Journal of Food Quality

Lipids and Food Quality

Lead Guest Editor: Domenico Montesano
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Editorial Board

Contents

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Editorial
Lipids and Food Quality

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Lipids represent a major component of food and important structural and functional constituents of cells in biological systems. The aim of this special issue is to provide a representation of the new analytical and qualitative aspects about food lipids. The main focus is on the state of the art in the various areas covered, with an indication of the current developments taking place and the problems and challenges that remain to be addressed. With this editorial, we launch a series of papers presenting new analytical methodology, health properties, and food safety related to lipids. The papers were selected through routine rigorous double-blind external peer review by qualified experts.

Lipids contribute to many desirable qualities to foods, including attributes of texture, structure, mouthfeel, flavour, and colour. Generally, the quality of food is closely related to the quality of their lipids. Very often, the degradability and alteration of lipids are the main causes of the loss of quality of food. This is why several researchers have investigated the lipid fraction of food not only to provide new knowledge about their composition but also to assess whether it was possible inhibiting or slowing down alteration processes or modifying the native composition by fortifying foods with some lipid classes with health properties. A very important topic is presented by the study of the composition of lipid fraction present in foods, in particular the ratio ω-3/ω-6, the % of saturated fatty acids (SFA), and the occurrence of *trans* fatty acids. In fact, wrong ω-3/ω-6 ratios, high quantity of SFA, and *trans* fats are associated with cardiovascular diseases and other undesirable health effects. Therefore, many health authorities, such as EFSA or FDA, have recommended the use of healthy foods without *trans* fatty acids and a lower consumption of saturated fatty acids. Currently, many researchers consider healthy a diet with a lipid fraction rich in ω-3 fatty acids and conjugated linoleic acid (CLA) due to their beneficial effects such as cardiovascular and anti-inflammatory ones. In this special issue, J. Arias-Rico et al. reviewed the possible health effects of ω-3 supplementation in poultry products. This phase 1 study was performed on 29 volunteers of whom fourteen participants (9 women and 5 men) consumed chicken and eggs supplemented with ω-3 fatty acids and fifteen participants (8 women and 7 men) consumed chicken and eggs non-supplemented with ω-3 fatty acids. Both groups participated for a period of 14 weeks. After 14 weeks, the supplemented group had an increase in HDL, reducing the atherogenic index. Generally, high intake of *trans* fat is closely related with chronic diseases such as cardiovascular disease and cancer. In consideration of this, Buitimea-Cantúa et al. investigated fat blends, produced by direct blending process of palm stearin (PS) with high oleic sunflower oil (HOSO) in different concentrations, concluding that the direct blending process of equal amounts of PS and HOSO was an adequate strategy to formulate a new zero-trans crystallized vegetable fats with characteristics similar to commercial counterparts with well-balanced fats rich in both ω-3 and ω-6 fatty acids. To date, many foods rich in ω-3 and ω-6 are known, but fish

are certainly the first source of these fatty acids. Generally, marine organisms bring lipids with a high nutritional value, rich in polyunsaturated fatty acids and with low levels of saturated ones and cholesterol. Pyz-Aukasik et al. determined fatty acid profile regarding the fat of farmed grass carp, bighead carp, Siberian sturgeon, and wels catfish. The total content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in 100 g of muscle tissue of the examined fish was very high particularly for bighead carp (488.67 mg) and Siberian sturgeon (619.06 mg). The ratios of ω-6/ω-3 in all the fish analyzed were always high or very high, between 0.44 and 1.72, and, similarly, the ratio PUFA/SFA was always very interesting (0.45–1.61). For these reasons, the authors concluded that the lipid fraction of the analyzed fish can be considered beneficial for human health. Another important topic concerning the study of food lipids is represented by extraction methods. The recovery and the quality of the lipid fraction from foods depend on the method used for its extraction; therefore, nowadays, the setting of the optimal extraction parameters is the focus of scientific research in the field of foods. Rosales-Garcia et al. performed the extraction of squalene from puffed *Amaranthus hypochondriacus* seeds by supercritical fluid extraction (SCFE). The authors determined squalene content and carried out the acidic profile of the extracts by GC-MS. The extract obtained by SCFE reached 460 g/kg of squalene in oily extract with the optimized parameters, confirming that this matrix is a rich source of squalene, a potent natural antioxidant. Heat treatment such as roasting and frying are two of the most common methods used industrially, and not only, to improve the sensory properties of many foods. However, these treatments are not always harmless; in fact, some nutritional alteration might be unfavorable and lead to negative health impacts upon consumption. Ghazzawi and Al-Ismail evaluate the effects of frying and roasting on different nuts (raw almonds, pine, cashew, and pistachio). The authors took into consideration the modifications, after the heat treatment, of the total phenol content, total flavonoids, oxidative stability, and of the acidic profile of the nuts' fat. In conclusion, the effects of roasting and frying have significantly influenced the fatty acid profile and the antioxidant activity of the matrices considered, and therefore, this work highlights the need to improve the knowledge on foods subjected to heat treatment. Sommella et al. determined the qualitative and quantitative variation of single fatty acids in cow milk samples before expiration date and within 28 days after expiration date in order to monitor how the profile of the lipid fraction is influenced by different physicochemical parameters. These authors concluded that, even after the expiration date, this matrix represents a rich source of fatty acids with potential substrates for the formulation of economically viable products and eco-friendly diesel-like fuels.

In light of these findings, all the papers published in this special issue represent exciting, innovative, and applicable approaches in the study of food safety and lipids quality, as well as emerging future research topics, in this multidisciplinary field. We hope that this special issue would attract major attention of the peers.

Acknowledgments

We are immensely grateful to the Editorial Board not only for the opportunity to edit this special issue but also for their constant help, suggestions, and guidance. With great pleasure, we extend our sincerest thanks to all the contributors for their excellent updated contributions. We would like to express our deep gratitude to all the scientific colleagues who helped us immensely by providing their valuable time to review these manuscripts. Finally, special thanks also go to all the editorial team.

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Research Article

Investigating Enzyme Activity of Immobilized *Candida rugosa* Lipase

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Candida rugosa lipase is a food-grade enzyme that is extensively utilized in the dairy processing industry for milk fat hydrolysis. The enzyme is mainly employed to modify the fatty acid chain length that results in the enhancement of flavors. The hydrolytic activities of *C. rugosa* lipase (fungal source) in its free and immobilized forms were investigated at different pH and temperature settings. The main objective of this study was to understand how different support materials (Celite-545, Sephadex G-25, and chitosan) and immobilization techniques alter lipase activity and stability. Our results indicated that hydrolytic activity increased significantly with immobilization on Celite-545. In general, immobilization resulted in considerable improvements in the stability of the enzyme with variations in pH and temperature. Immobilization on Celite-545 led to the highest catalytic efficiency. Remarkable improvements in the recovery and reusability of the immobilized lipases were noted. Comparatively, the acetone immobilization procedure resulted in higher activities than alcohol immobilization. In conclusion, the activity of *C. rugosa* lipase was enhanced most significantly when immobilized on Celite-545 using acetone as an adsorption solvent.

1. Introduction

During the last two decades, the use of lipases in the food industry has increased due to the increased attention in the products that are more natural. The major application of lipases in food industries involves biocatalysis of lipids especially fats and oils. They are mainly used for enhancing the flavor components during the production process of various products such as cheese, butter, salad dressings, sauces, and soups. The potential of lipases to work on a broad spectrum of substrates and their stability when subjected to wide ranges of pH, temperature, and organic solvents are chief reasons for their use since these properties support catalysis with lowered side reactions and reduce costs for waste treatments. Also, food flavor components synthesized with solvent-free techniques have superiority (compounds with no solvent impurities) for being safe in making delicious foods. Moreover, lipase-mediated reactions involving ester interchange can be utilized for attaining appropriate flavor esters [1].

C. rugosa lipase is a lipolytic food-grade enzyme that is synthesized using the controlled fermentation technique. This enzyme catalyzes the hydrolysis reaction of triglycerides, resulting in the production of mono- and diglycerides, glycerol, and free fatty acids. Lipase from *C. rugosa* is distinctive in its reactivity where it displays no positional specificity and results in effectual fatty acid hydrolysis from all three positions in the triglyceride. Lipase is also utilized for catalyzing esterification and acidolysis reactions (transesterification reaction) that result in the production of fatty acid esters and structured lipids, respectively. This lipase shows wide substrate specificity on the fats and oils of both vegetable and animal origins. *C. rugosa* lipase aids in the flavor development in Italian cheese, ripening

acceleration in Cheddar cheese, flavor improvement in processing blue cheese, and enhancing creamy flavor in butter oil. The enzyme principally results in the synthesis of short-chain fatty acids (C_4 and C_6) which are the prime components for tangy sharp flavor [2].

Reactions involving *C. rugosa* lipases are more environment-affable relative to some bulk chemical syntheses since the operation of lipase-catalyzed reactions nearly resembles that of natural metabolic pathways. Lower activation energies of lipase-mediated processes allow them to convert their reactants to end products by utilizing mild temperature and pH conditions, thus resulting in lower energy consumption. The enzyme is also stable in organic solvents and does not require any cofactors.

Despite its beneficial and productive uses, the employment of *C. rugosa* lipases for commercial industrial purposes faces some challenges. The harsh conditions of industrial processes usually lead to destabilization, thereby shortening its industrial lifespan. Other drawbacks include sensitivity to process controls, low solidity, and the tendency to be constrained by high concentrations of reactants [3]. Krajewska [4] stated that the strenuous recovery and reuse of an enzyme is also a challenging task since the powdered form of enzyme can form an emulsion, which results in significant loss, with their recovery from the reaction system being very low [5].

In addition, the interactions between amino acid residues in the core of a protein's sequence are not fully optimized and as such only attain the minimal prerequisites for their required functioning [6]. This suggests the need for methods that can improve the functionality of the enzyme. One such method is enzyme immobilization. Immobilization improves the stability and ease of enzyme recovery after a reaction and thus contributes to reduced production cost since the enzyme can be reused [7]. Although the technique of enzyme immobilization has become widespread, some commercial lipase enzyme products still exist in the free form—not immobilized [8, 9]. The immobilization has the potential for improving the activity, specificity, and stability of lipases as well as the ease of separating the enzyme from reaction products. In addition, the reusability of the immobilized enzyme is bound to reduce operational cost [10].

The properties of the support material matrix are predominant attributes that control the efficaciousness of the immobilization process. The matrix materials can modify the mechanism and the partitioning of the reactant and product components that are present in the reaction mixture and, because of which, they would influence the chemical properties, stability, and the activity of the enzyme. Three support materials were used in the study: Celite 545, Sephadex G-25, and chitosan.

Celite 545, also known as diatomaceous earth, is a naturally occurring rocky substance. It exists in the earth as a soft siliceous rock that can be effortlessly disintegrated into fine powder. Generally, the color of the material ranges from off-white to whitish. Due to its high porosity, the density of the material is very low. Its characteristic chemical composition includes silica (80–90%), alumina (2–4%), and iron oxide (0.5–2%) along with some fossil remains of diatoms, which are a type of algae. The material is hydrophobic in nature. Sephadex is a beaded material that is utilized for gel filtration techniques. It is synthesized by crosslinking dextran (a polysaccharide) with epichlorohydrin. The degree and the extent of the crosslinking reaction can be controlled, resulting in variations in the degree of polymer gelation. Hence, five different types are available based on their sizes, with G-10 consisting of smaller molecules and G-75 of the larger ones. The research was studied on Sephadex G-25 which is relatively smaller of all types. This support material is hydrophobic and generally available in three different particle sizes: fine, medium, and coarse. Chitosan is a carbohydrate molecule made of D-glucosamine and *N*-acetyl glucosamine linked through β-1,4 linkages. It is a hydrophilic substance that is available in fine powder form. These support materials were chosen in order to understand the *C. rugosa* lipase activity when immobilized on both hydrophilic and hydrophobic support materials. The research was performed on the basis of the previous study [11], in which the LIP1 enzyme which is an isoform of *C. rugosa* lipase was studied, when immobilized on the two hydrophobic supports Celite and Sephadex.

The main objective of this study was to immobilize *C. rugosa* lipase, a commercially available enzyme, on different support materials by using physical adsorption techniques. The supports materials were Celite 545, Sephadex G-25, and chitosan. Acetone and alcohol immobilizations were utilized for physical adsorption of the lipase to the support materials [12]. The effects of pH and temperature variations on the hydrolytic activity of the immobilized enzymes were examined. The variations exhibited by immobilized lipases in the esterification and acidolysis reactions were investigated as was the reusability of the immobilized lipases.

2. Materials and Methods

2.1. Materials. *Candida rugosa* lipase, Celite 545, Sephadex G-25 (medium), chitosan (low molecular weight), glycerol, caprylic acid, and sodium hydroxide (purity > 98%) were purchased from Sigma Aldrich (St. Louis, MO). Sodium phosphate was obtained from Fisher Scientific. Extra virgin olive oil (Filippo Berio) was purchased from a local grocery store. All organic solvents (technical grade) were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Immobilization Procedure

2.2.1. Ethanol Immobilization. Twelve grams of support materials was first mixed with 20 mL of ethanol for 18 hours at static condition and room temperature (20°C). Wet supports were then mixed with a 60 mL of sodium phosphate buffer (pH 7) and 3 g of the enzyme, keeping them in a shaking water bath at 30°C and 120 rpm for 12 hours. The immobilized lipase on the support was then filtered under vacuum, dried at 35°C–40°C for 72 hours, and stored at 4°C [13].

2.2.2. Acetone Immobilization. The lipase enzyme was immobilized on three different carriers (Celite 545, Sephadex G-25, and chitosan) using the acetone adsorption technique described by Wang et al. [14] and Lumor and Akoh [11].

Briefly, 3 g of the enzyme was dissolved in 60 mL of 10 mM sodium phosphate buffer (pH 6). The resulting solution was mixed with 12 g of the carrier. Then, 240 mL of cold acetone (−20°C) was added. The mixture was stirred for 30 min at room temperature (20°C) before it was filtered by suction, washed with 60 mL of cold acetone (10°C), and dried in an oven (25°C) for 72 hours. The powdered form was stored at 4°C.

2.3. Method of Analysis

2.3.1. Protein Content. The protein content was estimated using the FlashEA Nitrogen/Protein Analyzer (CE Elantech, Inc., Lakewood, NY) at USDA-ERRC, Wyndmoor, PA. After obtaining the percentage of nitrogen in the sample, a protein factor (6.25) was used to calculate the percentage of protein [15]. The samples to be analyzed were weighed in tin plates which were loaded in the analyzer along with a blank tin plate. The machine was calibrated with three different weights of aspartic acid. The samples were then heated, using a dynamic flash combustion technique which is a modified Dumas test that converts the samples to elemental gases. The percentage of nitrogen was then estimated by a detector.

2.3.2. Hydrolytic Activity. The hydrolytic activity of the lipase enzyme was assayed by preparing 200 mL of 10 mM sodium phosphate buffer of varying pH with 15% w/v of the olive oil emulsion. Gum arabic was added to the mixture at 5% w/v to act as an emulsifier. About 10 mL of the resulting emulsion was then incubated with 500 mg of each lipase at varying temperatures for 1 hour. After incubation, the products of reaction were titrated against 0.5 M NaOH with 1% phenolphthalein as an indicator. The amount of fatty acid released was estimated by calculating the difference in the volumes of titration (titer values) between samples and the blank. The time course analysis was also performed. All reactions were performed in triplicates. The activity was calculated using the following formula:

$$\text{specific hydrolytic activity} = \frac{(V * M)}{W * t}, \quad (1)$$

where V is the difference in the titer values between the blank and samples, M is the molarity, and W is the weight of lipase. The specific activity of the lipase was calculated by estimating the ratio of hydrolytic activity to the amount of protein.

2.3.3. Esterification Activity. Glycerol (1 g) and caprylic acid (1.27 g $C_8H_{16}O_2$) were chosen as reactants which were incubated with free lipase and its immobilized forms at 10% w/w. The reaction was carried out for 12 hours in screw-cap test tubes in an orbital shaking water bath at 40°C with a speed of 200 rpm. In order to pace up the reaction rate, the water produced in the reaction was absorbed by the addition of a total of 30% (w/w) of molecular sieves (4 Å in diameter, 8–12 mesh). The reaction was halted by the addition of 1 mL of methanol. After the reaction, the products from the screw-cap test tubes were titrated against 1.0 M sodium hydroxide with 1% phenolphthalein as an indicator. All reactions were performed in triplicates. Esterification activity is defined as the amount of enzyme that is consumed per minute per milligram of lipase in the reaction. The esterification activity was calculated using the following formula:

$$\text{specific esterification activity} = \frac{(V * M)}{W * t}, \quad (2)$$

where V is the difference in the titer values between the blank and samples, M is the molarity, and W is the weight of lipase.

2.3.4. Acidolysis Reaction. Structured lipids were produced in triplicates by an acidolysis reaction. One gram of olive oil and 0.3 g of caprylic acid were combined in screw-cap test tubes prior to adding lipase enzymes at 10% by weight of total reactants and placing them in an orbital shaking water bath at 45°C and 200 rpm for 12 hours. Thin-layer chromatography (Fisher Scientific, Pittsburgh, PA) separated the triacylglycerol bands. The bands corresponding to TAGs were scrapped off and modified to fatty acid methyl esters (FAMEs) using the boron trifluoride (BF_3) method: 2 mL of 0.5 M NaOH in methanol was added to the TAG bands at 90°C in screw-cap test tubes for 10 min followed by the addition of 14% BF_3 in methanol for another 10 min. FAMEs were extracted with 2 mL of hexane and then analyzed in parallel with a FAME standard (Supelco 37 component FAME Mix; Supelco, Bellefonte, PA), using the Agilent Technology 6890N Gas Chromatograph (Agilent Technologies, Inc., Wilmington, DE). The amount of caprylic acid that is incorporated after the reaction was estimated by analyzing different amounts of FAMEs which were integrated by an online computer. The area of the sample peak and the internal standard peak in the gas chromatogram were used to calculate the amount of fatty acids. The ratio of the incorporated amount of caprylic acid to the total amount of fatty acids present in the olive oil was established to determine the percentage of incorporated caprylic acid.

3. Results and Discussion

3.1. Protein Content. The percentage of protein in the lipase-immobilized support materials is an indication of the degree of immobilization achieved in our study. The percentage of protein (Table 1) found in the free form of *C. rugosa* lipase was 8.17%, which is consistent with the findings of Gitlesen et al. [16] and Ozturk [2] who reported 9.2% and 8.67% of protein, respectively. Based on our results (Table 1), the protein content of the samples was dependent on the type of the support material and the method of immobilization. Acetone-immobilized samples yielded a higher protein content than alcohol-immobilized samples, which could be due to the difference in solvent polarity. Differentiation on the basis of the support material used revealed that lipase immobilized on Celite-545 resulted in the highest amount of protein, whereas lipase immobilized on chitosan resulted in the least amount of protein, with lipase immobilized on Sephadex G-25 yielding an intermediate amount. The very low protein content of the lipase immobilized on chitosan may have been due to chitosan's hydrophilic nature. Lipase,

Table 1: Percentage of protein after lipase immobilization on different support materials.

Enzyme	Percentage of protein
Free enzyme	8.17
Celite-acetone	7.18
Sephadex-acetone	5.35
Chitosan-acetone	2.04
Celite-alcohol	6.04
Sephadex-alcohol	4.83
Chitosan-alcohol	1.41

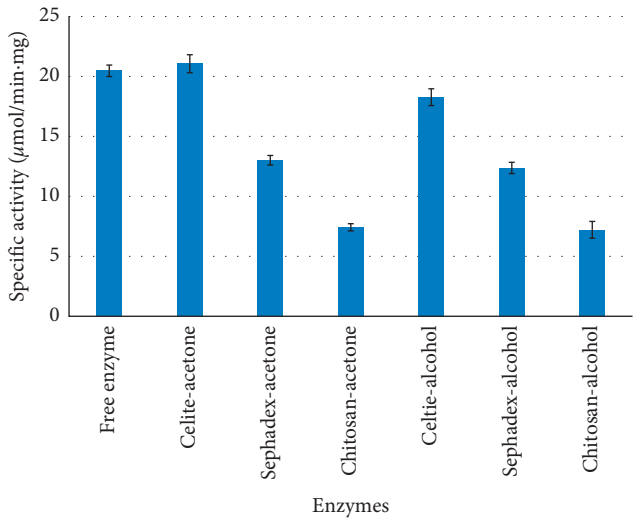

Figure 1: Graphical representation of specific hydrolytic activities of all enzymes (pH 7.0, temperature 45°C, and [S] = 1 mg/mL).

being hydrophobic in nature, binds readily to hydrophobic support materials such as Celite or Sephadex. Therefore, we concluded that the applications of acetone and Celite-545 were the most suitable for the immobilization process.

3.2. Hydrolytic Activity. The hydrolytic activity of lipases was calculated by titrating the reaction products with 0.5 M NaOH solution to determine the amount of fatty acids released. The specific hydrolytic activities of Celite-immobilized lipase samples were significantly higher than those of other immobilized support materials or even the free enzyme (Figure 1). Lipase immobilized on Sephadex G-25 demonstrated a slightly increased specific activity compared to the free enzyme. Various authors suggested that the immobilization of lipases with appropriate support materials has proven to enhance the catalytic properties [17]. According to Patrick [17], physical adsorption on matrix materials not only improves the stability of enzymes but also promotes the catalytic efficiency by improving the interfacial specificities and substrate binding ability.

Comparing the hydrolytic activity of the different immobilization methods, acetone immobilization resulted in higher specific activities on all immobilized supports than alcohol immobilization. The specific activities of alcohol-immobilized samples did not differ significantly from the free enzyme activities. Wu et al. [18] studied the effect of different polar solvent treatments during immobilization of lipases from three microbial sources, namely, *C. rugosa*, *Mucor javanicus*, and *Rhizopus oryzae*, on support materials. They were immobilized on Amberlite support materials by physical adsorption using different polar solvents that included acetone and ethanol along with 17 other organic solvents. They also found that acetone-immobilized samples demonstrated the highest activities among all other solvents, while ethanol-immobilized samples showed significantly lower values. This could be due to the lower polarity of acetone when compared to ethanol and suggests that acetone is a better solvent for physical immobilization of support materials.

3.3. Effect of pH on Hydrolytic Activity. The surrounding microenvironment can control enzyme activity. The pH of a solution plays a pivotal role in achieving an optimum amount of fatty acids during the hydrolysis reaction because the tertiary protein structure is controlled by hydrogen bonding interactions among the R groups of amino acids. The ionization of these R groups can be altered by a small change in the pH value, disrupting the original native conformation, thereby resulting in the loss of enzymatic activity. The product inhibition becomes significant, causing a decrease in the reaction rate. Hence, every enzyme has a favorable pH range that preserves the native structural conformation and therefore its activity [19]. Generally, immobilization modifies the optimal pH of the enzyme, which may be beneficial.

In this study, the differences in hydrolytic activity of all enzymes as a function of pH were investigated. As plotted in Figures 2 and 3, *C. rugosa* lipase in its free form was less active at both lower (acidic) and higher (basic) pH values, potentially due to the structural changes caused by the variation in pH. According to Akova and Ustun [20], the cysteine amino acid residues of the enzyme can be moderately damaged due to β-elimination in alkaline solutions, whereas in the acidic solutions, the easily breakable peptide bonds that are adjacent to the aspartic acid residues are hydrolyzed. Montero et al. [21] reported a higher probability of protein aggregation in an acidic pH, specifically higher molecular weight proteins because of their hydrophobic nature. The enzyme in its free form was most active at pH 7.0, as also reported by Fadiloglu and Söylemez [22] and Ozturk [2]. For enzyme immobilization on Celite, the highest hydrolytic activity was observed at pH 7.0 for both acetone-and alcohol-immobilized enzymes, respectively. The optimal pH levels for lipases immobilized on Sephadex with acetone and alcohol were 6.5 and 6.0, respectively, and the optimal pH for lipase immobilized on chitosan were 7 and 6.5, respectively. Overall, it can be concluded that the activity of enzyme immobilized on Celite by acetone immobilization was comparatively stable at acidic and alkaline pH. This gives us an advantage of utilizing the enzyme for various reactions that may require varied pH conditions.

