. The most notorious change due to heating is the increase in the proportion of myristic (14:0) and the polyunsaturated fatty acids, and the decrease of stearic (18:0) and especially oleic (18:1n-9) acids. The unexpected increase in the polyunsaturated fatty acid proportion of the heated oils seems to be in contradiction with the lower "cloud time." A possible interpretation of these changes in the composition could lie in the fatty acid distribution pattern of the triacylglycerols, as was reported for a nonidentified frog species (3). Palmitic (16:0) and palmitoleic (16:1) acids are concentrated in the *sn*-1 position, while stearic (18:0) is concentrated at both the *sn*-1 and *sn*-2 positions. Linoleic (18:2) and linolenic (18:3) acids are preferentially attached to the *sn*-2 position, and oleic acid (18:1) seems to be equally distributed among the three positions. The calculated fatty acid composition from these positional distribution data is rather similar to our oil for the main components, with the exception of linolenic acid. Because the bullfrogs used in this study were subjected to a controlled diet during their development, and the oil was extracted from the fat-storage organ, the 1,2,3-random distribution hypothesis of the fatty acids in the triacylglycerol molecules is suitable (13). Based on this, the calculated mole percentage of triolein in the BFO would be 15.6%, a relatively high figure due to the even distribution of oleic acid in the triacylglycerols. Triolein has a melting point of –5°C; so, as a first approximation, it would be melted at the temperature of the extraction in the nonheated oil. Because it is the main triacylglycerol molecule, it would serve as a vehicle for solubilization of other triacylglycerols, especially those of the same carbon number. In other words, mutual solubility effects would be present and may explain the fatty acid composition obtained for the nonheated oil.

Stabilization of BFO with natural substances. Among the flavonoids, results presented here demonstrate an antioxidant

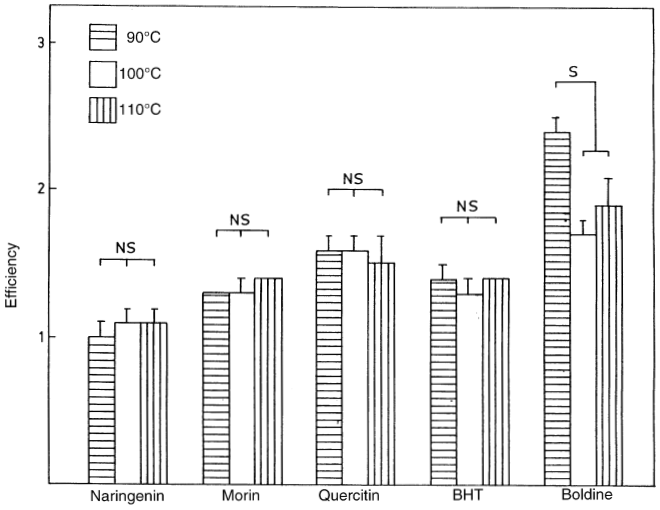

FIG. 2. Efficiency, measured as the ratio of the induction periods of stabilized bullfrog oil and the oil with no antioxidant added, for the different antioxidants assayed at 90, 100, and 110°C. NS, not significant ($P < 0.05$), *S*; significant ($P < 0.05$). BHT, butylated hydroxytoluene.

effect of morin and quercitin on BFO oxidation. Similar results have been obtained for these flavonoids on thermal autoxidation of refined and deodorized palm oil (14) and for spontaneous (15,16) and Fe^{2+}-induced oxidation of sardine oil (16). Naringenin did not show any antioxidative effects, which is in accordance to previous reports that showed little antioxidant effect for this flavonoid on different experimental models (14,17).

Boldine showed the highest antioxidative effect among the antioxidants tested. Similar results were observed on spontaneous (15,18) and Fe^{2+}-induced oxidation of sardine oil (18) and biological membranes (19).

BHT, a synthetic antioxidant widely used in the fats and oils industry, showed an intermediate antioxidative effect, similar to that of morin ($P < 0.05$), and less than that of quercetin and boldine ($P < 0.05$).

The relative efficiency of the different antioxidants tested is graphically shown in Figure 2. For the flavonoids and for BHT, no significant differences were observed at each temperature tested ($P < 0.05$), but boldine showed a different efficiency depending on the temperature.

BFO with all antioxidants tested except boldine showed a linear relationship for the dependence of log (IP) on temperature (Table 4). The slopes obtained for all antioxidants except boldine are within the range obtained for pure (20) and stabilized vegetable oils (21), with a mean value of 0.03 per °C, but they showed significant differences within them ($P < 0.05$). The values of the intercepts represent the logarithms of IP at 0°C and could be taken as a relative stability criterion (8). Interestingly, extrapolation of the IP values at 0°C showed that naringenin would have a prooxidant effect, morin would have no net effect, and only BHT and quercitin would show an antioxidant effect (boldine was not considered because of its deviation from linearity, but according to the tendency obtained,

it would show an enhanced antioxidant effect). However, these conclusions should be taken with caution because they were not obtained in the temperature range studied.

The results of this work showed that boldine is an effective antioxidant agent against BFO oxidation. Previous works have shown its efficiency in protecting highly unsaturated lipid systems (15,18,19), and this work showed that it is also effective in protecting more saturated oils. The difference obtained in the antioxidative efficiency at different temperatures for boldine may be due to a different mechanism of action of this alkaloid, probably in the scavenging action on different radicals. Hydroxyl radicals are known to be highly reactive, contrary to superoxide radicals (22). While flavonoids are equally efficient in scavenging hydroxyl and superoxide free radicals, boldine is only efficient in scavenging the more reactive hydroxyl free radicals (7). Thus, for the same concentration of antioxidant, boldine would be more effective in scavenging highly reactive radicals than the flavonoids assayed. Besides, it has also been suggested that boldine may interact with the peroxyl radicals, producing a boldine-derived substance that may be more active than the parent compound (7). Any of these hypotheses may explain the higher antioxidative activity of boldine.

In addition, the slight differences obtained in the temperature coefficients would indicate different antioxidative effects when extrapolating experimental data to the ambient or subambient temperatures at which the oil will be stored.

Possible uses of BFO. The results of this work show the chemical characterization of BFO and extraction, fractionation, and stabilization of BFO on a laboratory scale. The simultaneous extraction–fractionation procedure has obvious advantages for possible industrial production of BFO. Usually, crude oil is fractionated in a separate process during oil refining. If the procedure reported in this work can be scaled up, it would be possible to obtain an oil partially fractionated in a single step, thus minimizing operational costs. Taking in account some possible use of this oil, such as a vehicle for liposoluble vitamins or as an ingredient in cosmetic products, the characteristics of the oil extracted would be adequate in a first approximation. Further research is needed to ascertain possible uses of this new oil source.

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