3.4. Effect of Temperature on Hydrolytic Activity. Temperature affects enzyme activity in two different ways. The mobility

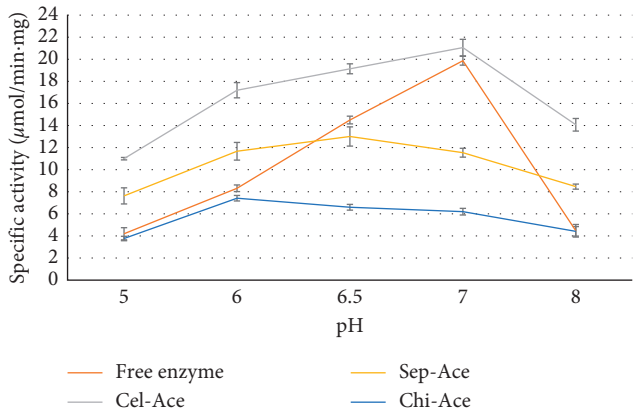

Figure 2: Specific hydrolytic activity of acetone-immobilized samples with varying pH values (temperature 45°C and [S] = 1 mg/mL).

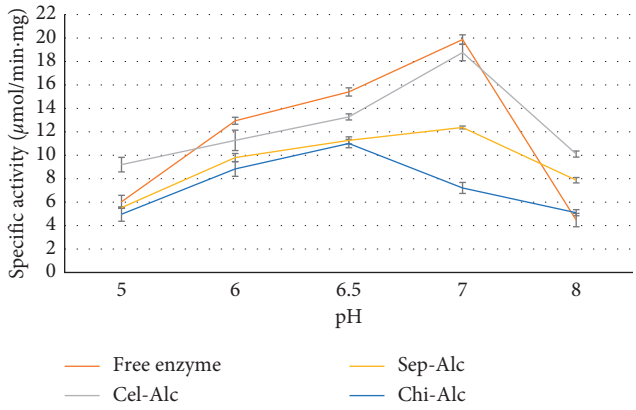

Figure 3: Specific hydrolytic activity of alcohol-immobilized samples with varying pH values (temperature 45°C and [S] = 1 mg/mL).

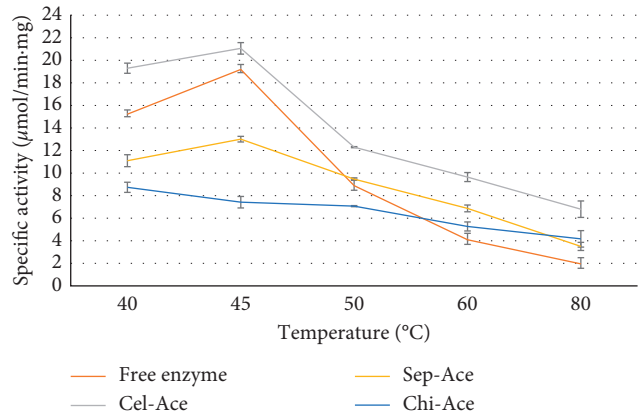

Figure 4: Specific hydrolytic activity of acetone-immobilized samples with varying temperatures (pH 7.0 and [S] = 1 mg/mL).

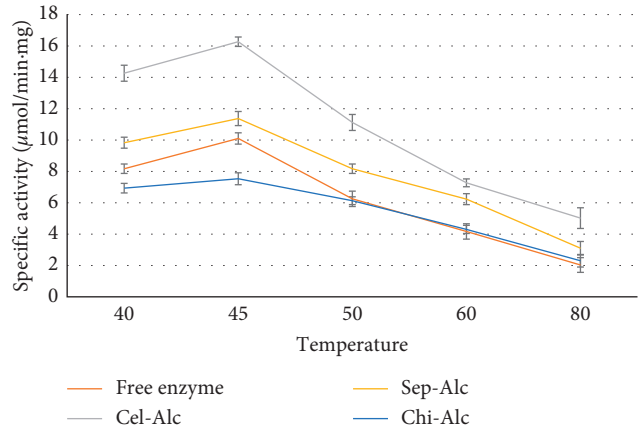

Figure 5: Specific hydrolytic activity of alcohol-immobilized samples with varying temperatures (pH 7.0 and [S] = 1 mg/mL).

of the substrate reactants increases when raising the temperature, but this substantial effect on the catalytic rate is limited as temperature-dependent enzyme denaturation becomes more significant. At an enzyme-specific temperature, an even small increase in the temperature can lead to so much denaturation that the loss of enzyme activity results in decreased product formations.

In this study, the specific hydrolytic activity of all enzymes was determined at different temperatures ranging from 40°C to 80°C. Except for lipase immobilized on chitosan using acetone adsorption, all lipases demonstrated the highest activity at 45°C (Figures 4 and 5), suggesting that immobilized enzymes were significantly more active at higher temperatures than the free enzyme. Unimmobilized *C. rugosa* lipase lost 80% of its activity at 80°C. Similar to unimmobilized C. rugosa lipase, around 72% of the specific hydrolytic activity was lost by both Sephadex-immobilized lipases at 80°C. The immobilization on Sephadex conferred less thermal stability to the lipase than immobilization on chitosan, which showed a 53% reduction in enzymatic activity at 80°C, and on Celite with approximately 60% reduction. Although immobilization on chitosan showed little activity, it conferred significant thermal stability to the lipase.

3.5. Timeline and Kinetics of Hydrolytic Activity. The rate at which an enzyme catalyzes a reaction is crucial to its progress. Every enzyme has its own rate phenomena in the conversion of substrates into products. Enzyme kinetics explains the catalytic behavior of enzymes. In this study, the effect of support materials on the rate of enzyme catalysis was measured through a time-course study of the hydrolysis reaction.

The hydrolytic activities of free and immobilized lipase enzymes were analyzed over an extended period of time until 16 hours to determine the progress of catalysis. As presented in Figures 6 and 7, the amount of fatty acids produced by all the samples increased with the reaction time. At a constant temperature and pH, changing the substrate concentration resulted in varying amounts of fatty acids. Two different substrate concentrations were chosen to explain enzyme kinetics, using the Michaelis–Menten equation. With two values for initial velocities and substrate concentrations, $V_{\max}$ and K_m values of the reactions could be established (Figure 8).

The Michaelis–Menten equation determines the rate of reaction:

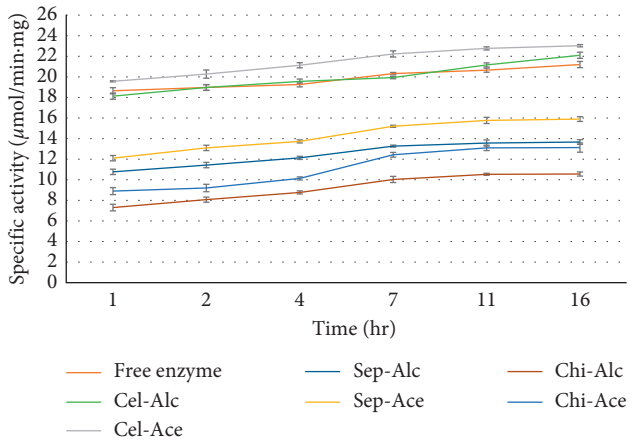

Figure 6: Timeline of hydrolysis reaction at [S] = 1 mg/mL (pH 7.0 and temperature 45°C).

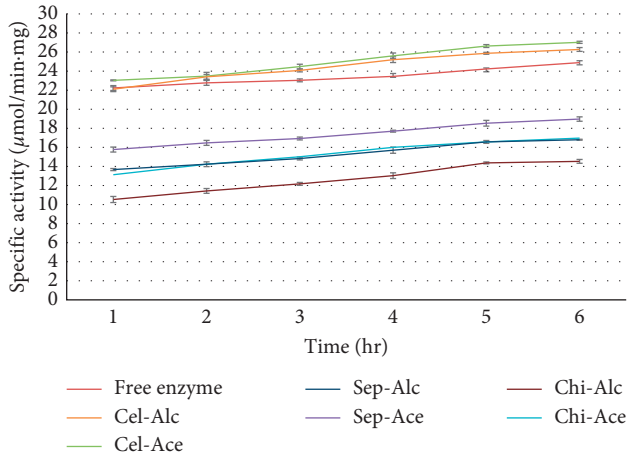

Figure 7: Timeline of hydrolysis reaction at [S] = 2 mg/mL (pH 7.0 and temperature 45°C).

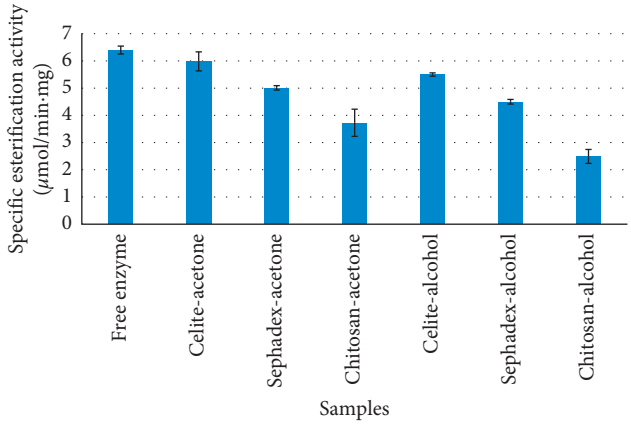

Figure 8: Specific esterification activities of all enzymes (pH 7.0, temperature 40°C, and time 12 hours).

$$V = \frac{V_{\max} * [S]}{K_m + [S]}, \quad (3)$$

Table 2: Variation in the Michaelis–Menten constants.

Enzyme	$V_{\max}$ (μM/min)	K_m (μM)	$V_{\max}/K_m$ ($\min^{-1}$)
Free enzyme	0.41	0.97	0.43a
Celite-acetone	0.50	0.87	0.56b
Sephadex-acetone	0.42	0.92	0.43a
Chitosan-acetone	0.25	1.19	0.21
Celite-alcohol	0.46	0.89	0.52c
Sephadex-alcohol	0.41	1.10	0.37
Chitosan-alcohol	0.23	1.25	0.19

where V = rate of the reaction, $[S]$ = substrate concentration, $V_{\max}$ = maximum rate that can be achieved, and K_m = substrate concentration at half of $V_{\max}$. K_m can also be defined as the substrate concentration at which half of the enzyme's active sites are utilized by the substrate. The higher the K_m value, the higher the amount of substrate required to saturate the enzyme and therefore the lower the affinity of that enzyme for the substrate. The $V_{\max}$ value indicates the maximum reaction speed.

The initial hydrolytic velocity of the samples at different substrate concentrations was estimated from the slope of the line. The two constants, $V_{\max}$ and K_m, of the Michaelis–Menten equation were determined for all enzymes by solving the two substituted linear equations with the two unknowns. The calculated $V_{\max}$ and K_m values of these samples are reported in Table 2.

The ratio of the Michaelis–Menten constants allowed the calculation of the catalytic efficiency of the enzymes. The catalytic efficiency of lipase immobilized on Celite supports was significantly higher than that of other enzymes, suggesting that the maximum rate of reaction can be achieved with a lower substrate concentration. Free lipase and Sephadex-immobilized lipase demonstrated similar catalytic efficiencies. Hence, immobilizing *C. rugosa* lipase on Sephadex could add to the cost of reaction if other factors such as pH and residual recovery are not considered. Chitosan-immobilized lipase, being less capable of binding to the support, resulted in lower catalytic efficiency and may not be suitable for immobilization of *C. rugosa* lipase with a physical adsorption technique.

3.6. Esterification Activity. Percentage esterification was estimated as the ratio of moles of caprylic acid consumed in the reaction to the number of moles of initial caprylic acid. The reaction between 1 g of caprylic acid and 1.27 g of glycerol in the presence of *C. rugosa* lipase was carried out at 40°C for 12 hours at pH 7. *C. rugosa* lipases in both free and immobilized forms were moderately successful in achieving appreciable ester conversion rates. Average percentages ranging from 28 to 72% esterification were achieved during 12 hours of this experiment (Table 3).

Although appreciable amounts of esters were formed, the immobilization procedure was not able to significantly improve specific activity. The specific esterification activity of free lipase and Celite-immobilized lipase did not differ significantly. Lipase immobilized on chitosan and Sephadex resulted in lower specific activities. Chitosan-immobilized lipase, being hydrophilic, has less lipase-binding capacity,

Table 3: Percentage ester conversions.

Enzyme	% ester conversions
Free enzyme	70.72
Celite-acetone	68.95
Sephadex-acetone	62.95
Chitosan-acetone	35.45
Celite-alcohol	66.25
Sephadex-alcohol	59.54
Chitosan-alcohol	28.86

resulting in lower esterification activity. The significantly lower specific esterification activities of Sephadex-immobilized lipases are not well understood and should be further investigated.

Lumor and Akoh [11] reported that *C. rugosa* lipase had no significant esterification activity at 60°C for 1 hour. Since the enzymes in this study showed considerable ester formation at 45°C for 12 hours, further optimization of reaction conditions might obtain even higher ester yields.

3.7. Acidolysis Reaction. Acidolysis reaction, one of the reactions catalyzed by lipase enzymes, can result in the development of novel structured lipids used to incorporate a desirable fatty acid into an oil/fat to improve its nutritional value. The goal here was to study how lipase immobilization affects this reaction, determining the degree of incorporation of caprylic acid into olive oil. Caprylic acid is not present in olive oil but is found in coconut oil, corn oil, and palm kernel oil and in trace amounts in grape seed oil. The acidolysis reaction of 0.3 g of caprylic acid and 1 g of olive oil was carried out at pH 7.0, at 45°C, and at 200 rpm for 12 hours.

The reaction did not yield significant results with about 0.03%–0.16% of the fatty acid per unit protein incorporated into olive oil samples (Table 4). Srivastava et al. [23] successfully incorporated oleic acid and palmitic acid into milk fat by a transesterification reaction in the presence of *C. rugosa* lipase. The reason for lower incorporation of caprylic acid by *C. rugosa* lipases could be due to fatty acid selectivity.

Although the enzyme incorporation of caprylic acid was low, acetone-immobilized Celite-545 was capable of incorporating a slightly higher percentage, while immobilization on the two other support materials resulted in lesser incorporation of caprylic acid when compared to the free enzyme. *C. rugosa* lipases seem more efficient in catalyzing the incorporation of long-chain unsaturated fatty acids such as oleic acid than medium-chain saturated fatty acids such as caprylic acid. Further study using long-chain unsaturated fatty acids might help us estimate the effect of immobilization.

3.8. Residual Activity. Although the use of enzymes in industrial production improves the quality of the reaction products, the expense of enzymes compared to chemical catalysts usually makes the enzymatic process unaffordable. By immobilizing enzymes, they can be reused and thereby minimize production cost. In this study, all the enzymes—both free and immobilized—were reused up to six times to determine residual activity. The enzyme in the

Table 4: Percentage of caprylic acid incorporated into olive oil samples.

Enzymes	% of caprylic acid incorporated	% of caprylic acid incorporated per mg of protein
Free enzyme	0.54 ± 0.04	0.15 ± 0.01^{a}
Celite-acetone	0.517 ± 0.09	0.16 ± 0.03^{b}
Sephadex-acetone	0.17 ± 0.02	0.087 ± 0.01^{c}
Chitosan-acetone	0.05 ± 0.01	0.053 ± 0.00^{d}
Celite-alcohol	0.35 ± 0.22	0.13 ± 0.11^{a}
Sephadex-alcohol	0.138 ± 0.01	0.08 ± 0.00^{c}
Chitosan-alcohol	0.025 ± 0.00	0.039 ± 0.00^{e}

Note. Different letters indicate statistically significant differences between the groups.

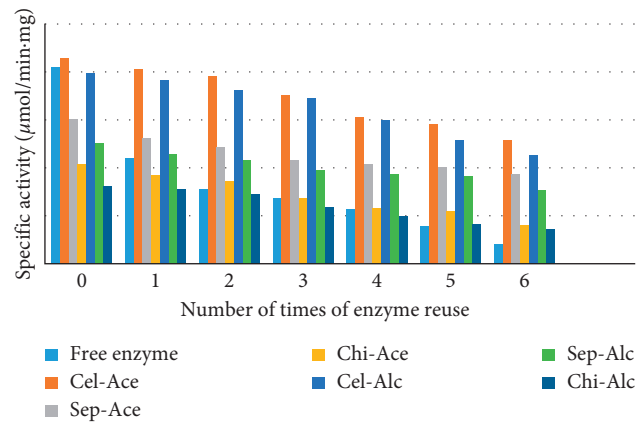

Figure 9: Residual enzyme specific activity (pH 7.0, temperature 45°C, and [S] = 1 mg/mL).

Table 5: Percentage loss of enzymatic activity after six successive uses.

Enzyme	% loss of activity
Free enzyme	99.30
Celite-acetone	38.21
Sephadex-acetone	61.07
Chitosan-acetone	40.05
Celite-alcohol	38.54
Sephadex-alcohol	55.35
Chitosan-alcohol	43.13

product mixture was filtered at room temperature and washed in two steps with the 10 mM sodium phosphate buffer solution followed by washing with *n*-hexane twice. Retaining and recovery of free enzyme from the mixture was not accurate due to its miscible nature. Hydrolytic activities of the enzymes were determined, and residual activity was calculated.

Free enzymes retained significantly less enzymatic activity than the immobilized enzymes (Figure 9) due to their inability to recover the powdery form of the free enzyme adequately after each reaction cycle. The free enzyme lost 99.3% of its activity after six successive cycles (Table 5), while losses in the enzymatic activities of the immobilized enzymes after 6 cycles ranged from 38.2% to 61.1%. Lipase

immobilized on Celite-545 and chitosan exhibited comparatively lower activity loss. Celite-immobilized lipase demonstrated intermediate residual activity. Sephadex-immobilized lipase lost around 55–60% of its activity. Although lipase immobilized on chitosan exhibited intermediate specific activities, residual performance in residual activity was the lowest. Chen et al. [24] reported similar results when they examined storage stability and reusability of immobilized enzymes and found that the immobilized enzymes showed higher stability after 10 cycles of reuse than the free enzyme. To determine the storage stability, the authors stored all enzymes in a phosphate buffer for 30 days and reported that free enzymes retained only about 30% of their activity, whereas the immobilized enzymes retained all of theirs. In another study, Alagoz et al. [25] immobilized *Candida* methylica formate dehydrogenase on glyoxyl agarose-, glyoxyl silica-, and aldehyde-functionalized Immobeads. After 5 cycles of reuse, the researcher reported that the free enzyme lost around 88% of activity, while the immobilized enzymes lost only 50–60%.

4. Conclusion

The current research examined the activity of *C. rugosa* lipase when immobilized using acetone or alcohol on three different support materials (Celite-545, Sephadex G-25, and chitosan). Of the two solvents, acetone was more effective in the immobilization process due to its lower polarity. The hydrolytic activities of the enzymes, both free and immobilized, under different pH and temperature conditions allowed to optimize the reaction conditions and to analyze the degree of stability provided by the support materials. *C. rugosa* lipase immobilized on the Celite-545 support material exhibited significantly greater activity than all the other immobilized enzymes in terms of hydrolytic reaction, fatty acid incorporation, and stability at varying pH and temperatures along with good recovery and reuse qualities. Sephadex G-25 did not perform well as expected. The specific hydrolytic activities of immobilized Sephadex G-25 were comparable to those of the free enzyme, suggesting that its use as a support material provides no economic benefit. Moreover, it did not exhibit either significant ester formation or thermal stability. Immobilized chitosan displayed very low activity values due to its less porosity. However, the support material conferred good stability on the enzyme concerning temperature variations. In conclusion, the activity of *C. rugosa* lipase was enhanced most significantly when immobilized on Celite-545 using acetone as an adsorption solvent. Per our study, the acetone-immobilized *C. rugosa* lipase can be utilized in the oil/fat industry for hydrolysis reactions to achieve an improved quality and quantity of hydrolysis products and reduced resource wastage and manufacturing costs.

Additional Points

Practical Application. C. rugosa lipase is used for producing glycerol and fatty acids which help in enhancing the flavor in food substrates such as milk, butter, cheese, ice, and cream. Even though the enzyme can be synthesized commercially, the higher cost is the prime factor to achieve successful application [26]. Immobilization improves the stability and ease of enzyme recovery after a reaction and thus contributes to reductions in production cost since the enzyme can be recycled. Although the technique of enzyme immobilization has become widespread, some commercial enzyme products still exist in the free form—not immobilized [9]. The immobilization of these enzymes, especially lipases, would be beneficial to the field of lipid chemistry. This has the potential for improving the activity, specificity, and stability of the enzyme as well as the ease of separating the enzyme from reaction products. In addition, the reusability of the immobilized enzyme is bound to reduce operational cost [10].

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Mrs. Bhagya Sri Kaja was the principal researcher, conducted the research, and drafted the manuscript. Dr. Stephen Lumor directed and assisted in the laboratory experiments and provided training to the principal researcher. Dr. Samuel Besong assisted with the results and overall application of the manuscript. Dr. Bettina Taylor provided suggestions to improve the project planning and manuscript editing and revision. Dr. Gulnihal Ozbay provided suggestions regarding the experimental approach and assisted with research write-ups, dissemination, and preparation of the manuscript.

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Research Article

Selected Parameters of Nutritional and Pro-Health Value in the Common Carp (*Cyprinus carpio* L.) Muscle Tissue

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This study analysed the effect of the type of production on the nutritional and health value of common carp muscle tissue, especially lipid-related indexes. The muscle tissue of common carps originating from three types of fish farms with different technological levels (i.e., intensive, low-intensive, and semiextensive) was studied. The type of production technology, especially the intensive breeding technique, has been shown to have a significant effect on dry weight, total protein, and total fat content in the common carp muscle tissue under study. The muscles of fish originating from these farms were characterised by the highest values of the mentioned indicators, which amounted to 24.6%, 18.74%, and 1.58%, respectively, for dry matter, protein content, and total fat content. The type of feed used in intensive, low-intensive, and semiextensive common carp breeding has been proven to have a highly significant effect on the fatty acid profile. As regards unsaturated fatty acids, monounsaturated fatty acids were dominant in all cases. The muscle tissue of fish cultured in farms with a semiextensive technological level was characterised by the highest content of unsaturated fatty acids. Moreover, the ratio of polyunsaturated fatty acids to saturated fatty acids was, in this case, the most favourable. Dietary indices of atherogenicity (AI) and thrombogenicity (TI) were studied as well. In all analysed cases, the values of these indices were very favourable and several times lower than for other animal fats.

1. Introduction

Consumption of fish in Central and Eastern Europe is still insufficient, which is a consequence of consumers' unsatisfactory knowledge and prices. Greater demand for this type of food is observed among people with university education and in circles where eating fish is a family tradition [1]. However, it is beyond doubt that increasing attention is being paid to food quality, both in Poland and elsewhere. The quality of fish muscle tissue, in terms of both the qualitative and the quantitative characteristics, including its nutritional value and chemical composition of muscles, can be modified by changing breeding conditions and, more specifically, feeding technologies.

Considerations of the pro-health effect of food must take into account the important role of animal origin fat, including polyunsaturated fatty acids and cholesterol in human nutrition. Fish, which is a food unmodified over the centuries and which can be regarded as one of humans' prime foods, is a valuable source of those nutrients [2, 3]. The spectrum of health-promoting effects of n-3 PUFAs on the cardiovascular system is very broad. Numerous studies have demonstrated that the consumption of a large number of foods providing a source of these acids, inter alia the application of a Mediterranean diet that is rich in fish, contributes to a reduction in the risk of the incidence of coronary heart disease and cardiac insufficiency. Moreover, the effectiveness of PUFAs in the course of coronary heart

disease and cardiac insufficiency and in the prevention of atrial fibrillation attacks was also confirmed. Experts agree on the nutritional recommendations concerning the consumption of PUFAs. In 2004, the International Society for the Study of Fatty Acids and Lipids recommended that 500 mg EPA and DHA should be consumed on a daily basis to prevent the occurrence of ischaemic heart disease. Similar recommendations were issued by the European Food Safety Authority (daily intake of 250 mg EPA and DHA for adults (EFSA 2010)) as well as by German, Austrian, and Swiss nutritional associations (DGE/ÖGE/SGE 2008) [4]. Polyunsaturated fatty acids play an extremely important role in the proper functioning of the human body. They provide a substrate for the synthesis of eicosanoids, that is, tissue hormones that regulate the functions of other hormones and neurotransmitters [5]. Fish fat abounds in C22:6 n-3 docosahexaenoic acid (DHA), C20:5 n-3 eicosapentaenoic acid (EPA), and C18:3 n-3 linolenic acid, whose beneficial effect on human health has been corroborated by research [6]. Much has been said about the benefits resulting from the consumption of polyunsaturated fatty acids in the context of the primary and secondary prevention of ischaemic heart disease; however, the most interesting studies are those probing the relationship between the diet and the risk of the occurrence of depression. Sánchez-Villegas et al. [7] studied the relationship between the consumption of lipids and the incidence of depression in the Mediterranean population. An increased risk of depression occurrence resulting from the increased consumption of *trans*-isomers of unsaturated fatty acids was demonstrated. At the same time, a weak negative correlation was observed between the consumption of MUFAs and PUFAs and the occurrence of such mental disorders. A sufficient supply of good quality fat has a protective effect on the human circulatory and nervous system. Fish muscle tissue also supplies large amounts of vitamins B, A, and D, and its proteins are easily absorbable and have a beneficial amino acid profile [8, 9].

Common carp, like the rainbow trout, is popular in many regions of Europe and it is one of the freshwater fish with a high nutritional value. Numerous studies of breeding, particularly the feeding of common carp, have shown the significant effect of the type of feed on the slaughter yield and quality of common carp muscle tissue, especially the content of protein, total fat, mono- and polyunsaturated fatty acids, and cholesterol [10, 11]. Therefore, this study attempted to determine the effect of the type of production on the nutritional and health value of common carp muscle tissue, especially the lipid-related indexes. The particular role of the common carp in human nutrition was confirmed by an experiment conducted by Adamkova et al. (2011) [12], who studied the effects of the incorporation of common carps into the diet as an element of secondary prevention in people suffering from ischaemic heart disease. Two representative groups of people (a test group and the control group) were subjected to testing. In the diet of the first one, 200 g of a fillet of common carp fed with a mixture with increased n-3 PUFA content (439 ± 146 mg/100 g of the fillet) was incorporated. The total energy consumption in both groups was the same. The results of laboratory tests carried out after four weeks of the experiment clearly indicated the positive effect of the consumption of common carp in the event of CVDs. Plasma lipid levels were significantly improved in the group of patients who had fish incorporated into their diet compared to the control group. A reduction in the total cholesterol level by 27% was demonstrated, with a decrease by 2% in the control group ($p < 0.001$). LDL cholesterol level decreased by 26% as compared to 4% ($p < 0.001$); the TG value in the plasma decreased by 26% as compared to 3% ($p < 0.001$). An increase in HDL cholesterol level by 30% in relation to 10% was observed ($p < 0.001$). Based on a POLKARD–SPOK study, Filipiak and Opolski [13] indicate the occurrence of disturbances in lipid metabolism in 78% of the population of patients at high risk of death due to CVDs. The data suggests that it is beneficial to enrich the diet with polyunsaturated fatty acids to improve the values of blood serum lipidogram through an increase in the consumption of common carp, particularly in Central European countries.

2. Material and Methods

Material from five fish farms in different locations in Poland was used in the study; the production technology applied in them was intensive (I) in 2 farms, low-intensive (NI) in 1 farm, and semiextensive (SE) in 2 farms. In order to avoid the effect of climate on the growth of common carps, which are poikilotherms, samples were taken from ponds situated in various parts of the country. Most farms were characterised by single-season production. Only one of the farms, with a semiextensive technological level, carried out multiseason production. Of the mentioned types of farms, three types of ponds were noted: commercial and fattening fish bins (I), only commercial (NI), and commercial I recreational and angling (SE). The stocking density in the farm applying the intensive production technology was 1,600 fish/ha, in the ponds with the low-intensive technological level was 1,315 fish/ha, and for the semiextensive production was 600 fish/ha. Particular farms were distinguished by production at a level of 1,002.5 kg/ha, 993 kg/ha, and 659 kg/ha, respectively, for the intensive, low-intensive, and semiextensive technological levels. On average, the weight of common carps harvested in the farm with intensive production technology was 1,159.5 g per fish; common carps harvested from the ponds with the low-intensive production technology had an average weight of 1,523 g per fish, whereas those from ponds characterised by semiextensive production technology had an average weight of 1,424 g per fish. The following types of feed were used in different farms:

(i) Cereal mixture—wheat, barley, and rye in the ratio of 3 : 1 : 1 or only fishing bait in semiextensive farms.

(ii) Cereal mixture (wheat, barley, and rye in the ratio of 2 : 1 : 1) or a cereal mixture (maize + wheat and barley in the ratio of 1 : 3) in low-intensive production technology.

(iii) Granulate Aller-Aqua, which comprises dried distiller grain, sunflower protein concentrate, soybean protein concentrate, fish meal, poultry meal, blood meal, feather meal, rapeseed oil, wheat, triticale, rape, soybean, vitamins, minerals, and amino acids, in intensive production technology.

Altogether, 75 fish were taken for the study, 15 consumer common carps of the *S* grade (0.8–1 kg) and *D* grade (1-2 kg). The fish were then killed, boned, and stored at −18°C.

In order to conduct a chemical analysis, a 5 cm wide boneless and skinless section was taken from a frozen fillet from the ventral to the dorsal side. The next stage involved grinding and homogenisation of a sample in a homogeniser (Type PRO350 BIOEKO, time: 40 minutes, speed: 11,000 RPM).

The dry weight was determined by drying samples to a constant mass, in accordance with the guidelines of AOAC [14]. Total ash was determined by drying, carbonising, and incineration [14]. Total protein content (nitrogen ×6.25) was determined by the Kjeldahl method [15].

Fat was extracted in accordance with the procedure proposed by Folch et al. [16]. Two-gram homogenised samples of muscle tissue were homogenised with 20 ml of methanol for 1 minute and with 40 ml of chloroform, also for 1 minute. The homogenate was filtered through a degreased filter. The remainder was washed with 60 ml of mixture of methanol and chloroform at a ratio of 2 : 1. The filtrate was then washed with 40 ml of chloroform and 20 ml of methanol and combined with 0.88% of sodium chloride at 25% of the filtrate volume. The mixture was shaken and left overnight for the layers to separate. The upper layer was collected with a vacuum pump and rejected. The lower layer was a lipid extract. A mixture of water and methanol in a ratio of 1 : 1 was added to it, in two replicates, in an amount of 1/4 of the amount of filtrate, and its top layer was each time discarded. The lipid extract was filtered through calcined sodium sulphate (VI) on a degreased filter and the solvent was then distilled off. The remainder was weighed in accordance with AOAC 2002 guidelines [17].

Methyl esters of fatty acids were prepared by the modified method developed by Peisker [18]. A 0.5 g sample of fat was put into an ampoule, 2 cm^3 of methylating mixture (methanol : chloroform : concentrated sulphuric acid, at a ratio of 100 : 100 : 1 (v/v/v)) was added, and the ampoule was sealed. The ampoules were heated in a water bath for two hours at 100°C. The fatty acids were analysed by gas chromatography on a 6890N Agilent Technologies chromatograph under the following conditions: capillary column with internal diameter of 0.32 mm, length 30 m (Supelcowax 10 liquid phase, film thickness of 0.25 μm), injector (split 50 : 1) (flow rate of 10 ml/min), injector temperature of 225°C, detector temperature of 250°C, and column temperature of 180°C. Fatty acids were identified through a comparison of retention times of the standards (a mixture of 37 acids) and peaks in the test sample.

The gross calorific value (W_{B}- kcal/100 g) of breeding common carp muscle tissue was calculated using physical energy equivalents from the following formula: $W_B = 5.65 * B + 9.45 * T + 4.15 * W$ [kcal/g], where B is the protein content in a sample, g/100 g; T is the fat content in a sample, g/100 g; W is the carbohydrates content in a sample, g/100 g; 5.65, 9.45, and 4.15 are physical energy equivalents for proteins, fats, and carbohydrates, respectively, kcal/g.

The net calorific value (W_{n}- kcal/100 g) of the carcasses was calculated using Atwater net equivalents, from the following formula: $W_N = 4 * B + 9 * T + 4 * W$ [kcal/g] [19], where B is the protein content in a sample, g/100 g; T is the fat content in a sample, g/100 g; W is the carbohydrates content in a sample, g/100 g; 4, 9, and 4 are Atwater net equivalents for proteins, fats, and carbohydrates, respectively, kcal/g. When the calorific values W_B and W_N were expressed in kJ/100 g, a conversion factor of 1 kcal = 4.19 kJ was applied. Since common carp muscle tissue contains only trace amounts of carbohydrates; this energy component was left out of the calculations [19].

The dietary atherogenic index (AI) and thrombogenic index (TI) were estimated with the formulae developed by Ulbricht and Southgate [20]. The atherogenic index was calculated from the formula AI = C12:0 + 4 C14:0 + C16:0/∑PUFA n-6 + ∑PUFA n-3 + ∑MUFA, where C12:0, C14:0, and C16:0 are the content of saturated acids: lauric, myristic, and palmitic (%); ∑PUFA n-6 are the total polyunsaturated fatty acids n-6 (%); ∑PUFA n-3 are the total polyunsaturated fatty acids n-3 (%); ∑MUFA are the total monounsaturated fatty acids (%). The thrombogenic index (TI) was calculated from the formula TI = C14:0 + C16:0 + C18:0/(0.5 ∗ C18:1) + 0,5 (MUFA-C18:1) + (0,5 ∗ PUFA n-6) + 3 ∗ PUFA n-3 + (PUFA n-3/PUFA n-6), where: C14:0, C16:0, and C18:0 are the content of saturated acids: myristic, palmitic, and stearic (%); MUFA is monounsaturated fatty acid; PUFA n-6 are polyunsaturated fatty acids n-6 (%); PUFA n-3 are polyunsaturated fatty acids n-3 (%).

The statistical analysis was performed using Statistica 12 software, with the t-test for samples independent from groups at $p \leq 0.05$.

3. Results

An analysis of the composition of common carp muscle tissue from fish farms of three types has shown a distinct effect of the method of feeding on the nutritional and energy value of the meat. Common carp muscle tissue from a farm which applied the intensive breeding technology had the highest calorific value (gross: 182 kcal; net: 102 kcal) followed by the material from farms where semiextensive production technology was applied (116 and 91 kcal, resp.). The lowest calorific value was recorded for the muscle tissue of common carp bred by the low-intensive technology; the gross calorific value of the product was 109 kcal and net calorific value was 88 kcal (Table 1). The net calorific value is an especially important parameter for living organisms; it is the amount of energy actually used by an organism for life processes. The muscles of fish fed by the intensive method also contained the highest percentage of dry matter (24.6%), protein (18.74%), and fat (1.58%) in the carcass, which obviously has its effect on the calorific value. Moreover, the content of dry matter is closely connected with the amount of fat in muscle tissue, as it is a component whose form does not change significantly during drying. Muscle tissue of fish from low-intensive breeding contained the lowest level of fat of all the samples: 0.56%. The dry matter content was 22.31% and the total protein content was 18.32% (Table 1). Despite the similarity of the feed used in low-intensive and semiextensive farms (cereal mixtures), the higher protein content in fish muscle tissue from the

Table 1: Calorific value and basic chemical composition in muscle tissue of common carp samples under study.

Item		Production technology		
		Intensive ($n = 30$)	Low-intensive ($n = 15$)	Semiextensive ($n = 30$)
Gross calorific value (kcal/100 g)	$\overline{x}$	182	109	116
Gross calorific value (kJ/100 g)	$\overline{x}$	764	458	487
Net calorific value (kcal/100 g)	$\overline{x}$	102	88	91
Net calorific value (kJ/100 g)	$\overline{x}$	428	370	382
Dry matter (%)	$\overline{x}$	24.6[a]	22.31[b]	21.98[b]
	SD	0.34	0.82	0.81
Total protein (%)	$\overline{x}$	18.74[a]	18.32[a]	17.94[a]
	SD	0.63	0.43	0.33
Total fat (%)	x	1.58[a]	0.56[b]	1.49[a]
	SD	0.96	0.11	0.68
Total ash (%)	$\overline{x}$	1.01[a]	1.11[a]	1.02[a]
	SD	0.03	0.24	0.07

[a,a]The same letters in a row denote the absence of significant statistical differences between mean values at $p \leq 0.05$. [a,b]Different letters in a row denote the presence of significant statistical differences between mean values at $p \leq 0.05$.

first type of breeding could be an effect of an addition of maize in the feed. This cereal contains similar levels of protein to other species, but it was an additional component of the feed, thereby increasing its consumption by fish. Regardless of the feed or production technology, monounsaturated fatty acids dominated in common carp muscle tissue (Table 2). A statistical analysis of the content of monounsaturated fatty acids has revealed significant differences in the content of MUFA in muscle tissue of common carp from farms where intensive and low-intensive production technologies were applied (Table 2). Oleic acid (C18:1 n-9) dominated among monounsaturated fatty acids in all cases. Only for C15:0 pentadecanoic acid were the differences not statistically significant. Statistically significant differences have been demonstrated between all of the fatty acids in the profiles of monounsaturated fatty acids in muscle tissue of fish which were given natural feed and bred by low-intensive technology. A comparison of intensive and semiextensive farms did not reveal any statistically significant differences for C20:0 eicosanoic acid. There were some statistically significant differences between the other fatty acids, which were significantly affected by differentiating fodder and high-protein mixtures, enriched with animal fats and natural plant feed (Table 2).

Intensive fish production enables one to provide fish with the right amount of feed as well as to achieve the desired dietary value of the finished product. The study found that common carp muscle tissue bred by this model is significantly richer in essential polyunsaturated fatty acids (PUFAs) (Table 2). An analysis of the fatty acid profile of muscle tissue of the common carp under study shows a large content of PUFA in muscles of all the fish (Table 2). C18:2 n-6, C20:5 n-3, C20:4 n-6, and C22:6 n-3 acids dominated among PUFAs in all samples. A comparison of fish from intensive and low-intensive breeding farms showed statistically significant differences in the content of fatty acids in most cases. No differences were observed in the content of the following fatty acids: C18:4 n-3, C20:1 n-9, C20:1 n-7, C20:3 n-3, C20:4 n-3, and C20:5 n-6. Not many significant differences were found between the fatty acid contents in common carp muscle tissue from the two extreme types of fish farms: intensive and semiextensive. Only for C18:2 n-6 or C20:1 n-9 acids were no statistically significant differences found. Only two cases with no statistically significant difference were identified in an analysis of common carp muscle tissue by the low-intensive and semiextensive method. Those were the content levels of fatty acids C20:2 n-9 and C22:6 n-3 (Table 2). It can be concluded from the results of the statistical analysis of the fatty acid profile of fish muscle tissue from three types of fish farms that the type of feed has a great effect on its composition. The contents of various profile components vary from one fish type to another. There are some other cases with no differences between them, but only on the farms where intensive and low-intensive production technology was applied. This arises from the similarity of feed used in these fish ponds and the small number of results with no statistically significant differences between farms: I-SE, LE-SE, which clearly shows the differences in the nutritional value of the feed and translates into the material composition. Regardless of the type of common carp production technology, the fish fat contained saturated and unsaturated fatty acids at a beneficial ratio (Figure 1). The ratio was 0.84 in common carp caught on intensive farms, 0.74 in the low-intensive ones, and 0.87 in semiextensive ones. An examination of the fatty acid profile in common carp muscles has shown that they are not only a source of PUFA n-3 and PUFA n-6; the values of AI and TI are considerably lower than for other animal fats (Figure 2). For example, the atherogenic index for cow milk is ca. 3-4. The values for fish muscle tissue are 0.43, 0.54, and 0.35 for intensive, low-intensive, and semiextensive technology, respectively. For the samples of muscles under study, the values were 0.46, 0.65, and 0.84 for intensive, low-intensive, and semiextensive breeding, respectively.

Table 2: Fatty acid profile in muscle tissue of common carp under study (%).

Fatty acids	Production technology		
	Intensive (n = 30)	Low-intensive (n = 15)	Semiextensive (n = 30)
Saturated fatty acids			
C14:0	1.59[a]	1.44[b]	0.84[c]
C15:0	0.3[a]	0.22[a]	0.1[b]
C16:0	19.02[a]	21.65[b]	18.3[c]
C17:0	0.28[a]	0.19[b]	0.13[c]
C18:0	5.05[a]	5.58[b]	6.40[c]
C20:0	0.11[a]	0.08[b]	0.11[a]
C22:0	0.19[a]	0.03[b]	0[c]
Total ratio UFA	26,54	29,19	25,88
Monounsaturated fatty acids			
C14:1	0.09[a]	0.08[b]	0.04[c]
C16:1 n-7	8.65[a]	10.29[b]	8.17[c]
C17:1	0.37[a]	0.25[b]	0.15[c]
C18:1 n-9	33.87[a]	39.99[b]	45.61[c]
C18:1 n-7	3.55[a]	2.96[b]	2.52[c]
C20:1 n-11	0.38[a]	0.32[a]	0.16[b]
C20:1 n-9	2.28[a]	2.1[a]	2.24[ab]
C20:1 n-7	0.10[a]	0.09[a]	0.05[b]
C22:1 n-11	0.37[a]	0.09[b]	0.06[c]
C22:1 n-9	0.14[a]	0.07[b]	0.05[c]
Total ratio MUFA	49,8	56,24	59,05
Polyunsaturated fatty acids			
C18:3 n-3	3.03[a]	2.06[b]	0.71[c]
C18:4 n-3	0.73[a]	0.52[a]	0.14[b]
C20:3 n-3	0.15[a]	0.14[a]	0.07[b]
C20:4 n-3	0.36[a]	0.28[a]	0.14[b]
C20:5 n-3	2.59[a]	1.34[b]	0.73[c]
C22:5 n-3	0.85[a]	0.38[b]	0.27[c]
C22:6 n-3	2.20[a]	0.96[b]	0.84[b]
Total ratio n-3	9,91	5,68	2,9
C18:2 n-6	10.59[a]	7.13[b]	9.92[a]
C18:3 n-6	0.32[a]	0.24[b]	0.37[c]
C20:3 n-6	0.44[a]	0.25[b]	0.33[c]
C20:4 n-6	1.55[a]	0.85[b]	1.06[c]
C22:5 n-6	0.14[a]	0.14[a]	0.22[b]
Total ratio n-6	13,04	8,61	11,09
Ratio n-3/n-6	0,74	0,66	0,26
C20:2 n-9	0.41[a]	0.29[b]	0.28[b]
Total ratio PUFA	23,36	14,58	15,08

[a.a]The same letters in a row denote the absence of significant statistical differences between mean values at $p \leq 0.05$. [a.b]Different letters in a row denote the presence of significant statistical differences between mean values at $p \leq 0.05$.

4. Discussion

The muscle tissue of common carp fed exclusively with cereal mixtures or fishing bait (i.e., food of a simple composition, without being enriched with animal protein or fat, therefore having a lower nutritional value) contained the smallest percentage of dry matter and protein. The percentage of protein in all carcasses did not deviate from its mean content in the muscle tissue of other fish species (13% to 25%) [21]. It is noteworthy that the protein of common carp, regardless of the production technology, contains essential amino acids in amounts much higher than in the standard protein [22]. This protein contains a higher percentage of amino acids such as phenylalanine, leucine, isoleucine, threonine, methionine, cystine, and valine, compared with beef, pork, or mutton [21, 23]. From a nutritional point of view, the protein of

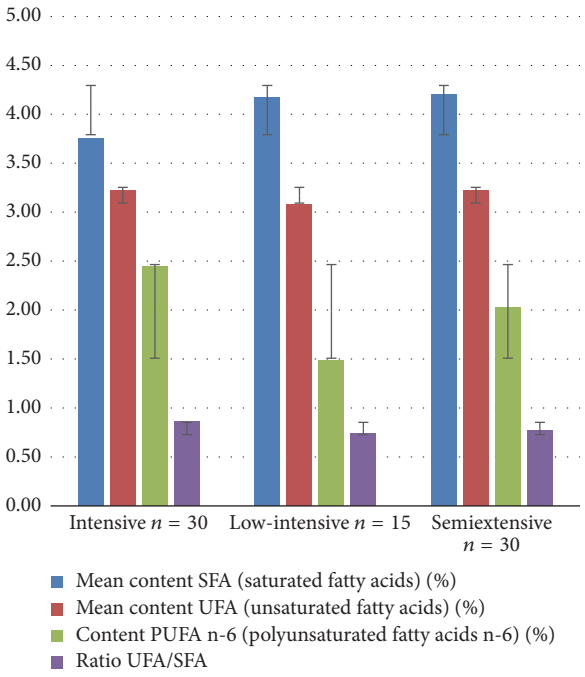

Figure 1: Selected parameters of fatty acid profile of carp muscle tissue under study.

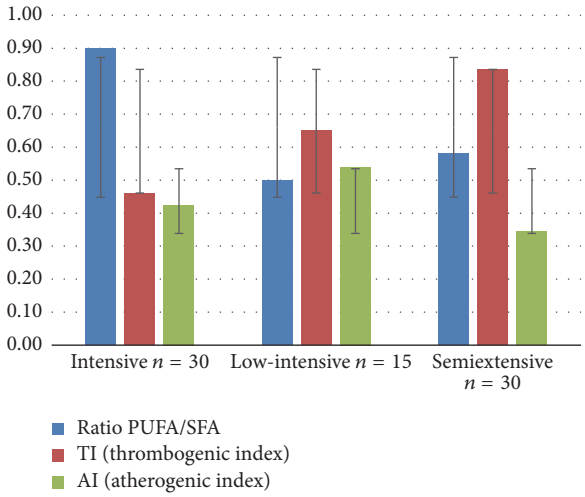

Figure 2: Selected parameters of fatty acid profile of carp muscle tissue under study (ctd.).

this fish species is regarded as complete. The composition of rainbow trout muscle tissue (another freshwater fish) is similar. A deficit of essential amino acids in the human diet has been shown to result in many adverse changes in the body. A sufficient supply of valine helps to maintain the right coordination of movements, the right body weight, function of dendritic cells, and a feeling of hunger. A deficit of leucine can lead to neurological disorders and a deficit of methionine impairs body immunity [24].

It has been shown in a number of studies that the type of feed given to fish affects the chemical composition of their muscle tissue. The fatty acid profile, affected by the type of feed, is very important in human nutrition. Unlike the meat of slaughter animals, fish fat is also a rich source of vitamins A and D [24]. Like marine fish, freshwater fish can be a rich source of essential fatty acids; when included in the diet, they supply the body with cardioprotective, hypotensive, and antitumour substances [11, 25]. The availability of omega-3 fatty acids for different human tissues depends on the diet and is of great importance both for their correct development and for prevention and treatment of chronic diseases [26]. Similar observations were included in the paper by Grela et al. [27], who analysed the composition of marine and freshwater fish muscle tissue. Their muscle tissue was found to contain the highest percentage of C16:0 palmitic (ca. 20%) and C18:0 stearic acid, although the content of the latter in the samples was much lower. Similarly, the highest percentage of palmitic acid in the fatty acid profile was found in muscle tissue of other freshwater fish of South Asia, rohu *(Labeo rohita)* of the Cyprinidae family and Mozambique tilapia *(Oreochromis mossambicus)* [28], as well as muscle tissue of common carp from Lake Beyşehir in Turkey and rainbow trout (15–18%) bred in Poland [24], which indicates that this fatty acid dominates the MUFA profile in fat of freshwater fish regardless of the climate and season [29]. Monounsaturated fatty acids reduce the level of "bad" cholesterol (LDL) and increase the level of "good" cholesterol (HDL) in blood [30, 31]. The same results were obtained in a study of the common carp muscle tissue composition conducted by Guler et al. [29]. They also demonstrated seasonal variability of the content of total fat in fish carcasses, with the highest content observed in winter (4.45%).

The preparation given to the fish contained 56% of protein and 11% of lipids. It is important to be able to model the lipid profile of food because fats are the main high-energy nutrient. It has been shown in numerous studies that high consumption of fat and its improper composition can stimulate the development of civilizational diseases. The fatty acid profile, especially the proportion of saturated to mono- and polyunsaturated acids, is of particular importance. Polyunsaturated essential omega-3 and omega-6 fatty acids are not synthesised in the human body and they must be supplied with food [32, 33]. The group's main representative ones include α-linolenic acid (C18:3) of the n-3 family; it is a precursor of C20:5 n-3 eicosapentaenoic (EPA) and 22:6 n-3 docosahexaenoic acid (DHA) and linoleic acid (C18:2 n-6), a precursor of C20:4 n-6 arachidonic acid (AA) [34]. Modelling of PUFA content of the muscle tissue of freshwater fish is of interest to numerous researchers. A similar experiment was conducted in the Czech Republic by Mraz and Pickova (2011) [35] who also analysed three systems of nutrition. In the first one, the fish had access to plankton; in the second one, the fish had access to plankton with the addition of cereals and rapeseed cake granules. The fatty acid profile in the white muscles of fish was then analysed. The muscles of fish which were not provided with supplements were characterised by a high content of n-3 PUFAs (in particular EPA and DHA). The supplementation with rapeseed cake granules resulted in the PUFA n-3 content being at a moderate level. The addition of cereals led to a high

content of oleic acid and a low content of n-3 polyunsaturated fatty acids.

Similar literature reports have also pointed to freshwater fish as a valuable source of omega-3 fatty acids and emphasised their significant role in the daily diet [36, 37]. Another method for modifying the composition of fatty acids is the application of the finishing feeding strategy. Since the composition of fish muscles is highly variable, it would be valuable if producers could produce raw material with a high and repeatable level of n-3 polyunsaturated fatty acid content. The difficulties primarily encountered by fish breeders who intend to enrich the feed with components providing n-3 PUFAs include the increasingly rare use of fish oil as well as a high price and low availability of algae and microorganisms. For this reason, the possibility is being considered of the application of finishing feeding developed with the following in mind: species of carnivorous fish and those with a medium fat content, such as the turbot *(Psetta maxima)*, fatty fish such as the Atlantic salmon *(Salmo salar)*, and lean fish such as the Atlantic cod *(Gadus morhua)* and the Murray cod *(Maccullochella peelii peelii)* [38]. Supplementation serves a significant role in nutrition. The content of n-3 HUFAs (highly unsaturated fatty acids) can be increased with the supplementation of ALA, taking advantage of the common carps' capacity for bioconversion of ALA into n-3 HUFAs [35]. An effect of feed modification on the fatty acid profile was studied by Menoyo et al. [39], who examined how an addition or total substitution of feed with linseed oil will influence the quality of muscle tissue of Atlantic salmon. It has been shown that feed can be fully substituted with linseed oil, with no effect on the productivity or sensitivity of muscles to oxidation of lipids. Linseed oil affected the metabolism of fatty acids in the liver, increasing the activity of glucose-6-phosphate dehydrogenase and accumulation of C20:4 n-6 eicosatetraenoic acid. This was accompanied by a decrease in the concentrations of C20:5 n-3 eicosapentaenoic and C22:6 n-3 docosahexaenoic acid in fish muscle tissue. According to the analysis of factors affecting the distribution of fatty acids within the muscle tissue of the common carp, carried out by Mraz and Pickova (2011) [35], a fish fillet is not uniform, and thus the distribution of fatty acids in the muscles varies. The highest lipid content was noted in the abdominal wall (approx. 30%) and in the red muscles (16-17%), while the lowest was in the white dorsal muscles (approx. 1-2%). The role of genetic factors must not be omitted. It was demonstrated that the fatty acid content of the muscle tissue is hereditary; moreover, there is a positive correlation between the body size (body length and weight) and the lipid content [40]. Consideration of the effect of external factors on the quality of fish muscle tissue should also take into account the climate. Çelik et al. [41] examined the composition of muscle tissue of zander *(Sander lucioperca)* and showed that a higher percentage of omega-3 fatty acids were present in muscle tissue of fish bred in a colder climate. Furthermore, Çağlak and Karsli [42] showed seasonal influences not only on the lipid index of the edible portion of zander, but also on higher content of aspartic acid, glutamic acid, and lysine in muscle tissue of these fish in autumn than in spring. Similar results were presented in a study conducted by Guler et al. [29], where a significantly higher omega-3 to omega-6 acid ratio was shown during a spawning period as well as in spring and in autumn. Pleadin et al. [43] investigated the impact of seasonal changes and the location of breeding farms in the Adriatic Sea on the chemical composition of sea brass *(Dicentrarchus labrax)* and sea bream *(Sparus aurata)*. The study was conducted in October 2012 and January 2013. They showed a significant influence of seasonality on moisture content and fat content in fish muscle tissue, while the location of farms did not significantly influence the indicators. The value of these relations is similar to other species of fish such as *S. schall* or *T. lineatus*, where PUFA/SFA ratios are 0.4 and 1.7, respectively. The lower values of the ratio of polyunsaturated to saturated fatty acids are characterised by muscle tissue of species such as *L. niloticus*, *B. bajad*, and *O. niloticus* [44].

There are two dietary indexes associated with the lipid profile: the atherogenic index and the thrombogenic index. Atherogenesis denotes the development of atherosclerotic changes in blood vessels, which result in the development of ischaemic heart disease. A negative lipid ratio in blood speeds up atherogenesis. Thrombogenic components denote particles which facilitate the formation of blood clots. Edible fats are classified into two types: atherogenic (i.e., those that favour the development of atherosclerotic changes) and antiatherogenic (which have an antiatherosclerotic effect). Most animal fats have an atherogenic effect due to the high content of saturated fatty acids and cholesterol. The other group includes mainly vegetable oils [45]. This means that the fat has much higher antiatherosclerotic and cardioprotective properties than milk fat and emphasises its pro-health value. The thrombogenic index, which for milk ranges from 3.75 to 4.71, is another parameter which indicates the beneficial properties of common carp fat [46]. The nutritional value of the common carps tested, given indicators such as the thrombogenicity index and the atherogenic index, was very favourable, as was the case with commercially important species of marine fish such as bream *(Sparus aurata)*, sea bass *(Dicentrarchus labrax)*, dentex *(Dentex dentex)*, and turbot *(Scophthalmus maximus)* [47].

The findings suggest that the muscle tissue of common carp and other freshwater fish can be a valuable replacement for marine fish in the diet. It is a rich source of PUFA in desired proportions. Moreover, there is a distinct, beneficial effect of intensive technology of fish breeding on selected parameters of lipid profile, the amount of components with the cardioprotective action, total amount of fat, and the associated content of fat-soluble vitamins. The differences in results for samples obtained from various types of farms arise from the type of feed given to the animals. The feed given in the semiextensive technology was a cereal mixture: wheat, barley, and rye in the ratio of 3 : 1 : 1, or only fishing bait; in the low-intensive technology it was a cereal mixture—wheat, barley, and rye in the ratio of 2 : 1 : 1—or a cereal mixture—maize + wheat and barley in the ratio of 1 : 3. The fish were given industrial feeds in the intensive production technology; these were made of the following ingredients: yeast, wheat gluten, fish meal, krill meal, fish oil, wheat, vitamins, minerals, and amino acids.

Their composition was much more diverse; it contained both complete animal protein and plant protein. An addition of fish oil in the right proportions, especially saturated fatty acids, allows for modification of the composition of fatty acids in the bodies of fed animals. Yeasts are used in animal feeding as a source of vitamin B, some bioelements, enzymes, and digestible protein. They have a beneficial effect on intestinal flora and stimulate the growth and development of young animals and their health and productivity [48].

5. Conclusions

(1) The type of production technology, especially intensive breeding technique, has a significant effect on dry weight and total fat in the common carp muscle tissue under study.

(2) The type of feed used in intensive, low-intensive, and semiextensive common carp breeding has a highly significant effect on the fatty acid profile. Regardless of the technology of common carp breeding, fish fat has a beneficial proportion of unsaturated (UFA) to saturated (SFA) fatty acids and is a rich source of polyunsaturated fatty acids (PUFAs).

(3) Low values of atherogenic index (AI) and thrombogenic index (TI) of common carp fat indicate its antiatherosclerotic and cardioprotective properties, which are much stronger than in milk fat. This confirms the pro-health properties of fat in common carp muscle tissue.

(4) Owing to its nutritional and pro-health value, muscle tissue of common carp, especially from intensive breeding, can be a complete substitute for marine fish in the human diet.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Evaluation of Consumption of Poultry Products Enriched with *Omega-3* Fatty Acids in Anthropometric, Biochemical, and Cardiovascular Parameters

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An alternative for prevention and treatment for cardiovascular diseases (CVD) is increasing the intake of bioactive compounds as *omega-3*. However, several countries as México do not consume regularly foods with high content of *omega-3*, mainly fish products due to cultural, social, and economic factors. Therefore, the addition of *omega-3* in other food sources could contribute to completing the requirements established of these fatty acids. To evaluate the effect of the consumption of poultry products supplemented with *omega-3* in healthy population, a phase 1, double blind randomized, controlled parallel-group trial was carried out. After 14 weeks, the supplemented group had an increase in HDL, reducing the atherogenic index. The supplementation with *omega-3* in poultry products could contribute to a cardioprotective effect. It is necessary to complete studies with a higher evaluation period to determine the improvement in anthropometric and cardiovascular parameters.

1. Introduction

The cardiovascular diseases (CVD) are a leading cause of morbidity and mortality worldwide. An estimate of 17.7 million people died from cardiovascular diseases in 2015. World Health Organization (WHO) showed that CVD are the leading cause of mortality in Mexico. Risk factors include tobacco and alcohol use, physical inactivity, unhealthy diets, elevated blood pressure, overweight/obesity, hyperglycemia, and hyperlipidemia [1–5]. Therefore, it is necessary to modify the lifestyles mainly by improving the diet. Several studies show that the consumption of rich food in nutrients improves health. The *omega-3* fatty acids offer a more suitable preventive or therapeutic option for many chronic diseases due to their biological activity [6, 7]. Various institutions had established recommendations for the consumption of *omega-3* as a protective factor against several diseases: the American Dietetic Association (ADA); International Society for the Study of Fatty Acids and Lipids (ISSFAL); French Agency for Food Environment and Occupational Health Safety *Omega-3* Report; European Society of Cardiology; Report FAO: FAT and Fatty acid in Human Nutrition; USDA Dietary Guidelines for Americans. These recommendations of *omega-3* intake differ from 250 mg up to 4 g/day, depending on whether it is for prevention [8, 9] or treatment of several pathologies [9–14]. In order to achieve this level of daily intake of *omega-3*, it is recommended to consume 600–660 g of fish minimally per week [9–11], in particular fatty fish such

as mackerel, lake trout, herring, sardines, albacore tuna, and salmon, or to consume the equivalent in fish oil commercially available as concentrated pharmaceutical preparations [15].

However, Mexico is not a country with a high consumption of fish products due to social, cultural, and economic situations [16]. Compared to the *per capita* consumption of fish products of countries like Spain, Norway, Japan, Myanmar, Korean Republic, Malaysia, Iceland, and Maldives at 43–165 kg/year, Mexico's intake of these products is quite low at 4 kg/year [17]. Therefore, according to other studies, the alternative for increasing intake of *omega-3* could include supplemented daily products such as dairy (yogurt, milk, and margarine), juices, chicken, and eggs [18, 19]. The objective of this research was to evaluate the effects of the consumption of eggs and chicken supplemented with *omega-3* on anthropometric, biochemical, and cardiovascular parameters in healthy population.

2. Material and Methods

2.1. Subjects and Study Design. The study was a phase 1, double blind randomized, controlled parallel-group trial where 29 volunteers of both sexes participated (17 women and 12 men with an average age of 32 ± 5.6 years old, an approximate weight of 74 ± 18 kg, and height of 1.64 ± 0.084 m). They complied with inclusion and exclusion criteria of study. Some participants were excluded from the study due to preexisting conditions, like hypertriglyceridemia and hypercholesterolemia, or because they did not complete the evaluations. At the baseline, participants were randomly assigned to the group that consumed supplemented poultry food (experimental group) or to the control group. Fourteen participants (9 women, 5 men) consumed chicken and eggs supplemented with *omega-3* fatty acids and fifteen participants (8 women and 7 men) consumed chicken and eggs nonsupplemented with *omega-3* fatty acids. Both groups participated for a period of 14 weeks. During this time, participants consumed the poultry products in different preparations; we recommended that they consume the poultry products with fat-free preparations (boiled, grilled, etc.).

The experimental group consumed 250 grams of chicken meat (≈228 mg of *omega-3*) and 3 eggs (≈1590 mg of *omega-3*) each week. The control group consumed the same amount of chicken meat and eggs but without treatment of supplementation. The amount of eggs consumption was calculated according to recommendations established in NOM-043-SSA2-2012 [20] and by the Organization INPROVO [21]. Anthropometric measurements, blood samples for biochemical determination, and cardiovascular function data were collected at the baseline and at the 14th week. Subjects followed their regular habits, as physical exercise and regular diet. They signed the informed consent to participate in the intervention. The study protocol was approved by the Ethic Committee of the Health Sciences Institute of the Autonomous University of the State of Hidalgo, Mexico.

2.2. The Chicken Product. A total of 200 poultry males and females (Cobb and Ross strain) were acquired for production of meat and 60 laying hens (Plymouth rock and Rhode Island strain) for production of eggs. Poultry were housed in stainless steel cages during the period of September to November 2016 and their care was conducted according to the University approved methods. The animals were fed (supplemented and nonsupplemented) with a commercial diet and water provided for ad libitum consumption for 21 days. The commercial diet for supplemented animals was added with 0.5–0.8 g/day of *omega-3* obtained of cod leaver oil. It is the recommended amount to avoid the fish odor and/or flavor in meat and eggs. After 21 days of growth of the animals, the collection of eggs and the obtaining of the meat began. The quality of the meat and eggs supplemented and nonsupplemented with *omega-3* was evaluated according to the established standard in NMX-FF-080-SCFI-2006 [22] and PROY-NOM-159-SSA1-2015 [23].

2.3. Anthropometric Measurements. The anthropometric measurements (weight, height, and waist circumference) were evaluated according to the recommendations established by the Secretary of Health (SSA) in Mexico [24–26]. The body mass index (BMI) was calculated according to the specifications of the World Health Organization (WHO) [27] and the body composition was measured by body densitometry using air-displacement via the Bod Pod®. All testing was done in accordance with the manufacturer's instructions [28] to obtain the values of fat mass (percentage and kilograms), fat-free mass (percentage and kilograms), and weight (kg).

2.4. Biochemical Parameters. Fasting blood samples were collected by venipuncture into vacutainer tubes serum [29], centrifuged at 6500 rpm for 15 minutes (centrifuge brand Hamilton Bell), and stored at −21°C for further analyses. Total cholesterol (TC, optimal value < 200 mg/dL), high density lipoprotein (HDL, optimal value > 40 mg/dL), triglycerides (TG, optimal value < 150 mg/dL), and glucose (optimal value 100 −125 mg/dL) were determined using commercially available kits (Spinreact®), considering the specifications of the clinical practices guide for diabetes and dyslipidemias [30, 31].

LDL (low density lipoprotein) cholesterol was determined according to the following formula [32]:

$$\text{LDL mg/dL} = \text{TC} - \left[\text{HDLc} + \left(\text{Tg}\right) 5\right]. \quad (1)$$

TC is total cholesterol, HDLc is high density lipoprotein cholesterol, and Tg is triglyceride.

The atherogenic index (AI) was calculated with the following formula:

$$\text{Castelli Index} = \frac{\text{Total cholesterol}}{\text{HDL cholesterol}}. \quad (2)$$

According with Castelli [33], a low atherogenic index was considered <4.5, 4.5 to 7 as moderate and >7 high.

2.5. Cardiovascular Function. Cardiovascular function was evaluated with the following parameters.

The heart rate was evaluated at rest using a pulsometer (MedStar®). The ranges were considered optimum with

Table 1: Anthropometric parameters of experimental and control group.

	Week 0	Week 14
Experimental group (n = 14)		
Body weight (Kg)	74.60 ± 19.20[a]	74.5 ± 19.7[a]
Height (m)	1.62 ± 0.09[a]	1.62 ± 0.09[a]
Body mass index (BMI)	28.1 ± 6.1[a]	28.0 ± 6.2[a]
Waist circumference (cm)	90.7 ± 16.2[b]	89.7 ± 16.2[a]
Body fat (%)	33.1 ± 9.6[b]	31.7 ± 9.8[a]
Body fat (Kg)	25.5 ± 11.9[b]	24.5 ± 12.3[a]
Fat-free mass (%)	66.9 ± 9.6[a]	68.2 ± 9.8[b]
Fat-free mass (Kg)	49.0 ± 10.5[a]	49.9 ± 10.6[b]
Control group (n = 15)		
Body weight (Kg)	83.8 ± 17.5[a]	83.2 ± 17.9[a]
Height (m)	1.65 ± .07[a]	1.65 ± 0.07[a]
Body mass index (BMI)	30.4 ± 4.7[a]	30.2 ± 5.1[a]
Waist circumference (cm)	96.6 ± 14.4[a]	95.2 ± 14.2[a]
Body fat (%)	37.2 ± 7.9[b]	35.5 ± 9.2[a]
Body fat (Kg)	31.8 ± 11.0[a]	30.1 ± 12.0[a]
Fat-free mass (%)	62.4 ± 7.6[a]	64.4 ± 9.2[a]
Fat-free mass (Kg)	52.0 ± 10.6[a]	53.0 ± 10.4[b]

[a,b]Different letters between column values (0 and 14th week) indicate significant difference ($p \leq 0.05$ Student's t-test).

values between 65 and 85 beats/minute and considered high with a rate above 85 beats per minute [34].

The arterial pressure was evaluated according to Clinical Practices Guide with an aneroid baumanometer (MedStar®) [34]. According to the American Health Association (2017) 120/80 mmHg is considered as optimum values [35].

2.6. Bruce Exercise Stress Testing. Bruce Exercise Stress test was evaluated according to the Bruce protocol, recording the electrical activity of the heart that occurs in each heart beat during physical exercise. The subjects performed the maximal exercise tests on an endless band (Wellch Allyn®), programed for increasing the angle of inclination and speed. The participants walked in an initial slope of 10% and a speed of 1.7 km/h; every 3 min the slope was increased 2% and speed to 2.5, 3.4, 4.2, 5.0, and 5.5 km/h [36–38]. In addition, every three minutes (0, 3, 6, 9, 12, and 15 minutes) the heart rate, blood pressure, and electrocardiographic segments were registered [PQ (120–200 milliseconds), QRS-interval (60–100 milliseconds), QT (360 milliseconds), and QTc (340–450 milliseconds)], until the individuals reached 80% heart rate, considered as the maximum of cardiac capacity. At the end of the test, the duration and recovery time in minutes were recorded. The test was ended when the subject showed exhaustion, fatigue, or inability to maintain a running cadence [39].

2.7. Statistical Analysis. Statistical analysis was performed using the SPSS statistical software (version 23). The Shapiro-Wilk test was applied to determine the distribution of the data of the variables in the normal curve to identify if the hypothesis would be verified with parametric or nonparametric test. To compare the existence of differences between week 0 and 14th of the intervention in the experimental and control group, a Student's t-test was used. Statistical significance was considered as p value ≤ 0.05 with 95% confidence intervals. In addition, unpaired t-test studies were done together with the analysis of variance between groups for independent samples. The effect size (Δ) was estimated between the means of the before-after observed differences in the treatment groups using Student's t-test.

3. Results and Discussion

No subjects reported any side effects derived from the intake of the poultry products consumed in the study. Participants indicted that supplemented poultry products presented intense flavor and texture softer in comparison with the control products and even with major sensorial characteristics in comparison with common commercial products.

The poultry products complied with high quality parameters according to Mexican specifications. The chicken meat was considered to be at the *"Extra"* category established for products of high quality (NMX-FF-080-SCFI-2006) [22] and the eggs were classified in the *"Extra Mexico"* category, which describes fresh and high quality products (PROY-NOM-159-SSA1-2015) [23].

3.1. Anthropometric Parameters. Table 1 shows the results of anthropometric measurements at the baseline and after the intervention period. In general, both study groups had a BMI higher than 25. The experimental group had an average BMI of ≈28.1 and values considered as overweight or preobesity and the control group had values of ≈30.4 ± 4.7 that indicate obesity, according to the WHO classification [40]. In addition, the participants of both groups had high values of fat percentage (33–37%) and a waist circumference higher than 80 cm (around 90.7 to 96.6 cm). These data coincide with

Table 2: Biochemical parameters of experimental and control group.

	Week 0	Week 14
Experimental group (n = 14)		
Glucose (mg/dL)	91.1 ± 12.7^{a}	98.5 ± 17.2^{a}
Cholesterol (mg/dL)	142.2 ± 47.4^{a}	146.1 ± 39.3^{a}
Triglycerides (mg/dL)	102.0 ± 19.1^{a}	116.0 ± 18.2^{a}
HDL (mg/dL)	$51.9 \pm 12.5^{a*}$	$60.1 \pm 18.9^{b*}$
LDL (mg/dL)	69.9 ± 45.5^{b}	62.7 ± 41.9^{a}
Atherogenic index	2.9 ± 1.1^{b}	$2.6 \pm 1.1^{a*}$
Control group (n = 15)		
Glucose (mg/dL)	97.9 ± 14.4^{a}	97.9 ± 12.2^{a}
Cholesterol (mg/dL)	137.3 ± 35.8^{a}	138.1 ± 45.9^{a}
Triglycerides (mg/dL)	112.9 ± 26.3^{a}	119.5 ± 25.2^{a}
HDL (mg/dL)	$72.2 \pm 21.7^{b*}$	$54.2 \pm 19.9^{a*}$
LDL (mg/dL)	42.5 ± 33.3^{a}	59.2 ± 52.2^{b}
Atherogenic index	2.0 ± 0.66^{a}	$3.3 \pm 3.4^{b*}$

^{a,b}Different letters between column values (0 and 14th week) indicate significant difference ($p \leq 0.05$ Student's t-test). $*$ indicates significant differences between groups in the same column ($p = <0.05$).

current trends (ENSANUT 2006 and 2012) that at least 70% of the Mexican population is overweight and obesity [41].

After the 14th week intervention, both groups improved their anthropometric measurements with a decrease in body fat and an increase in fat-free mass, which could be due to the healthier production conditions of the poultry products (both supplemented and nonsupplemented) in comparison to the conditions of commercial products. The group who consumed supplemented foods with *omega-3* had a decrease in waist circumference after the intervention period, but there was no statistical significance between groups. It is possible that longer periods of study would be necessary to establish a significant difference between the two study groups. Other studies showing the improvement of anthropometric variables with the consumption of *omega-3* supplement foods or *omega-3* supplements (doses of ≈2 g/day) had intervention periods of 4-5 months [42, 43]. These studies had established that the supplementation with *omega-3* could inhibit the differentiation of preadipocytes and an increase of the apoptosis of these cells and/or the regulation of sympathetic nervous system and production of leptin and adiponectin causing the regulation of body fat which leads to changes in the distribution of the ratio of fat-free mass [43, 44].

3.2. Biochemical Parameters. At the beginning of the study, the participants had optimum biochemical parameters (glucose, triglycerides, cholesterol, HDL, and LDL) in accordance with the Procedures Manual of the Secretary of Health (SSA) in Mexico [29, 30] and these were maintained in normal conditions until the end of the intervention (Table 2). The statistical analysis showed differences with HDL (high density lipoprotein) levels between the groups at the beginning of the study, which could be affected by several individual factors of the study population (age, diet, stress, tobacco, alcohol, physical activity, etc.) [33, 45, 46], which must be considered in future supplementation studies. At the end of study, the concentrations of HDL showed a tendency to increase in experimental group (51.9 to 60.1 mg/dL) and LDL (low density lipoprotein) decreased (69.9 to 62.7 mg/dL) causing the reduction of atherogenic index (2.6 to 2), while the control group showed a contrary behavior. The value of the means difference of HDL was 25.47 mg/dL (CI 95% 4.076–46.882, p: 0.021). This represented an increase of 42.5% of the HDL at baseline concentration. These values reinforce the result that there was a positive impact of the supplementation. The effect of the *omega-3* had been found in other studies with supplement foods (yogurt, butter, and pate with fish oil) [47–49] accompanied by a significant reduction of LDL and an increase of HDL in blood.

Some authors have established that LDL reduction and HDL increase correlated with intake of *omega-3* could be due to a variety of mechanisms. The consumption of marine *omega-3* fatty acids could have a relatively neutral effect on LDL and HDL through targeted effects on specific transcription factors and nuclear receptors. Such is the case of PPAR (peroxisome proliferator-activated receptor) that increase HDL by enhancing reverse cholesterol transport [50]. According to some research, the fraction PON1 (paraoxonase), Clusterine, ApoAI, and ApoCIII present in HDL allow regulation in oxidation mechanisms of lipids metabolism [51, 52] and anti-inflammatory process [53] and decrease in proatherogenic lipoproteins (LDL, VLDL) [51, 54]. These functions play an important role in the prevention of atherosclerosis development and other cardiovascular diseases [12, 51, 52, 55]. In addition, other studies show that *omega-3* significantly reduce LDL due to the enzymatic inhibition of *acyl*-CoA: 1,2-diaglycerolacyltransferase impacting hepatic synthesis of triglycerides and LDL [49, 56], protecting the dysfunction of endothelial cells inhibiting lipogenesis and favoring lipolysis, increasing mitochondrial dynamics, and therefore causing a decrease of chronic diseases.

3.3. Cardiovascular Function Parameters. At the beginning of the study, the experimental and the control group reached

Table 3: Cardiovascular function parameters of experimental and control group.

	week 0	week 14
Experimental group (n = 14)		
Test duration (min)	9.8 ± 2.1[a]	10.3 ± 1.9[a]
Recovery time (min)	4.5 ± 1.7[a]	4.0 ± 1.3[a]
Electrocardiogram		
PQ (ms)	157.3 ± 17.2[a]	158.7 ± 15.8[a]
QRS (ms)	91.5 ± 11.8[a]	89.0 ± 9.0[a]
QT (ms)	413.0 ± 31.4[a]	404.1 ± 31.1[a]
QTc (ms)	408.7 ± 31.0[a]	416.5 ± 20.3[a]
Heart rate (beats/minute)		
Before test	82.4 ± 11.1[b]	74.3 ± 10.1[a]
At the test end	135.7 ± 15.9[a]	152.2 ± 15.0[b]
Blood pressure mm/Hg		
Systolic before test	110.5 ± 11.1[a]	106.1 ± 9.2[a]
Systolic at the test end	132.2 ± 11.1[a]	134.6 ± 12.4[a]
Diastolic before test	78.5 ± 7.4[b]	72.8 ± 6.1[a]
Diastolic at the test end	84.2 ± 12.8[b]	74.2 ± 7.3[a]
Control Group (n = 15)		
Test duration (min)	10.6 ± 3.7[a]	10.6 ± 3.5[a]
Recovery time (min)	4.8 ± 1.7[a]	4.5 ± 1.6[a]
Electrocardiogram		
PQ (ms)	158.0 ± 17.4[a]	156.4 ± 16.4[a]
QRS (ms)	98.2 ± 12.4[a]	96.5 ± 12.5[a]
QT (ms)	407.6 ± 36.1[a]	403.2 ± 4.0[a]
QTc (ms)	417.3 ± 22.2[a]	416.8 ± 23.4[a]
Heart rate		
Before test	74.3 ± 11.1[a]	78.2 ± 13.9[b]
At the test end	139.0 ± 19.1[a]	144.1 ± 22.1[a]
Blood pressure mm/Hg		
Systolic before test	109.6 ± 9.3[a]	107.0 ± 12.2[a]
Systolic at the test end	131.0 ± 12.5[a]	134.0 ± 25.6[a]
Diastolic before test	76.0 ± 8.7[a]	74.6 ± 7.4[a]
Diastolic at the test end	81.3 ± 7.4[a]	77.6 ± 10.4[a]

[a,b]Different letters between column values (0 and 14th week) indicate significant difference ($p \leq 0.05$ Student's t-test).

80% of their cardiac capacity at 10 minutes of physical effort without significant changes after the 14th week of study. In addition, the time range of the electrocardiographic segments (PQ, QRS, QT, and QTc) in both groups was normal at each evaluation time, discarding the presence of cardiac arrhythmias or other abnormalities that could affect the performance of the stress test during the evaluation period. The basal heart rate and arterial pressure of the study groups had normal ranges; however, after the 14th week only the experimental group showed a decrease in both parameters changing from normal ranges to optimum (heart rate, diastolic pressure before and after the test) (Table 3). The participants of the experimental group had less fatigue and less heart strain at the 14th week having a normalizing effect on the diastolic pressure according with other studies [57–59]. However, these changes were not statistically significant between the experimental group and the control group. Previously, we have mentioned that these differences could become significant if the time study had been extended.

Several studies [57–59] had shown less cardiac effort after supplementation with *omega-3*, which could be due to several mechanisms, mainly due to the function of these polyunsaturated fats on the permeability and fluidity of the cell membrane improving nervous impulse and muscular contraction. Other authors [60] have established that the aldosterone secretion, increased nitric oxide, and a higher production of prostaglandins are related to a reduction of platelet aggregation contributing with anti-inflammatory

process which allows the regulation of blood pressure and thus the improvement of cardiovascular function.

4. Conclusion

The consumption of supplemented poultry products with *omega-3* increased the HDL concentration and decreased atherogenic index related with cardiovascular diseases improving the overall health status in the test population. Therefore, poultry products supplemented with *omega-3* could be a viable alternative in populations where the consumption of fish products is low. Hence, it is important to replicate these studies with the extension of the time study and the inclusion of other foods.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Modification of Lipid Profile in Commercial Cow Milk Samples before and after Their Expiration Date: Evaluation of Storage Crucial Parameters and Possible Environmentally Friendly Disposal Alternatives

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Milk waste is considered a highly polluting material and its disposal is an economic and environmental problem for the dairy sector. Despite this, it can be turned into a source of nutraceutical products and biodiesel substrate. The objective of this study was to determine the qualitative and quantitative variation of single fatty acids in cow milk samples before expiration date and within 28 days after expiration date in order to monitor how the profile of the lipid fraction is influenced by different physicochemical parameters. It was shown that lipolysis in milk is a process independent of time but dependent on the values of pH and total titratable acidity, while crucial parameters for the lipid oxidation are temperature and time of exposure to atmospheric oxygen. All of these factors are at the basis of the efficacy of milk storage conditions. Moreover, our data demonstrate that milk, several weeks after its expiry date, is a rich source of fatty acids that may be recovered as potential substrates for the formulation of economically viable products and eco-friendly diesel-like fuels.

1. Introduction

Milk is considered a complete food, with true nutraceutical properties. It contains a wide range of bioactive compounds that exert healthy properties to both neonates and humans [1] such as proteins, sugars, mineral salts, vitamins, and lipids. Nutraceutical formulations are often based on milk bioactive peptides, which are usually latent and become active when released after proteolysis of the parent proteins [2]. Nevertheless, lipids represent another important component of milk, being able to influence the physical, organoleptic, and nutritional properties of dairy products and playing an important role in human health promotion and diseases prevention. However, lipid composition of dairy products undergoes far-reaching changes during storage, which can reduce the shelf life and quality of milk and modify the overall flavor [3]. For these reasons, milk is increasingly subjected to quality control and safety assessment [4] through the evaluation of the chemical composition and purity as well as levels of different microorganisms which are responsible for

the lipolysis and lipid oxidation phenomena. Lipolysis in milk consists in the hydrolysis of triglycerides into free fatty acids (FFA) and partial glycerides. This process can be mediated by the lipoprotein lipases (LPLs) naturally occurring in milk or by the microbial lipases from psychrotrophic bacteria contaminating the raw milk during cold storage [5, 6]. Since LPLs are relatively unstable to heat, pasteurization inactivates most of the enzyme, so that lipolysis rate is significantly decreased in pasteurized homogenized milk [7]. The release of short- and medium-chain FFA (from C_4 to C_{10}) and their subsequent conversion to other acids and/or ethyl esters by microbial lipases produce detrimental features such as rancid smell and taste and functionality defects [8].

Milk fatty acid composition is a key factor determining its storage condition. Cow milk, similar to sheep and goat milk, contains almost 3 times less C14:0 (myristic) acid and 2 times more C16:0 (palmitic) acid than buffalo milk [9]. It has been reported that cow milk fat in comparison to goat milk fat contains 54.6% less C6:0 acid, 69.9% C8:0, 80.2% C10:0, and 56.3% CLA and 75% more C4:0 acid [9]. Donkey milk contains several times more saturated fatty acids (SFAs) (C8:0, C10:0, and C12:0), twice less of C14:0 and C16:0 fatty acids, and ten times less of (C18:0) stearic acid (1.12%) than cow. In donkey milk, the amount of C18:1 (oleic) acid is 3 times smaller than in milk of other species. Among common species, donkey milk is one of the richest in polyunsaturated fatty acids (PUFAs), C18:2, and C18:3 (linoleic and linolenic) [9].

Milk fatty acid composition mainly depends on the nutrition of the animals [10, 11]. Actually, many strategies have been adopted in the attempt to adjust the fatty acid profile of milk in order to ameliorate its nutritional quality and to increase its benefits on human health. Dairy cows have been fed on oils and mixture of vegetable fats to increase the content of PUFAs in milk [12, 13]. Nevertheless, any increase in PUFA content had negative effects on milk, making it more susceptible to oxidation [14]. In fact, lipid oxidation in milk is highly influenced by its content of unsaturated fatty acids at a rate that especially depends on the degree of unsaturation (Timmons et al. (2001) [15]). Therefore, dairy products from cows fed on diets rich in PUFAs are prone to oxidation if no precautions against oxidation are taken, such as adding natural antioxidants to the raw milk [16, 17]. Moreover, numerous nutraceutical formulations for the treatment of oxidative stress conditions [18] and cardiovascular diseases [19] are based on natural phytochemical extracts such as polyphenols [20, 21] and carotenoids also recovered from food by-products [22] together with PUFA from dairy wastewater, characterized by high fat content. In large dairy industries, while cleaning the equipment, the residual butter and related fats are washed out and collected in the effluent treatment plant. This by-product is usually disposed as solid waste or incinerated. However, this strategy is not economically advantageous and generates pollutants. One possible solution could be to convert the dairy wastewater into biodiesel and use it as an alternative fuel [23]. Recently, global attention has been focusing on food industry waste products as fat sources for developing safe, alternative fuels that are economically viable and environmentally friendly [24]. Biodiesel, which is defined as a non-petroleum-based diesel fuel, is a mono-alkyl (methyl, propyl, or ethyl) ester of long chain fatty acids. Biodiesel is regarded as an alternative fuel with similar performance characteristics to petroleum diesel. It is produced by reacting fats and oils (e.g., vegetable oils and animal fats) with alcohol in the presence of a catalyst. The feedstock for biodiesel is largely triglyceride oils which may be edible vegetable oils such as soybean (commonly used for biodiesel production in the United States) and rapeseed (canola) oil (major feedstock for its production in Europe). However, the global emphasis has been focusing on the production of biodiesel from low-cost feedstock, such as waste cooking oil (WCO), which is more economical and environmentally friendly [25]. Similarly, other fat-based waste products may be taken into consideration for the production of alternative fuels such as dairy waste.

In the light of these considerations, the objective of this study was to determine the qualitative and quantitative variation of single fatty acids in cow milk samples before expiration date and within 28 days after expiration date in order to monitor how the profile of the lipid fraction is influenced by different physicochemical parameters.

2. Materials and Methods

2.1. Materials. Acetonitrile, clarifying reagent for dairy products, ethylenediaminetetraacetic acid, *N*,*N*-dimethylformamide, *n*-hexane, 4-nitrophenol, 4-nitrophenyl butyrate, phenolphthalein, phenylmethanesulfonyl fluoride, 2-propanol, sodium sulfate, sodium hydroxide, and Tris-buffered saline (TBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sampling and Sample Preparation. Commercial samples of bovine milk (skimmed, semiskimmed, and whole) from Centrale del latte di Salerno (CdLdS) (Salerno, Campania, Italy) were analyzed; samples were preserved at room temperature and the corresponding fatty acids were extracted at different weeks after expiration date. The lipid fraction was obtained from milk by liquid-liquid extraction (LLE). An aliquot of 15 mL of milk was treated with 50 mL of a mixture of isopropanol and *n*-hexane (3 : 2 v/v). This solution was stirred vigorously for 10–15 min and the hexane phase was recovered. The extraction procedure was repeated twice and the combined hexane fractions were dehydrated first with 15 mL of 0.47 M Na_2SO_4 solution and then treated with Na_2SO_4 (3 g) for final dehydration. After solvent removal under vacuum, the lipid extract was dried at 40°C to constant weight. An aliquot of 50 μg of lipid extract was dissolved in dry toluene (1 mL) and then treated with 1 M sodium methoxide in dry methanol (1 mL) to convert fatty acids and complex lipids into their corresponding methyl esters (FAMEs) and analyzed by GC-MS.

2.3. Gas Chromatography-Mass Spectrometry Analysis. Gas chromatography-mass spectrometry analyses (GC-MS) were carried out employing an Agilent 6850 Series II apparatus coupled to an Agilent Mass Selective Detector (MSD) 5273. A fused silica HP-5MS capillary column was used (30 m

× 0.25 mm i.d., 0.33 μm film thickness). MS detection was performed using an ionization voltage of 70 eV, an electron multiplier energy of 2000 V, and a scan range of 40–550. Injector and detector temperatures were set at 250 and 280°C, respectively. Column temperature was set at 140°C for 10 min, followed by a first ramp of 15°C/min until 200°C. This temperature was maintained for 1 min; then a second ramp of 10°C/min was used until 230°C. After 1 min, a third ramp of 0.4°C/min was applied until the temperature of 233°C. After 3 min, the final ramp of 0.5°C/min was used till reaching the final temperature of 238°C and maintained for further 2 minutes. Total analysis time was 41.50 min. Gases flow rates (White Martins) were 30 mL/min for hydrogen, 30 mL/min for nitrogen, and 250 mL/min for synthetic air. Each injection (1.2 μL) was performed in duplicate. The peak areas of FAMEs were determined using ChromQuest 4.1 software.

2.4. Acidity and pH Analysis. The pH value and total acidity were determined in commercial milk samples (skimmed, semiskimmed, and whole milk) at different weeks after their expiration date. The pH values were measured using a digital pH meter of a glass electrode (BASIC 20, Crison). Prior to use, the pH meter was calibrated with standard buffer solution (pH 4 and 7). Total titratable acidity (TA) was determined according to the AOAC method [26]. TA was determined by measuring the volume (mL) of 0.25 N sodium hydroxide required to titrate 100 mL of milk sample, using phenolphthalein as an indicator (five drops). The milk sample was titrated to a faint pink color that persisted after vigorous shaking (end-point). The volume of titrant consumed corresponded to the amount of lactic acid present in the sample. The results were expressed in Soxhlet-Henkel degree (°SH).

2.5. Total Lipase Activity Assay. A spectrophotometric method was developed in order to determine the total activity of the lipase (TLA) in milk samples, thus determining the amount of free fatty acids (FFA) liberated from the triglycerides contained in bovine milk [27]. Through this method it is possible to quantify the level of *p*-nitrophenol ($A_{\max}$ 400 to 420 nm) released after the lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate, used as a substrate. The analysis was performed on different milk samples (skimmed, semiskimmed, and whole milk) at different weeks after their expiration date. The lipase activity was assayed by detecting *p*-nitrophenol on a Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., San Jose, California). The analyses were carried out at room temperature and the absorbance was measured at 420 nm. *p*-Nitrophenol was liberated by incubating 500 μL of milk sample with 50 μL of 0.05 mM *p*-nitrophenol butyrate standard solution and 2 mL of 0.05 M Tris buffer, pH 7.6, at 37°C for exactly 30 min. The reaction was terminated by incubation at 37°C for 10 min with 400 μL of a solution composed by 3 : 1 (v/v) 0.06 M EDTA, pH 7.6, in NaOH 2 N and phenylmethanesulfonyl fluoride (PMSF) 0.06 M in dimethylformamide. This solution was diluted with 2 mL of clarifying reagent solution at 37°C for 5 min to render casein micelles and fat globules soluble and allow direct spectrophotometric measurements without preliminary separation. The final solution was analyzed at 420 nm against corresponding solvent blank that was prepared in the same assay conditions, except that lipase activity inhibitors were added to the milk sample before adding the substrate solution. Lipase activity was determined by comparing sample A_{420} values with those of a standard curve prepared with *p*-nitrophenol.

2.6. Data Analysis. The experimental results were expressed as mean ± standard deviation (SD, $n \geq 3$). Statistical analysis of data was performed by Student's *t*-test or by two-way ANOVA followed by the Tukey-Kramer multiple comparison post hoc test. The level of significance (α-value) was 95% ($P < 0.05$). The degree of linear relationship between two variables was measured using the Pearson product moment correlation coefficient (R). Correlation coefficients (R) were calculated using Microsoft Office Excel.

3. Results and Discussion

3.1. Lipase Activity in the Milk Samples. Lipases are an important group of enzymes, being associated with fat metabolism. In particular, these enzymes hydrolyse the glycerol esters of fatty acids at the oil/water interface and are responsible for the production of undesirable rancid flavors [28]. The activity of lipase in milk is highly correlated to its acidity, a determining factor for assessing the quality of milk products. There are two main conventions for expressing acidity in dairy products: titratable acidity and pH. Titratable acidity (TA) plays a fundamental role in all phases of milk coagulation. This includes the aggregation rate of para-casein micelles and the reactivity of rennet. TA also influences the rate of syneresis and determines the suitability of milk for cheese making. The pH of milk affects not only the enzymatic reactions but also its colloidal stability. Table 1 shows the pH and TA values of 15 commercial milk samples analyzed at several weeks after their expiry date. The results show that pH values were in the range of 6.71–4.23 in skimmed milk, 6.72–4.28 in semiskimmed milk, and 6.72–4.61 in whole milk. The values of TA were in the range of 6.36–58.15 in skimmed milk, 6.17–52.22 in semiskimmed milk, and 6.17–56.75 in whole milk. This decrease in pH was attributed to increased lactic acid levels produced by fermenting bacteria. TA also increased for the same reasons.

Both the pH values and the degree of acidity did not change in a regular manner over time. This could probably be ascribed to the different types of fermentation (both acidic and putrefactive) which can occur in milk, since its initial microbial load is heterogeneous. However, as shown in Table 1, TA was inversely correlated with the lipase activity level ($R = 0.9178$, 0.8545 and 0.8392 for skimmed, semiskimmed, and whole milk, resp.) that has been demonstrated to be highly sensitive to pH. Most of lipases in milk have pH optimum values of 8.5–9.0, while other lipases have pH optimum values ranging from 6.5 to 7.9. In the milk samples analyzed, lipase activity was significantly low at acidic pH values. In fact, at pH values of 6.71, 5.85, 4.91, 4.33, and 4.23, the amount of *p*-nitrophenol generated in skimmed milk was 70.8%, 57.1%, 18.3%, and 1.7%, respectively.

Table 1: pH, titratable acidity, and lipase activity of skimmed, semiskimmed, and whole milk samples at four weeks after their expiration date.

	Milk samples	$T0$	Week I	Week II	Week III	Week IV
pH value*	Skimmed milk	6.71 ± 0.01^{a}	4.33 ± 0.01^{d}	4.23 ± 0.01^{e}	4.91 ± 0.01^{c}	5.85 ± 0.02^{b}
	Semiskimmed milk	6.72 ± 0.02^{a}	5.51 ± 0.01^{b}	4.55 ± 0.01^{c}	4.28 ± 0.01^{e}	4.41 ± 0.01^{d}
	Whole milk	6.72 ± 0.02^{a}	5.38 ± 0.05^{b}	4.61 ± 0.01^{e}	5.22 ± 0.02^{c}	4.73 ± 0.01^{d}
Titratable acidity*,#	Skimmed milk	6.36 ± 0.03^{e}	35.57 ± 0.49^{b}	58.15 ± 0.46^{a}	28.93 ± 0.40^{c}	16.70 ± 0.01^{d}
	Semiskimmed milk	6.17 ± 0.03^{e}	23.41 ± 0.40^{d}	52.22 ± 0.08^{a}	37.00 ± 0.50^{c}	41.66 ± 0.31^{b}
	Whole milk	6.17 ± 0.10^{e}	30.20 ± 0.05^{c}	56.75 ± 0.03^{a}	20.56 ± 0.47^{d}	45.45 ± 0.19^{b}
Lipase activity*,§	Skimmed milk	0.024 ± 0.007^{a}	0.0044 ± 0.0030^{d}	0.0004 ± 0.0005^{e}	0.0137 ± 0.0120^{c}	0.017 ± 0.011^{b}
	Semiskimmed milk	0.011 ± 0.003^{a}	0.0037 ± 0.0021^{b}	0.0008 ± 0.0005^{c}	0.0011 ± 0.0004^{c}	0.001 ± 0.005^{c}
	Whole milk	0.006 ± 0.001^{a}	0.0012 ± 0.0011^{c}	0.0001 ± 0.0005^{d}	0.0054 ± 0.0010^{b}	0.001 ± 0.001^{c}

*Milk samples were analyzed in triplicate. Values are expressed ± SD. #Data were expressed in Soxhlet-Henkel degree. §Data were expressed as μmol of *p*-nitrophenol/mL reaction mixture. Means in the same row followed by different inline letters (a, b, c, d, and e) are statistically different according to Tukey's HSD test ($P < 0.05$).

Conversely, the percentage of lipase activity in whole milk decreased by 10.0%, 80.0%, 83.3%, and 98.3%, corresponding to increasing TA values. In contrast to other enzymes, the rate at which a lipase-catalyzed reaction proceeds is not governed by the substrate concentration at the interface between the lipid substrate and the aqueous phase of an emulsion, but it is dependent on the amount of free fatty acids, which are released by disruption of the milk fat globule, exposing the lipid substrate to the lipase [29].

3.2. Fatty Acid Profile of Milk before Expiration Date. The fatty acid composition of skimmed, semiskimmed, and whole milk samples before their expiration date is reported in Table 2. The saturated fatty acids accounted for 64.3, 47.3, and 42.9% of total fatty acids in whole, semiskimmed, and skimmed milk, respectively. The most abundant among saturated fatty acids was palmitic acid (16:0) (43%), followed by myristic acid (14:0) (16%), stearic acid (18:0) (17.5%), and short-chain fatty acids (C4:0–C10:0) (15%) in all milk samples. Monounsaturated fatty acids represented 31.0, 46.8, and 51.7% of total fatty acids in whole, semiskimmed, and skimmed milk, respectively. Oleic acid (18:1) was the main monounsaturated fatty acid, accounting approximately for 77.5%. Polyunsaturated fatty acids accounted for 4.7, 5.9, and 5.4 of total fatty acids in whole, semiskimmed, and skimmed milk, respectively. Among them, the main constituents were dihomo-gamma-linolenic acid (20:3*n*-6) (28.4%) and conjugated linoleic acid (CLAc9t11) (23.8%). The ratio between *n*-6 and *n*-3 fatty acids in whole and semiskimmed milk was 2,1 : 1, while in skimmed milk it was 2,3 : 1.

3.3. Fatty Acid Profile after Expiration Date. Analysis of milk samples carried out at different weeks after their expiration date showed a gradual decrease in their amount of unsaturated fatty acids, while unchanged quantities of saturated fatty acids were detected. After first, second, third, and fourth weeks, the percentage of degradation of both monounsaturated and polyunsaturated fatty acids of whole and semiskimmed milk was 25%, 44%, 58%, and 69%, respectively (Tables 3 and 4). The degradation process of some unsaturated fatty acids in skimmed milk was not constant as that observed for the other samples (Table 5). The amount of *trans*-9 *cis*-12 octadienoic acid after one week was unchanged, while alpha-linolenic acid remained constant up to two weeks. Linolelaidic, heptadec-10-enoic and *cis*-vaccenic acids decreased by 20% at first week, 40% at second week, and 60% at third and fourth weeks.

On the basis of the above-mentioned data, it is evident that the amount of unsaturated fatty acids decreased over time, apparently independent of pH, titratable acidity, and type of milk. On the contrary, crucial parameters for the fatty acid degradation were storage temperature and time of exposure to atmospheric oxygen, at the base of the lipid oxidation process in milk samples.

Knowing how to dispose of waste milk properly is an essential part of dairy industry management. Serious problems can develop when waste milk is handled incorrectly. These include damage to the environment and, potentially, a failed wastewater treatment system. In fact, improper handling of waste milk is the main reason why wastewater systems fail. Due to its high organic compound and phosphorus content, milk can promote the growth of algae and aquatic plants which increase the oxygen demand and the likelihood of fish death. Several milk disposal methods have been recently proposed in order to face the environmental pollution question, mainly: feeding to livestock, use as a soil fertilizer, and manufacturing soaps.

Actually, the employment of waste milk as a source for alternative biofuels has recently gained general interest. Lateef et al. [30] have shown that inclusion of waste milk as a cosubstrate during biohydrogen production from cow manure could improve hydrogen production that is readily turned into methane, the final product of anaerobic digestion process. Nevertheless, biodiesel which is derived

Table 2: Fatty acids composition (MG/G total fatty acids) of the commercial samples of bovine milk (skimmed, semiskimmed, and whole) from Centrale del Latte di Salerno before their expiration date.

Fatty acids	Whole milk	Semiskimmed milk	Skimmed milk
*Saturated**	*589.04 ± 0.58*^{a}	*111.31 ± 0.30*^{b}	*2.99 ± 0.24*^{c}
Butyric (C4:0)	34.25 ± 0.74^{a}	6.39 ± 0.21^{b}	0.17 ± 0.05^{c}
Caproic (C6:0)	21.29 ± 0.39^{a}	3.97 ± 0.27^{b}	0.11 ± 0.06^{c}
Caprylic (C8:0)	11.77 ± 0.27^{a}	2.19 ± 0.32^{b}	0.06 ± 0.07^{c}
Capric (C10:0)	22.46 ± 0.37^{a}	4.19 ± 0.43^{b}	0.11 ± 0.08^{c}
Lauric (C12:0)	25.52 ± 0.42^{a}	4.76 ± 0.54^{b}	0.13 ± 0.09^{c}
Tridecanoic acid (C13:0)	0.84 ± 0.57^{a}	0.16 ± 0.05^{b}	0.004 ± 0.001^{c}
Myristic (C14:0)	93.78 ± 0.66^{a}	17.51 ± 0.76^{b}	0.48 ± 0.40^{c}
Pentadecanoic (C15:0)	13.80 ± 0.71^{a}	2.58 ± 0.88^{b}	0.07 ± 0.01^{c}
Palmitic (C16:0)	251.70 ± 0.85^{a}	46.99 ± 0.92^{b}	1.28 ± 0.47^{c}
Margaric (C17:0)	8.07 ± 0.74^{a}	1.51 ± 0.84^{b}	0.04 ± 0.03^{c}
Stearic (C18:0)	103.90 ± 0.68^{a}	19.40 ± 0.74^{b}	0.53 ± 0.21^{c}
Arachidic (C20:0)	1.66 ± 0.51^{a}	1.66 ± 0.65^{a}	0.008 ± 0.003^{b}
*Monounsaturated**	*283.68 ± 0.44*^{a}	*110.13 ± 0.56*^{b}	*3.61 ± 0.43*^{c}
Myristoleic (C14:1)	8.99 ± 0.32^{a}	3.49 ± 0.47^{b}	0.09 ± 0.04^{c}
Palmitoleic (C16:1)	12.13 ± 0.26^{a}	4.70 ± 0.50^{b}	0.12 ± 0.05^{c}
Heptadec-10-enoic acid (C17:1)	5.60 ± 0.18^{a}	2.17 ± 0.33^{b}	0.05 ± 0.06^{c}
Elaidic (C18:1*n*-9t)	25.51 ± 0.25^{a}	9.89 ± 0.27^{b}	0.27 ± 0.04^{c}
Oleic (C18:1*n*-9c)	216.70 ± 0.34^{a}	84.05 ± 0.11^{b}	2.92 ± 0.87^{c}
Vaccenic acid (C18:1*n*-7t)	9.47 ± 0.47^{a}	3.78 ± 0.57^{b}	0.10 ± 0.04^{c}
cis-Vaccenic acid (C18:1*n*-7c)	5.28 ± 0.50^{a}	2.05 ± 0.63^{b}	0.056 ± 0.004^{c}
*Polyunsaturated**	*42.88 ± 0.63*^{a}	*13.97 ± 0.70*^{b}	*0.38 ± 0.35*^{c}
Linolelaidic (C18:2*n*-6t)	5.56 ± 0.72^{a}	1.81 ± 0.81^{b}	0.05 ± 0.04^{c}
trans-9, *cis*-12 octadienoic acid (C18:2t9c12)	2.54 ± 0.92^{a}	0.83 ± 0.09^{b}	0.02 ± 0.01^{c}
Gamma-linoleic (C18:2*n*-6)	8.01 ± 0.84^{a}	2.61 ± 0.79^{b}	0.07 ± 0.03^{c}
Alpha-linolenic (C18:3*n*-3)	3.82 ± 0.79^{a}	1.24 ± 0.68^{b}	0.03 ± 0.07^{c}
Dihomo-gamma-linolenic acid (C20:3*n*-6)	11.99 ± 0.60^{a}	3.91 ± 0.57^{b}	0.11 ± 0.05^{c}
Conjugated linoleic acid (CLAc9t11)	10.18 ± 0.53^{a}	3.32 ± 0.46^{b}	0.09 ± 0.04^{c}
Conjugated linoleic acid (CLAt10c12)	0.78 ± 0.04^{a}	0.25 ± 0.03^{b}	0.007 ± 0.001^{c}

*Milk samples were analyzed in triplicate. Values are expressed ± SD. Means in the same row followed by different inline letters (a, b, and c) are statistically different according to Tukey's HSD test ($P < 0.05$).

from triglycerides by transesterification has been attracting considerable attention as a renewable, biodegradable, eco-friendly, and nontoxic fuel [24]. Biodiesel is currently more expensive than pure diesel, since costs of biodiesel production are highly dependent on the costs of feedstock [31, 32]. At present, partially or fully refined and edible-grade vegetable oils such as soybean, rapeseed, sunflower, and palm oil are the predominant feedstock for biodiesel production, which obviously results in the high price of biodiesel. Therefore, waste edible oil such as waste cooking oil seems to be a good substitute in order to reduce cost [33]. Our data (Tables 3–5) demonstrated that expired milk is still rich in those fatty acids that are the main constituents of the waste cooking oils used for the biodiesel production, such as palmitic, myristic, and oleic acids [34]. What is more, a significant amount of these glycerides in the expired milk already occurs in a hydrolysed form so that minor quantities of catalysts than employed for waste cooking oils for the esterification and transesterification reactions would be necessary, partially solving an economical and environmental question.

4. Conclusion

In conclusion, the present study showed that lipolysis in milk is a process independent of time but dependent on the values of pH and TA, while crucial parameters for the lipid oxidation are temperature and time of exposure to atmospheric oxygen. All of these factors are at the base of the efficacy of milk storage conditions. Moreover, our data demonstrated that milk several weeks after its expiration date is a rich source of fatty acids that may be recovered as a potential substrate for the formulation of alternative diesel-like fuels.

Table 3: Fatty acid profile (MG/G total fatty acids) of whole milk samples at different weeks after their expiration date.

Whole milk	*T*0	Week I	Week II	Week III	Week IV
Monounsaturated[*]					
Myristoleic (C14:1)	8.99 ± 0.32^{a}	6.74 ± 0.49^{b}	5.06 ± 0.23^{c}	3.79 ± 0.36^{d}	2.84 ± 0.32^{e}
Palmitoleic (C16:1)	12.13 ± 0.41^{a}	9.10 ± 0.55^{b}	6.82 ± 0.34^{c}	5.12 ± 0.48^{d}	3.84 ± 0.32^{e}
Heptadec-10-enoic acid (C17:1)	5.60 ± 0.58^{a}	4.20 ± 0.32^{b}	3.15 ± 0.45^{c}	$2.36 \pm 0.88^{c,d}$	1.77 ± 0.17^{d}
Elaidic (C18:1*n*-9t)	25.51 ± 0.60^{a}	19.13 ± 0.32^{b}	14.35 ± 0.56^{c}	10.76 ± 0.93^{d}	8.07 ± 0.27^{e}
Oleic (C18:1*n*-9c)	216.70 ± 1.18^{a}	162.53 ± 0.62^{b}	121.89 ± 1.05^{c}	91.42 ± 1.01^{d}	68.57 ± 0.93^{e}
Vaccenic acid (C18:1*n*-7t)	9.47 ± 0.72^{a}	7.10 ± 0.73^{b}	5.33 ± 0.66^{c}	4.00 ± 0.13^{d}	3.00 ± 0.22^{e}
cis-Vaccenic acid (C18:1*n*-7c)	5.28 ± 0.86^{a}	$3.96 \pm 0.48^{a,b}$	$2.97 \pm 0.74^{b,c}$	$2.23 \pm 0.14^{c,d}$	1.67 ± 0.24^{d}
Polyunsaturated[*]					
Linolelaidic (C18:2*n*-6t)	5.56 ± 0.76^{a}	$4.17 \pm 0.74^{a,b}$	$3.13 \pm 0.83^{b,c}$	$2.35 \pm 0.28^{c,d}$	1.76 ± 0.33^{d}
trans-9, *cis*-12 octadienoic acid (C18:2t9c12)	2.54 ± 0.64^{a}	$1.91 \pm 0.66^{a,b}$	$1.43 \pm 0.37^{b,c}$	$1.07 \pm 0.37^{c,d}$	0.80 ± 0.36^{d}
Gamma-linoleic (C18:2*n*-6)	8.01 ± 0.53^{a}	6.01 ± 0.59^{b}	4.51 ± 0.58^{c}	3.38 ± 0.46^{d}	2.53 ± 0.21^{e}
Alpha-linolenic (C18:3*n*-3)	3.82 ± 0.41^{a}	$2.87 \pm 0.40^{a,b}$	$2.15 \pm 0.49^{b,c}$	$1.61 \pm 0.45^{c,d}$	1.21 ± 0.27^{d}
Dihomo-gamma-linolenic acid (C20:3*n*-6)	11.99 ± 0.37^{a}	8.99 ± 0.33^{b}	6.74 ± 0.31^{c}	5.06 ± 0.55^{d}	3.79 ± 0.46^{e}
Conjugated linoleic acid (CLAc9t11)	10.18 ± 0.22^{a}	7.64 ± 0.28^{b}	5.73 ± 0.22^{c}	4.29 ± 0.68^{d}	3.22 ± 0.68^{d}
Conjugated linoleic acid (CLAt10c12)	0.78 ± 0.34^{a}	$0.59 \pm 0.16^{a,b}$	$0.44 \pm 0.32^{b,c}$	$0.33 \pm 0.23^{b,c}$	0.25 ± 0.22^{c}

[*] Milk samples were analyzed in triplicate. Values are expressed ± SD. Means in the same row followed by different inline letters (a, b, c, d, and e) are statistically different according to Tukey's HSD test ($P < 0.05$).

Table 4: Fatty acid profile (MG/G total fatty acids) of semiskimmed milk samples at different weeks after their expiration date.

Semiskimmed milk	*T*0	Week I	Week II	Week III	Week IV
Monounsaturated[*]					
Myristoleic (C14:1)	3.49 ± 0.23^{a}	2.62 ± 0.32^{b}	1.96 ± 0.21^{c}	$1.47 \pm 0.37^{c,d}$	1.10 ± 0.63^{d}
Palmitoleic (C16:1)	4.70 ± 0.34^{a}	3.53 ± 0.47^{b}	2.64 ± 0.32^{c}	$1.98 \pm 0.47^{c,d}$	1.49 ± 0.74^{d}
Heptadec-10-enoic acid (C17:1)	2.17 ± 0.45^{a}	1.63 ± 0.14^{b}	1.22 ± 0.17^{c}	$0.92 \pm 0.26^{c,d}$	0.69 ± 0.53^{d}
Elaidic (C18:1*n*-9t)	9.89 ± 0.56^{a}	7.42 ± 0.25^{b}	5.56 ± 0.56^{c}	4.17 ± 0.75^{d}	3.13 ± 0.94^{d}
Oleic (C18:1*n*-9c)	84.05 ± 1.05^{a}	63.04 ± 0.37^{b}	47.28 ± 0.32^{c}	35.46 ± 0.64^{d}	26.59 ± 0.85^{e}
Vaccenic acid (C18:1*n*-7t)	3.78 ± 0.66^{a}	2.84 ± 0.44^{b}	2.13 ± 0.33^{c}	$1.59 \pm 0.53^{c,d}$	$1.20 \pm 0.77^{c,d}$
cis-Vaccenic acid (C18:1*n*-7c)	2.05 ± 0.74^{a}	1.54 ± 0.35^{a}	$1.15 \pm 0.63^{a,b}$	$0.86 \pm 0.46^{a,b}$	0.65 ± 0.63^{b}
Polyunsaturated[*]					
Linolelaidic (C18:2*n*-6t)	1.81 ± 0.83^{a}	$1.36 \pm 0.46^{a,b}$	$1.02 \pm 0.44^{a,b}$	$0.76 \pm 0.32^{a,b}$	0.57 ± 0.50^{a}
trans-9, *cis*-12 octadienoic acid (C18:2t9c12)	0.83 ± 0.17^{a}	0.62 ± 0.05^{a}	$0.47 \pm 0.57^{a,b}$	$0.35 \pm 0.29^{a,b}$	0.26 ± 0.17^{b}
Gamma-linoleic (C18:2*n*-6)	2.61 ± 0.28^{a}	1.96 ± 0.14^{b}	1.47 ± 0.63^{c}	$1.10 \pm 0.16^{c,d}$	0.83 ± 0.46^{d}
Alpha-linolenic (C18:3*n*-3)	1.24 ± 0.49^{a}	0.93 ± 0.17^{a}	$0.70 \pm 0.71^{a,b}$	0.52 ± 0.24^{b}	0.39 ± 0.32^{b}
Dihomo-gamma-linolenic acid (C20:3*n*-6)	3.91 ± 0.31^{a}	2.93 ± 0.22^{b}	$2.20 \pm 0.83^{b,c}$	$1.65 \pm 0.37^{c,d}$	1.24 ± 0.30^{d}
Conjugated linoleic acid (CLAc9t11)	3.32 ± 0.12^{a}	2.49 ± 0.25^{b}	1.87 ± 0.21^{c}	$1.40 \pm 0.43^{c,d}$	1.05 ± 0.18^{d}
Conjugated linoleic acid (CLAt10c12)	0.25 ± 0.02^{a}	$0.19 \pm 0.04^{a,b}$	$0.14 \pm 0.14^{a,b}$	$0.11 \pm 0.08^{b,c}$	0.08 ± 0.03^{c}

[*] Milk samples were analyzed in triplicate. Values are expressed ± SD. Means in the same row followed by different inline letters (a, b, c, d, and e) are statistically different according to Tukey's HSD test ($P < 0.05$).

Additional Points

Practical Applications. The present work can contribute to clarifying the factors that are at the base of the efficacy of milk storage conditions. Data regarding the milk fatty acid profile after its expiration date may encourage the recovery and use of this waste product as a potential substrate for the formulation of alternative diesel-like fuels.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Table 5: Fatty acid profile (MG/G total fatty acids) of skimmed milk samples at different weeks after their expiration date.

Skimmed milk	*T*0	Week I	Week II	Week III	Week IV
Monounsaturated[*]					
Myristoleic	0.09 ± 0.02^{a}	0.07 ± 0.04^{a}	0.05 ± 0.03^{a}	0.04 ± 0.04^{a}	0.03 ± 0.07^{a}
Palmitoleic	0.12 ± 0.03^{a}	0.09 ± 0.05^{a}	0.07 ± 0.07^{a}	0.05 ± 0.05^{a}	0.04 ± 0.06^{a}
Heptadec-10-enoic acid	0.05 ± 0.05^{a}	0.04 ± 0.02^{a}	0.03 ± 0.03^{a}	0.02 ± 0.01^{a}	0.02 ± 0.04^{a}
Elaidic	0.27 ± 0.06^{a}	$0.20 \pm 0.06^{a,b}$	$0.15 \pm 0.07^{b,c}$	$0.11 \pm 0.01^{c,d}$	0.09 ± 0.07^{d}
Oleic	2.92 ± 0.07^{a}	2.19 ± 0.07^{b}	1.64 ± 0.14^{c}	1.23 ± 0.22^{d}	0.92 ± 0.04^{e}
Vaccenic acid	0.10 ± 0.08^{a}	0.08 ± 0.02^{a}	0.06 ± 0.01^{a}	0.04 ± 0.06^{a}	0.03 ± 0.05^{a}
cis-Vaccenic acid	0.06 ± 0.04^{a}	0.04 ± 0.03^{a}	0.03 ± 0.02^{a}	0.02 ± 0.07^{a}	0.02 ± 0.03^{a}
Polyunsaturated[*]					
Linolelaidic	0.05 ± 0.05^{a}	0.04 ± 0.02^{a}	0.03 ± 0.02^{a}	0.02 ± 0.02^{a}	0.02 ± 0.08^{a}
trans-9, *cis*-12 octadienoic acid	0.02 ± 0.05^{a}	0.02 ± 0.02^{a}	0.01 ± 0.01^{a}	0.01 ± 0.01^{a}	0.01 ± 0.01^{a}
Gamma-linoleic	0.07 ± 0.04^{a}	0.05 ± 0.01^{a}	0.04 ± 0.02^{a}	0.03 ± 0.01^{a}	0.02 ± 0.08^{a}
Alpha-linolenic	0.03 ± 0.03^{a}	0.02 ± 0.02^{a}	0.02 ± 0.02^{a}	0.01 ± 0.01^{a}	0.01 ± 0.07^{a}
Dihomo-gamma-linolenic acid	0.11 ± 0.02^{a}	$0.08 \pm 0.02^{a,b}$	$0.06 \pm 0.03^{b,c}$	$0.05 \pm 0.08^{b,c}$	0.03 ± 0.03^{c}
Conjugated linoleic acid	0.09 ± 0.02^{a}	$0.07 \pm 0.04^{a,b}$	0.05 ± 0.01^{b}	0.04 ± 0.07^{b}	0.03 ± 0.01^{b}
Conjugated linoleic acid	0.007 ± 0.004^{a}	0.005 ± 0.004^{a}	0.004 ± 0.001^{a}	0.003 ± 0.001^{a}	0.002 ± 0.001^{a}

[*] Milk samples were analyzed in triplicate. Values are expressed ± SD. Means in the same row followed by different inline letters (a, b, c, d, and e) are statistically different according to Tukey's HSD test ($P < 0.05$).

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Research Article

Fatty Acid Profile of Fat of Grass Carp, Bighead Carp, Siberian Sturgeon, and Wels Catfish

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The objective of this study was to determine fish species differences in fatty acid profile of the fat of farmed grass carp, bighead carp, Siberian sturgeon, and wels catfish so as to compare the consumer health benefits they provide. Fatty acid composition range was as follows: saturated fatty acids (SFA), 16.32%–32.96%; monounsaturated fatty acids (MUFA), 41.84%–55.31%; and polyunsaturated fatty acids (PUFA), 13.4%–26.31%. The total content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in 100 g of muscle tissue of the fish examined in the current study was 62.61 mg for grass carp, 150.51 mg for wels catfish, 488.67 mg for bighead carp, and 619.06 mg for Siberian sturgeon. The ratios of n-6/n-3 (0.44–1.72) and PUFA/SFA (0.45–1.61) in the fat of the fish analyzed were beneficial.

1. Introduction

Higher nutritive value of fish is well documented in the studies of many authors [1–7]. It comes mainly from easily digestible protein and fat it contains [8]. In recent years, increasing attention has been focused on the significance of fatty acids in human nutrition. Fish fat is characterized by an advantageous composition of fatty acids. The assessment of fatty acid profile includes the level of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and n-3 and n-6 family as well as the ratio of n-6 to n-3 and PUFA to SFA. Furthermore, the amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from the n-3 family and arachidonic acid (AA) from the n-6 family is of great importance. These acids are essential for normal growth and development of organism as well as health promotion and disease prevention [9–15].

A number of authors evaluated fatty acid profile of marine or farmed fish along with consumer health benefits provided by dietary inclusion of the studied fish [8, 16–22]. However, the data on nutritive value of fat of the selected fish species from the fishing farms in eastern Poland are limited.

The objective of the present study was determination of fish species variation in fatty acid profile of fat in important farmed food fish such as grass carp *(Ctenopharyngodon idella)*, bighead carp *(Aristichthys nobilis)*, Siberian sturgeon *(Acipenser baerii)*, and wels catfish *(Silurus glanis)* and comparison of their health-promoting benefits for consumers.

2. Materials and Methods

2.1. Samples and Analysis. Research material included muscle tissue of grass carp *(Ctenopharyngodon idella)* ($n = 6$), bighead carp *(Aristichthys nobilis)* ($n = 6$), Siberian sturgeon *(Acipenser baerii)* ($n = 6$), and wels catfish *(Silurus glanis)* ($n = 6$). The aforementioned fish species were caught at the fish farms located in eastern Poland. Grass carp and bighead carp are herbivorous fish, whereas Siberian sturgeon and wels catfish are predatory fish. The fish ate natural alimentation which was in pond and did not receive any industrial feed.

After fish harvesting and killing, the body weight and length of each fish were measured. Within an hour, the fish were transported at 0°C–4°C to the laboratory, where they were gutted and filleted. A laboratory sample consisted of a pair of fillets from each fish, minced twice and then homogenized.

Following lyophilization of the tissues, lipids were extracted with a Soxhlet extractor (VELP SCIENTIFICA SER 148 Solvent Extractor) [23]. To obtain fatty acids for analysis, 100 mg of fat was collected. Fatty acid esters were obtained according to international standards [24, 25]. The samples obtained were analyzed using a Varian 3800 gas chromatograph with a FID detector and a CP-Wax 52CB WCOT Fused Silica capillary column, with length of 60 m and an inner diameter of 0.25 mm. The initial temperature for the analysis was 120°C for 5 minutes and the final temperature was 210°C. The injector temperature was 260°C and the detector temperature was 260°C. The hydrogen flow rate was 30 ml/min, air 300 ml/min, and helium 1.4 ml/min. The volume of the injected sample was 1 μl. The results for the percentage content of fatty acids in the sample were obtained using Star GC Workstation Version 6.30.

The content of fatty acids expressed in mg/100 g muscle tissue was calculated using a conversion factor of 0.956 and the total lipid content of fish muscle tissue [26].

2.2. Statistical Analysis. The results presented as means ± SD were submitted to analysis of variance (MANOVA) at 0.05 significance level, using Statistica 10. The mean values were compared by Tukey's test.

3. Results and Discussion

The mean body weight and length were 2.47 ± 0.22 kg and 58.67 ± 1.75 cm for grass carp, 2.64 ± 0.19 kg and 50.58 ± 0.92 cm for bighead carp, 2.17 ± 0.82 kg and 79.83 ± 9.02 cm for Siberian sturgeon, and 2.71 ± 0.15 kg and 74.17 ± 1.17 cm for wels catfish.

As for the fish analyzed in this study, a fat content (%) in the muscle tissue was 10.84 ± 2.98 for Siberian sturgeon, 9.42 ± 2.21 for bighead carp, 3.33 ± 1.83 for wels catfish, and 3.07 ± 0.63 for grass carp. According to the Polish Standard [27], Siberian sturgeon and bighead carp were fat fish, while wels catfish and grass carp were medium fat fish.

Fatty acid profiles of fat of grass carp, bighead carp, Siberian sturgeon, and wels catfish are given in Table 1. The total saturated fatty acids (SFA) ranged from 16.32% up to 32.96%. Grass carp and bighead carp had significantly more saturated fatty acids as compared to Siberian sturgeon and wels catfish. Saturated fatty acids levels reported by other authors in the same fish species were different from those obtained in the present research. Ljubojević et al. [28] indicated a higher SFA content in bighead carp (32.82%) and wels catfish (30.22%) but lower in grass carp (28.72%). Badiani et al. [29] showed a higher SFA level in sturgeon (25.99%). Four fish species discussed in this study contained less SFA compared to other freshwater fish species like pike, zander, bream, tilapia, and pangasius, whose SFA level was between 36.28% and 42.18% [22, 30]. Palmitic acid (C16:0) was the dominant SFA in the analyzed fish, from 10.50% in wels catfish up to 24.66% in grass carp, contributing 64.34%–78.61% of the total saturated fatty acid (SFA). Palmitic acid was also found as the major SFA in the lipids of many freshwater fish species with percentage 16.83%–29.19% [22, 30]. Stearic (C18:0) and myristic acid (C14:0) followed palmitic acid with regard to the quantity in the saturated fatty acid group determined in the fat of grass carp, bighead carp, wels catfish, and Siberian sturgeon. The content of these acids was as follows: 1.59%–5.48% and 1.57%–2.50%, respectively. Alike, stearic acid and myristic acid were the next highest in the saturated fatty acid group of the fish from the Vistula Lagoon such as bream, eel, roach, perch, and pike perch ranging within 3.62%–6.02% and 1.55%–4.03%, respectively [8]. Monounsaturated fatty acids (MUFA) were found most abundant in the fat of the studied fish species; their level in wels catfish, Siberian sturgeon, and bighead carp was comparable (54.08%–55.31%) but significantly higher than in grass carp (41.84%). The aforementioned fish species showed the greatest amount of oleic acid (C18:1 n-9) within the range 29.35%–47.71% making up 69.12%–88.20% of the total monounsaturated fatty acids (MUFA). Palmitoleic acid (C16:1 n-9) varying within 2.22%–10.90% and vaccenic acid (C18:1 n-7) 2.74%–4.42% followed in order of the most abundant acids. The quantity of other fatty acids identified in this group was below 1%. Ljubojević et al. [28] highlighted a higher content of monounsaturated fatty acids in grass carp (50.6%) but lower in bighead carp (33.48%) and wels catfish (41.43%) as compared to the levels established in the same fish species in the present research. Badiani et al. [29] reported that MUFA content in sturgeon maintained level between 43.08% and 51.82% and the value close to the upper limit was determined in the present studies on this fish species. Like this research, oleic acid (18:1 n-9) in these fish species had the highest level in the group of monounsaturated fatty acids ranging between 24.00% and 38.83% [28]. In other freshwater fish species such as zander, pike, bream, and carp, oleic acid (18:1 n-9) also prevailed ranging from 16.01% up to 32.63%, whereas a content of monounsaturated fatty acids was lower (27.46%–48.57%) than that obtained in the present research in bighead carp, Siberian sturgeon, and wels catfish [22]. A level of polyunsaturated fatty acids (PUFA) in the fat of Siberian sturgeon, grass carp, and wels catfish was significantly higher (24.24%–26.31%) compared to bighead carp (13.4%). As for grass carp, Siberian sturgeon, and wels catfish, linoleic acid (C18:2 n-6) was most abundant, at the level of 9.56%–15.15% whereas linolenic acid (C18:3 n-3) dominated in bighead carp—3.52%. The PUFA n-6 content was the highest in wels catfish (16.63%) comparable to Siberian sturgeon and grass carp (14.00% and 13.64%, resp.), while the lowest content was in bighead carp (4.11%). The PUFA n-3 level was similar in four fish species varying between 9.29% and 10.94%. The other studies [28, 29] indicated a lower PUFA n-6 content in sturgeon (4.31%) and wels catfish (11.18%), while being higher in bighead carp (9.31%). Regarding grass carp, it was comparable (13.63%) to those obtained for these fish species in the present research. In these studies, PUFA n-3 content was lower in grass carp (7.46%) and higher in bighead carp, sturgeon, and wels catfish (24.54%, 18.09%,

Table 1: Fatty acid composition (%) of the fat extracted from analyzed fish (mean value ± SD).

Fatty acid	Grass carp	Bighead carp	Siberian sturgeon	Wels catfish
C12:0	0.04 ± 0.01	0.10 ± 0.04	0.01 ± 0.01	0.03 ± 0.01
C13:0	0.02 ± 0.01	0.07 ± 0.02	ND	0.01 ± 0.00
C14:0	1.76 ± 0.36	2.50 ± 0.25	1.79 ± 0.10	1.57 ± 0.14
C15:0	0.28 ± 0.08	0.64 ± 0.06	0.20 ± 0.01	0.18 ± 0.01
C16:0	24.66 ± 3.00	21.15 ± 1.69	14.44 ± 0.49	10.50 ± 0.68
C17:0	0.42 ± 0.18	0.63 ± 0.09	0.14 ± 0.03	0.18 ± 0.05
C18:0	5.48 ± 1.25	4.69 ± 0.49	1.59 ± 0.22	3.22 ± 0.31
C20:0	0.15 ± 0.03	0.20 ± 0.03	0.16 ± 0.03	0.45 ± 0.05
C22:0	0.13 ± 0.07	0.05 ± 0.02	0.04 ± 0.02	0.16 ± 0.02
C24:0	0.02 ± 0.01	0.01 ± 0.00	ND	0.02 ± 0.01
$\sum SFA$	32.96^{a}	30.04^{a}	18.37^{b}	16.32^{b}
C14:1	0.07 ± 0.03	0.08 ± 0.04	0.02 ± 0.00	0.02 ± 0.01
C15:1	0.09 ± 0.05	0.03 ± 0.01	0.01 ± 0.00	0.03 ± 0.01
C16:1n-7	0.49 ± 0.12	0.61 ± 0.26	0.26 ± 0.16	0.29 ± 0.14
C16:1n-9	8.66 ± 3.43	10.90 ± 0.75	3.44 ± 0.36	2.22 ± 0.45
C17:1	0.34 ± 0.08	0.92 ± 0.09	0.15 ± 0.06	0.1 ± 0.01
C18:1n-7	2.74 ± 0.43	4.42 ± 0.39	3.08 ± 0.20	3.60 ± 0.32
C18:1 n-9	29.35 ± 5.21	38.23 ± 2.03	47.71 ± 0.65	47.70 ± 2.52
C20:1n-7	0.10 ± 0.05	0.12 ± 0.11	0.05 ± 0.04	0.12 ± 0.01
$\sum MUFA$	41.84^{a}	55.31^{b}	54.72^{b}	54.08^{b}
C18:2 n-6	9.56 ± 0.98	2.14 ± 0.40	12.41 ± 0.63	15.15 ± 0.95
C20:2 n-6	0.40 ± 0.18	0.10 ± 0.04	0.29 ± 0.23	0.45 ± 0.11
C18:3n-3	7.91 ± 3.60	3.52 ± 0.77	3.36 ± 0.32	4.19 ± 0.48
C18:3n-6	0.20 ± 0.07	0.24 ± 0.06	0.68 ± 0.06	0.21 ± 0.03
C20:3n-6	0.75 ± 0.27	0.29 ± 0.12	0.15 ± 0.10	0.42 ± 0.06
C20:4n-6	2.72 ± 0.69	1.34 ± 0.40	0.48 ± 0.03	0.4 ± 0.10
C20:5n-3	1.10 ± 0.50	2.92 ± 0.80	2.38 ± 0.25	1.63 ± 0.42
C22:5n-3	0.86 ± 0.30	0.56 ± 0.24	0.79 ± 0.13	0.85 ± 0.24
C22:6n-3	1.08 ± 0.35	2.29 ± 0.66	3.7 ± 0.37	3.01 ± 0.81
$\sum PUFA$	24.62^{a}	13.4^{b}	24.24^{a}	26.31^{a}
$\sum n\text{-}6$	13.64^{a}	4.11^{b}	14.00^{a}	16.63^{c}
$\sum n\text{-}3$	10.94^{a}	9.29^{a}	10.23^{a}	9.68^{a}
n-6/n-3	1.24	0.44	1.37	1.72
PUFA/SFA	0.75	0.45	1.32	1.61

ND: not detected. Means in the same row with different superscript letters were significantly different, $p < 0.05$.

and 17.21%, resp.) [28, 29]. In other freshwater fish species, the PUFA n-6 and PUFA n-3 level was differentiated and ranged within 8.46%–16.32% and 5.01%–24.85%, respectively [22, 30]. The PUFA n-6 and n-3 values obtained in the present studies on four fish species were found within these ranges.

The ratio of PUFA n-6 to n-3 and PUFA to SFA is of great importance for good health. Nutritionists emphasize the significance of maintaining a low n-6 to n-3 ratio in diets to prevent arteriosclerosis [8]. Values higher than the maximum are harmful to health and may contribute to cardiovascular diseases development [31]. The UK Department of Health recommends the maximum n-6 to n-3 ratio of 4.0 [32]. The ratio n-6 to n-3 in the fish examined in the current study was 0.44 for bighead carp, 1.24 for grass carp, 1.37 for sturgeon, and 1.72 for wels catfish and, thus, it did not exceed the recommended maximum dietary ratio of 4.0 [32]. The reports available in the literature present the ratio of PUFA n-6 to n-3 in freshwater fish within the range of 0.21–2.78 [17, 28]. Another important factor in the prevention of cardiovascular disease is the ratio of PUFA to SFA [8]. A minimum value of PUFA to SFA ratio recommended is 0.45 [32], whereas these ratios in the fish examined were equal to or higher than the recommended minimum value of 0.45 [32], that is, 0.45 for bighead carp, 0.75 for grass carp, 1.32 for sturgeon, and 1.61 for wels catfish. Ljubojević et al. [28] and Özogul et al. [17] reported the PUFA to SFA ratio for freshwater fish within the range 0.66–1.56. The nutritive value of fish depends on the content of fatty acids that are beneficial to health [8]. The highest EPA level (eicosapentaenoic acid) was noted in the fat of bighead carp (2.92%) followed by Siberian sturgeon (2.38%), wels catfish (1.63%), and grass carp (1.10%). A DHA level (docosahexaenoic acid) was close to

Table 2: Fatty acid content (mg/100 g) in the muscle tissue analyzed fish (mean value ± SD).

Fatty acids	Grass carp	Bighead carp	Siberian sturgeon	Wels catfish
SFA	957.65 ± 177.49	2668.58 ± 475.65	1899.40 ± 478.74	512.12 ± 267.45
MUFA	1267.43 ± 397.33	5099.14 ± 1216.44	5923.41 ± 1589.00	1818.23 ± 967.92
PUFA	711.20 ± 179.92	1243.29 ± 467.41	2514.57 ± 694.70	849.91 ± 514.22
n-3	316.48 ± 129.40	865.73 ± 339.10	1052.46 ± 258.02	315.87 ± 208.90
n-6	394.72 ± 63.66	377.57 ± 132.98	1462.11 ± 443.06	533.73 ± 30.62
EPA	31.62 ± 14.55	273.83 ± 115.00	243.51 ± 54.60	55.56 ± 44.93
DHA	30.99 ± 8.72	214.84 ± 89.93	375.55 ± 73.18	94.95 ± 57.31

that in Siberian sturgeon and wels catfish (3.7% and 3.01%), lower in bighead carp (2.29%), and the lowest in grass carp (1.08%). In other freshwater fish species, EPA content varied from 0.65% up to 20.15%, whereas DHA level varied from 0.72% up to 27.08% [17, 33, 34]. The EPA and DHA values determined in the present research for grass carp, bighead carp, Siberian sturgeon, and wels catfish fall within these ranges. The percentage share of fatty acids in the fat does not reflect the concentration of these acids expressed as mg/100 g in muscle tissue. The fatty acids expressed in mg/100 g fish muscle tissue allow for determination of nutritive and a properly balanced diet. For wider information, the contents of SFA, MUFA, PUFA, and n-3 and n-6 family as well as EPA and DHA in the muscle tissue of the analyzed fish are presented in Table 2. In the fish species analyzed, EPA content was between 31.62 and 273.83, while DHA content was from 30.99 up to 375.55 mg/100 g muscle tissue. The least amount of these acids (including EPA + DHA mg/100 g) was found in the grass carp muscle tissues (62.61), with higher concentrations found in the muscle tissues of wels catfish (150.51) and markedly higher ones in the muscle tissue of bighead carp (488.67) and Siberian sturgeon (619.06). The level of EPA + DHA mg/100 g also varied among other farmed fish species and reached 24.8 in sutchi catfish, 70.8 in tilapia, 214.5 in carp, and 1804.0 in trout [3]. The majority of recommendations have been issued on the basis of the amount of EPA + DHA together, without specific guidance for each fatty acid [12]. The European Food Safety Authority recommends the intake of 250 mg/day EPA + DHA [35]. The results obtained in this study indicated that the recommended dose 250 mg/day of EPA + DHA was available in about 40 g of sturgeon, 50 g of bighead carp, 170 g of wels catfish, and 400 g of grass carp. Arachidonic acid (AA) (C20:4 n-6) concentration was the highest in grass carp (2.72%) followed by bighead carp (1.34%), whereas it was the lowest and comparable in Siberian sturgeon and wels catfish (0.48 and 0.4%, resp.). Ljubojević et al. [28] indicated a lower arachidonic acid content in grass carp (1.61%) and a higher one in bighead carp and wels catfish (4.05% and 3.55%, resp.) compared to the levels established in these species in the present studies. According to Badiani et al. [29], arachidonic acid concentration in cultured sturgeon oscillated between 0.44% and 1.16% and the values determined for this fish species in the current research were found within this range. A content of arachidonic acid in other farmed fish species such as trout, sutchi catfish, carp, and tilapia varied between 0.5% and 1.9% [3], while for carp, bream, pike, and zander it varied between 2.52% and 4.57% [22]. The 1.4 : 1–2 : 1 proportion of DHA (docosahexaenoic acid) to AA (arachidonic acid) proves beneficial for pregnant women [12] and this proportion was determined in the bighead carp fat (2.29 : 1.34). As for grass carp, Siberian sturgeon, and wels catfish, these acids were found at a different ratio, that is, 1.08 : 2.72, 3.7 : 0.48, and 3.01 : 0.4, respectively.

The analysis of fatty acid profile of the fat of grass carp, bighead carp, Siberian sturgeon, and wels catfish analyzed in the present studies as well as the research results presented by other authors showed the differences in concentration of fatty acids. The effect of species, age, diet, water salinity, season, and geographical location on the fatty acid profile of fish fat is well evidenced by other authors [1, 16, 36–39] and that explains the differences in fatty acid profiles of fish fat.

4. Conclusion

Fatty acid profile of the fat of the examined fish depended on the fish species. The fish species analyzed in the present research make a source of essential unsaturated fatty acids. The ratios of n-6 to n-3 fatty acids and polyunsaturated fatty acids to saturated fatty acids (PUFA/SFA) in the fat of the fish examined proved to be advantageous. The results have shown that the investigated fish species may constitute a healthy addition to the human diet.

Additional Points

Practical Applications. This study has documented effect of fish species on the fatty acid profile and nutritive value of fat of the muscle tissue of four species of important food fish. The obtained research results could serve as reference to nutritionists or dieticians.

Disclosure

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

A Comprehensive Study on the Effect of Roasting and Frying on Fatty Acids Profiles and Antioxidant Capacity of Almonds, Pine, Cashew, and Pistachio

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The aim is to evaluate the effects of frying and roasting on nuts. Frying and roasting were performed according to the local Jordanian home-made cuisine, and the nuts under experiment were raw almonds, pine, cashew, and pistachio. Nuts samples were roasted at 110°C for 16 minutes and fried at 175°C for 2.5 minutes. The results show that both roasting and frying of nuts did not affect the flavonoids content except for roasted pistachios where significant rise of flavonoids content was detected. Total phenolic content showed no significant differences except for pine nuts in which it increased significantly in both roasting and frying. Oxidative stability, presented by 1,1-diphenyl-2-picryl-hydrazyl (DPPH), was significantly different in all nuts except for pistachio nuts that have shown no differences. Fatty acids profile, presented by saturated fatty acids (SFA), oleic acid (OL), and essential fatty acids (EFA), was affected significantly by roasting and frying, especially for SFA in almonds and pine nuts and α-linoleic acid (ALA) contents of pine. In conclusion, the effects of roasting and frying on the aforementioned nuts species were positive for fatty acids profile and antioxidants activity.

1. Introduction

Consuming nuts is a very common phenomenon across different European countries, with its highest consumption rate in Mediterranean regions [1]. The consumption of nuts (raw or processed) has increased over the last decades due to their availability, reasonable price, and the rise in the nutritional health awareness [2]. The availability of legumes and nuts in Jordan has increased from 40 to 60 g/person/day. Additionally, the Food Based Dietary Guidelines for Arab Countries in 2013 recommended regular consumption of nuts.

The consumption of nuts yields beneficial effects on health due to their (1) desirable lipid profile, which is higher in unsaturated fatty acids (USFA) than saturated fatty acids (SFA), and (2) high antioxidants contents [3]. Epidemiological and interventional studies have shown how frequent consumption of nuts can enhance cardiovascular health by decreasing serum levels of low-density lipoprotein- (LDL-) cholesterol and risk of developing type II diabetes [4, 5]. Expressly that plant-derived omega 3 polyunsaturated fatty acids (PUFA) α-linolenic acid (ALA) (which is high in nuts) is deliberated as the precursor of the longer chain fatty acid in the body and hence the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Clinical trials and prospective studies identified well the last two fatty acids for their beneficial effects on prothrombotic cardiovascular risk factors [6].

Additionally, nuts have also been shown to promote weight maintenance when consumed as the source of fat in moderate diets [7]. This is a healthier practice instead of eating high fat content snacks from unhealthy sources such high energy condense food items (fried food, confectionary snacks, etc.) which are known to contain SFAs and trans-fatty acids [8]. Oxidation of fat and fat-containing foods is responsible for the deterioration in the food quality and nutritive value. Additionally, the oxidation of PUFA in food may be related to diseases such as atherosclerosis, diabetes, and cancer [9].

Moreover, nuts contain certain bioactive compounds which act as antioxidants (such as a variety of phenolic compounds and flavonoids), free radical scavengers, and metal chelates that may also play a role in the reduction of the risk for the development of chronic diseases. Phytochemical compounds are available in almonds [10], pistachio, cashew, and pine nuts. Chen and Blumberg demonstrated that flavonoids and phenols intake can decrease the risk of a number of chronic diseases including cardiovascular diseases, hypercholesterolemia, and diabetes mellitus due to their attributes of being antioxidant and anti-inflammatory and antiproliferation [11]. These antioxidants exist in all parts of the plant; nuts (seeds) are one of the richest sources [12].

Roasting and frying are two of the most common methods of nuts' thermal processing in order to enhance their sensory properties [13–15]. Although there are several commercial products, for example, nut bars or snacks that contain roasted and fried nuts, there is a lack of a scientific comparison of the differences in their nutrients content. Such nutritional alteration might be unfavorable and lead to negative health impacts upon consumption. Roasting and frying not only can change the obvious rawness of nuts but also can subsequently have chemical changes on nuts. More than 50% of nuts' energy comes from the fat content of nuts [6]; therefore it is important to study the chemical and bioactive changes that take place due to frying and roasting.

In Jordan, one of the common and traditional ways to present the local cuisine (rice-based meals) is to top rice with fried nuts: pine, almonds, peanuts, cashew, and pistachio. Nuts are also introduced as raw, roasted, fried or as a part of desserts in special occasions and ceremonies.

To the authors' best knowledge, there is no work found in the field of analyzing the chemical changing of the raw nuts after roasting or frying according to Jordanian culture. In relevant studies, roasted nuts were tested, but not fried nuts. This study is aimed to investigate the effects of roasting as well as frying on the content of total SFAs, monounsaturated fatty acids (MUFA) presented by oleic acid (OL), essential fatty acids (EFA), oxidative stability presented by 1,1-diphenyl-2-picryl-hydrazyl (DPPH), and bioactive compounds presented by flavonoids and phenolic. Roasted and fried nuts which were experimented with in this study were obtained from two major stores in Amman, Jordan.

2. Materials and Methods

2.1. Raw and Roasted Nuts. Dehulled raw and commercially roasted nuts (almonds, pine, cashew, and pistachio) (4 kg each) were purchased from two major local nuts stores in Amman. Commercially, the roasting of nuts in Jordan is carried out using a rotating drum roaster at approximately 110°C for 16 minutes. The following samples were used for the analysis: Indian cashew *(Anacardium occidentale)*, European pine syn. Scots pine *(Pinus sylvestris)*, pistachio *(Pistacia vera "Kerman")* from Aleppo, and almond *(Prunus amygdalus)* from India. Identical cultivar of each type of the raw nuts was roasted in each store to apply similar roasting conditions of the sold one and to assure that the same nuts cultivars are used from the two local nuts stores. The raw and roasted nuts were ground and sieved to obtain fine powder. The samples were then stored at −20°C until they were experimented with in this study.

2.2. Pan Frying of Raw Nuts. Identical raw nuts from each species were used from the two local nuts stores and pan frying of dehulled raw nuts (almonds, pine, cashew, and pistachio) (100 g) was carried out at 175°C for 2.5 minutes using refined corn oil as heating medium. Fried nuts were ground, sieved, and stored at −20°C until they were experimented with in this study. This process was carried out for the four nut types studied in this paper.

2.3. Fat Extraction. About 50 g of ground raw, roasted, and fried samples (almonds, pine, cashew, and pistachio) were soaked in petroleum ether for about 24 hours and then filtered. The petroleum ether phase was evaporated using a rotary evaporator under vacuum at a temperature not exceeding 40°C for 15 minutes. The lipid fraction was stored at −20°C in a freezer for additional analysis.

2.4. Nuts Extraction. The powder of raw, roasted, and fried nuts' (almonds, pine, cashew, and pistachio) extraction was carried out according to the method of [16]: 10 g of each sample was suspended in 100 ml of 90% ethanol and continuously shook for 2 hours. After filtration, the samples were vacuum evaporated. The extract was then recovered with 2 ml of 90% ethanol and assayed for its antioxidant activity, total phenolic compounds, and flavonoids.

2.5. Total Polyphenols Content (TPC). Total polyphenols content was carried out according to the methods of [16]. Samples of each extract (0.4 ml) were mixed with 2 ml of Folin-Ciocalteu reagent (diluted 10 times). After 3 minutes, 1.6 ml of 7.5% sodium carbonate was added. The absorbance was read at 750 nm after 30 minutes of incubation at room temperature (26 ± 2°C). Blank sample of gallic acid was used as a reference in spectrophotometric analysis. A standard curve was prepared and the results were expressed as mg gallic acid equivalent/g sample.

2.6. Total Flavonoids Content. 1 ml of the extract was added to 1 ml of aluminum trichloride (2% w/v). After 15 minutes of incubation, the absorbance was measured at 430 nm and the results were expressed as mg quercetin equivalents per mg sample. A standard curve was prepared by using a blank sample of pure quercetin acid as a reference in spectrophotometric analysis.

2.7. Free Radicals Scavenging Activity (DPPH). The antioxidant activity of the sample extracts was evaluated using 2,2-diphenyl-1-1 picrylhydrayl (DPPH) radical according to the method of [17]. An aliquot of 30 μl of the sample extracts was added to 0.5 ml of DPPH solution (25 mg/l) diluted to 5 ml of methanol. A control without extract was also prepared. The mixture was shaken vigorously and allowed to stand for 45 minutes in the dark and the absorbance was measured at

515 nm. The antioxidant activity of the extract was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance sample} - \text{Absorbance control}}{\text{Absorbance control}} * 100. \quad (1)$$

2.8. Vitamin E Determination. Vitamin E was determined following the method used by [18]. The analysis of vitamin E was carried out using a Knauer HPLC System (Germany). The determination was carried out following the method of Gimeno et al. (2000) [18]. The oil sample of raw, roasted, and fried peanut was diluted in hexane (1 : 10). An aliquot of 200 μl was transferred to a test tube containing 600 μl of methanol and 200 μl of the internal standard solution (300 mg/ml of α-tocopherol acetate in ethanol). The mixtures were mixed, centrifuged at 3000 g for 5 min, and then filtered through a 0.45 mm pore size filter. Fifty micrometers was directly injected into the Knaur HPLC system. The mobile phase was methanol-water (96 : 4, v/v) and the elution was performed at a flow rate of 2 ml/min. The analytical column was Venusil XBP, C18 (2) (Agelant Technologies, USA), and was kept at 45°C. Detection was performed at 292 nm using Knauer UV detector (model Smartline 2500, Germany).

2.9. Determination of Fatty Acids Profile. Fatty acid methyl esters (FAMEs) of the nuts samples were prepared according to the method outlined in [17]. The prepared FAMEs were studied using capillary Gas Liquid Chromatography (GLC) analysis [19]. The prepared methyl esters were analyzed using capillary GLC column (Restek, Rtx-225, USA, cross-bond 90%-cyano-propyl-methyl-poly-siloxane, 60 m and 0.25 μm df) immediately after esterification by injection 1 μl of the hexane layer through the injection port of the GLC (model GC-2010, Shimadzu, Inc., Kyoto, Japan). The GLC condition was at 15°C/min and held at this temperature for 10 minutes, and then it was increased from initial column oven temperature 165°C to 180°C at 1°C/minute and then further increased to 220°C at 3°C/min and held at this temperature for 10 minutes. Injector temperature was 240°C and the flame ionization detector temperature was at 250°C, flow rate of He was 0.8 ml/min, and the split ratio used was 80 : 1. Fatty acid identification was carried out by ingestion standard fatty acids (Sigma, USA). SFAs were detected as the sum of palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0), and lignoceric acid (C24:0). Monounsaturated fatty acids (MUFA) were detected by OL only as it is the predominant MUFA fatty acids in nuts. PUFA were detected by the sum of essential fatty acids (EFA) alpha-linolenic acid LA (an omega-3 fatty acid) and linoleic acid ALA (an omega-6 fatty acid).

3. Statistical Analysis

Each type of nuts from each store was tested separately; then the results altogether were averaged. It is worthy to mention that very close results were obtained from the two stores due to (1) the identical cultivars species that was used by the authors of this paper and because (2) similar conditions of nuts roasting and frying were applied in the two major local nuts stores as well. All tests were done three times and then subsequently averaged. Statistical analyses were performed using SPSS (Statistical Program for Social Sciences) version 17.0 for Windows. All analyses were conducted in triplicate, and data reported as means ± standard deviation (SD) and differences between means were considered significant at p value < 0.05 differences between means were considered significant at p-value < 0.05. Fatty acids' area [μV·s] from the chromatogram was completed and calculated by using Microsoft® Office Excel 2007 (12, 0, 4518, 1014) MSO.

4. Results and Discussion

The bioactive compounds of nuts such as the antioxidants capacity, phenols, flavonoids, vitamin E, and fatty acids are associated with numerous health benefits and are affected by heat treatments. Therefore, this study is aimed to investigate the effects of roasting and frying on the content of total saturated fatty acids, essential fatty acids, oxidative stability, and bioactive compounds presented by flavonoids and phenolic acids. Quercetin and gallic acid were used only as standards to expressed total phenolic and total flavonoids contents. Table 1 demonstrates the antioxidant capacity expressed by total flavonoids, total phenolic, and percentage of DPPH inhibition. Our results were not too distant in conclusion to other results from the literature apart from different units were used [20, 21].

4.1. Antioxidants Capacity. The antioxidant capacity and high phenol content of nuts whether raw or roasted/fired indicate that the associated health benefits would not be restricted to the lipidic fraction. They also explain that the importance of the stability of phenol compounds preserves from eventual thermal degradation and guarantees their antioxidant potential. Nuts are not a major source of dietary carotenoids [11]. Among the four types of tested nuts, the carotenoids contents were not detected or neglected. Therefore, the majority of the antioxidant capacity in nuts applies on DPPH, flavonoids, and phenolic acids. Table 1 shows that the tested nuts are known for the high content of gallic and quercetin acid which dabble as an antiproliferative, antimutagenic, and antioxidant [20].

Many factors have influenced the levels of phenol compounds in nuts such as the environmental factors, soil composition, and maturation level [20]. Table 1 demonstrates that roasting and frying (i.e., heat treatments) have led to insignificant consequences on total flavonoids among tested raw nuts with the exception of pistachio that showed onefold increase on roasted ones (raw = 6.71 and roasted = 13.74, $p < 0.05$).

This work shows that heat treatments have no significant influence on the total phenolic content expressed by gallic acid in cashew, pistachio, and almonds except for pine which have shown a significant increase on the total phenolic compounds ($p < 0.05$). A similar effect has been also noticed in phenolic acid (gallic acid) for pine nuts. The increase of

Table 1: Effect of roasting and frying on antioxidant activity as compared to raw nuts.

	Raw	Roasted	Fried
	Total flavonoids content (mg quercetin acid equivalent/g sample)		
Cashew	4.25 ± 1.41^{a}	5.20 ± 0.34^{a}	6.94 ± 2.57^{a}
Pistachio	6.71 ± 1.17^{a}	13.74 ± 1.58^{b}	6.85 ± 2.40^{a}
Almond	6.49 ± 2.43^{a}	4.58 ± 1.16^{a}	10.60 ± 1.23^{a}
Pine	4.58 ± 0.24^{a}	6.06 ± 1.03^{a}	7.95 ± 0.44^{a}
	Total Phenolic content (mg gallic acid equivalent/g sample)		
Cashew	5.39 ± 1.88^{a}	6.73 ± 0.63^{a}	7.09 ± 0.78^{a}
Pistachio	6.74 ± 1.72^{a}	10.50 ± 3.12^{a}	10.46 ± 3.45^{a}
Almond	5.87 ± 1.55^{a}	8.46 ± 1.38^{a}	8.24 ± 1.85^{a}
Pine	3.46 ± 0.35^{a}	7.62 ± 2.07^{b}	6.82 ± 1.35^{b}
		DPPH Inhibition (%)	
Cashew	80.87 ± 8.07^{b}	59.99 ± 5.12^{a}	59.45 ± 7.71^{a}
Pistachio	78.51 ± 6.92^{a}	74.20 ± 5.61^{a}	75.60 ± 7.61^{a}
Almond	59.50 ± 7.13^{b}	78.1 ± 4.61^{b}	31.64 ± 3.87^{a}
Pine	48.36 ± 8.15^{a}	63.2 ± 3.32^{ab}	72.3 ± 4.82^{b}

Values within the same row with different superscript letters are significantly different ($p < 0.05$).

total phenolic content as a result of roasting and frying could be due to the Maillard reaction which results in formation Maillard derivative such as pyrroles and furans that may react with Folin-Ciocalteu reagent [22]. However, roasted nuts have had 14% more total polyphenols than raw nuts and the percent of free polyphenols decreased [23]. Moreover, studies on almonds and hazelnuts showed an increment in antioxidant activity as an effect of roasting [24, 25].

Nuts' antioxidant capacities were evaluated by the DPPH radical scavenging method, which is based on the measurement of the reduction ability of antioxidants towards the radical DPPH [26]. Free radicals scavenger activity shows different manipulating on different nuts. While heat treatments significantly increase the DPPH activity in pine nuts, such treatments decrease the DPPH activity in cashew nuts ($p < 0.05$). Roasting almonds have increased the DPPH, but frying almonds DPPH was noticed to have decreased. The results indicated that heat treatments significantly increased DPPH values in pine nuts, as opposed to cashews, in comparison to raw ones ($p < 0.05$). Roasting increased DPPH in almonds against the raw peers.

According to Açar and colleagues, roasting nuts may destroy some bioactive compounds, but it can also form antioxidant compounds through the Maillard reaction. However, the total antioxidants capacity after roasting is the result of the thermal degradation of naturally occurring antioxidant compounds and the formation of new Maillard reaction products having antioxidant activity. Chandrasekara and Shahidi suggested that roasting cashews at high temperature-short time enhances effectively its antioxidant activity [Chandrasekara and Shahidi, 201] which is shown to be true as well in this study.

An increase in those compounds is believed to give the plausible taste in roasted and fried nuts rather than the raw one. Raw nuts are known to have a tart-like taste but once roasted or fried this specific taste can disappear. One of the explanations stems from the increase in flavonoids and phenols.

4.2. Fatty Acids Profile. The literature shows how healthy nuts consumption can be due to their fatty acids composition. Around 62% of nuts energy is coming from fat [27]. Fatty acids composition of nuts is beneficial because SFA is low and MUFA content is significantly higher than SFA, which has also been demonstrated in this work for specific nut types (SFA = ranges 7–18% while MUFA (OL) ranges 40–70% of total fat). The type of dietary fat intake affects plasma cholesterol level more than the total fat intake [27]. The high content of MUFAs and PUFAs is considered healthy fats in nuts oil content which can counterbalance the unfavorable SFAs [28].

Table 2 demonstrates the effect of roasting and frying on fatty acids profiles expressed by total SFA, MUFA presented by OL, and EFA expressed by (ALA and LA). Heat treatments have shown no alterations on fatty acids for pistachios. This suggests that roasting and frying have no effects on fatty acids profile content in pistachios. Almond nuts' fatty acids were all significantly affected when fried. Almonds' SFA and EFA (ALA and LA) have increased when fried, whereas OL has significantly decreased.

Heat treatments have had a significant effect on pine nuts' fatty acids profile. Our results show that pine nuts' EFA and SFA significantly increased when fried (an explanation might be due to the presence of vegetable oil median used) but as the frying oil median was removed from the nuts surface by blister paper immediately after frying; thus another explanation might be that nuts contain pores during the frying process that might absorb the oil median inside the nuts. On the other hand, roasting pine nuts significantly abridged LA to the half while doubling OL content ($p <$

Table 2: Effect of roasting and frying on fatty acids profile as compared to raw nuts.

	Fatty acids profile		
	Raw	Roasted	Fried
		Saturated fatty acids%	
Cashew	17.83 ± 0.44[a]	18.77 ± 0.58[b]	17.78 ± 0.45[a]
Pistachio	11.92 ± 1.40[a]	10.37 ± 1.11[a]	11.27 ± 0.77[a]
Almond	7.2 ± 0.07[a]	7.63 ± 0.18[b]	8.15 ± 0.27[c]
Pine	7.09 ± 0.01[a]	7.74 ± 0.04[b]	8.99 ± 0.06[c]
		Oleic acid%	
Cashew	60.52 ± 1.14[b]	62.27 ± 0.89[b]	57.18 ± 0.27[a]
Pistachio	60.86 ± 5.14[a]	61.55 ± 1.64[a]	61.57 ± 5.34[a]
Almond	69.31 ± 0.18[b]	68.37 ± 0.22[b]	65.1 ± 0.53[a]
Pine	39.82 ± 0.06[a]	68.76 ± 0.5[b]	38.29 ± 0.19[a]
		Linoleic acid%	
Cashew	21.51 ± 0.96[b]	18.61 ± 0.18[a]	24.77 ± 0.50[c]
Pistachio	26.88 ± 6.29[a]	27.90 ± 0.68[a]	26.76 ± 5.61[a]
Almond	23.41 ± 0.28[a]	23.94 ± 0.05[a]	26.69 ± 0.48[b]
Pine	52.68 ± 0.10[b]	23.44 ± 0.54[a]	52.16 ± 0.11[b]
		α-linolenic acid%	
Cashew	0.15 ± 0.01[a]	0.35 ± 0.24[a]	0.27 ± 0.15[a]
Pistachio	0.33 ± 0.07[a]	0.36 ± 0.03[a]	0.40 ± 0.06[a]
Almond	0.052 ± 0.00[a]	0.053 ± 0.00[a]	0.066 ± 0.01[b]
Pine	0.30 ± 0.01[b]	0.06 ± 0.00[a]	0.56 ± 0.01[c]

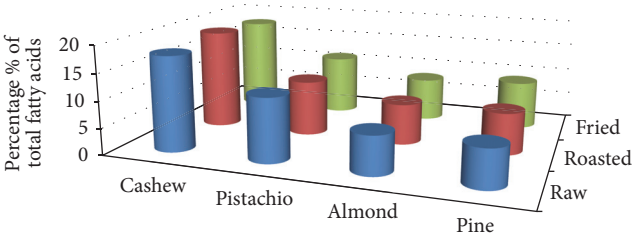
Values within the same row with different superscript letters are significantly different ($p < 0.05$); % SFA +OL + ALA + LA = 100%.

0.05). Cashew nuts showed no impact in ALA, but LA has significantly increased when fried as well as roasted.

ALA has the proinflammatory actions of the n-6 eicosanoids [6]. Therefore, due to the balance of n-6 and n-3 PUFA in the diet and its critical effect on cardiovascular disease CVD, the intake of fried almonds and pine should be controlled. The results shown in this work identify how roasted pine nuts can be a better diet balance with respect to the aforementioned health factors. In order to evaluate the oxidative quality of commercial fried nuts, Marmesat and colleagues demonstrated that frying oil changes the composition of fatty acids by the incorporation of substantial contents of the frying fat. High temperature of fat (during frying) may exert some effect on the quality and stability of the oxidative of the oil used in the frying process [6].

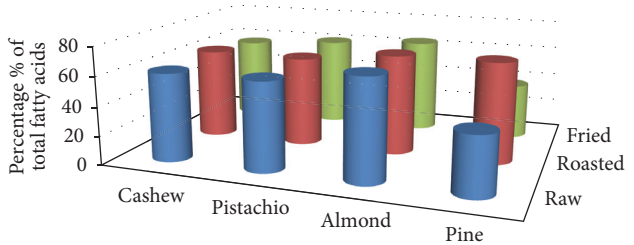
The high content of MUFAs and PUFAs is considered healthy fats in nuts oil content which can counterbalance the unfavorable SFAs [28]. Accordingly, nuts have positive impacts as far as CVD is concerned by preventing LDL oxidation, mediated by fatty acids profile and antioxidants content [21].

Figures 1–4 simplified the heat treatments effects among each type of nuts in regard to their fatty acids content. Table 3 summarizes the impacts of heat treatments on fatty acids content among cashew, pistachio, pine, and almonds to simplify the recommendation according to fatty acids type. Table 3 demonstrates ascending ranking of the tested nuts' values which are derived and shown in Table 2. There were no differences recorded in cashew nuts' fatty acid profile when roasted or fried. Cashews are the richest in SFA while



	Cashew	Pistachio	Almond	Pine
Raw	17.83	11.92	7.2	7.09
Roasted	18.77	10.37	7.63	7.74
Fried	17.78	11.27	8.15	8.99

Figure 1: Saturated fatty acids%.



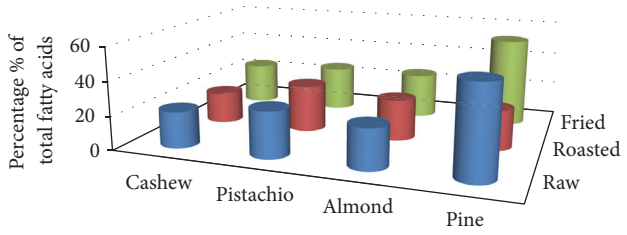
	Cashew	Pistachio	Almond	Pine
Raw	60.52	60.86	69.31	39.82
Roasted	62.27	61.55	68.37	68.76
Fried	57.18	61.57	65.1	38.29

Figure 2: Oleic acid%.

Table 3: Appraisal between the percentage of fatty acids content among raw and heat-treated nuts.

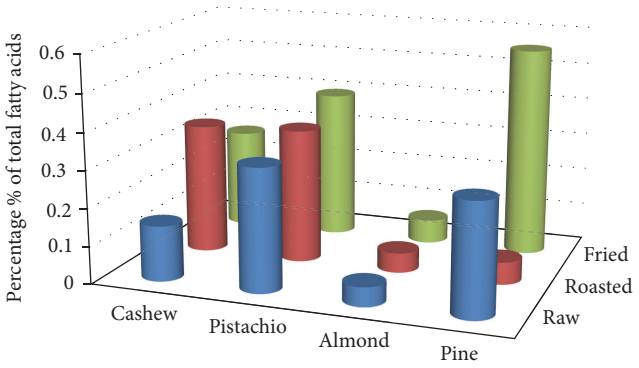
	Raw	Roasted	Fried
SFA	Cashew 17.8%	Cashew 18.7%	Cashew 17.8%
	Pistachio 11.9%	Pistachio 10.4%	Pistachio 11.3%
	Almonds 7.2%	Almonds 7.6%	Almonds 8.1%
	Pine 7.2%	Pine 7.7%	Pine 9%
OL	Almond 69.3%	Pine 68.7%	Almond 65.1%
	Pistachio 60.9%	Almond 68.4%	Pistachio 61.5%
	Cashew 60.5%	Cashew 62.3%	Cashew 57.2%
	Pine 40%	Pistachio 61.5%	Pine 38.3%
LA	Pine 52.7%	Pistachio 27.9%	Pine 52.2%
	Pistachio 26.9%	Almond 24%	Pistachio 26.8%
	Almond 23.4%	Pine 23.4%	Almond 26.7%
	Cashew 21.5%	Cashew 18.6%	Cashew 24.8%
ALA	Pistachio 0.33%	Pistachio 0.36%	Pine 0.56%
	Pine 0.3%	Cashew 0.035%	Pistachio 0.4%
	Cashew 0.15%	Pine 0.06%	Cashew 0.27%
	Almond 0.05%	Almond 0.05%	Almond 0.06%

% represents the percentage out of total fat content.



	Cashew	Pistachio	Almond	Pine
Raw	21.51	26.88	23.41	52.68
Roasted	18.61	27.9	23.94	23.44
Fried	24.77	26.76	26.69	52.16

Figure 3: Linoleic acid%.



	Cashew	Pistachio	Almond	Pine
Raw	0.15	0.33	0.052	0.3
Roasted	0.35	0.36	0.053	0.06
Fried	0.27	0.4	0.066	0.56

Figure 4: α-Linolenic acid%.

almonds and pine are the lowest with near values to each other. Cashews' OL did not differ as previously stated in this section of this work. Raw and fried almonds are the highest in MUFA (expressed by OL), while raw and fried pine nuts were the lowest. Roasted pine nuts were the highest in MUFA content.

In regard to EFA, pine nuts and pistachios have appeared to be the richest sources of it while almonds and cashews were, in contrast, the poorest. The last finding of this paper showed that the heat treatments among cashews and almonds were ineffective with respect to their EFA.

5. Conclusion

Nuts generally contain essential nutrients that enhance the antioxidant activity and contribute to health benefits. However, the way of serving, processing, and consuming nuts could affect these nutrients. Indeed, heat-treated nuts (roasted/fried) resulted in a significant positive and/or negative changes of nuts properties, varied according to nuts type. Altogether, heat treatment of nuts could have an impact that cannot be ignored; therefore, we recommend consuming different nuts as raw or as heat-treated in spite of high content of calories as an alternative to nonnutrient high-calories food.

6. Further Work

It has been stated that properties of nuts might depend on temperature and time of roasting and frying. This can motivate a future work for conducting similar analysis while taking into consideration roasting/frying temperature, time, and other processing factors. Different effects of roasting/frying

on various nut species would also be a useful area for further study to have an accurate explanation.

Additional Points

Research Limitations/Complications. Nuts were brought from local stores that might experience different circumstances with respect to storage/processing factors and the values of the active compounds could be affected.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Squalene Extraction by Supercritical Fluids from Traditionally Puffed *Amaranthus hypochondriacus* Seeds

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Extraction of squalene, a potent natural antioxidant, from puffed *A. hypochondriacus* seeds was performed by supercritical fluid extraction (SCFE); besides, to have a blank for comparison, extraction was performed also by Soxhlet method using organic solvents (hexane). Chemical proximal composition and seed morphology were determined in raw, puffed, and SCFE-extracted seeds. Extracts were obtained with a 500 mL capacity commercial supercritical extractor and performed between 10 and 30 MPa at 313, 323, and 333 K under constant CO_2 flow of 0.18 kg CO_2/h during 8 h. The squalene content was determined and the fatty acids present in the extracts were identified by GC-MS. The extract obtained by SCFE from puffed amaranth seeds reached 460 ± 28.1 g/kg squalene in oily extract at 313 K/20 MPa.

1. Introduction

Amaranth is an ancient crop widely harvested in the Americas, which is appreciated by its nutritional benefits and its religious ancient significance [1]. *Amaranthus hypochondriacus* is one of most important amaranth species and it is harvested in Mexico since pre-Columbian times to current days [2]. One common way to commercialize amaranth seeds is through swelling them by a puffing process [3]. Puffed amaranth seeds are obtained by heat that produces water vaporization and steam accumulation, which increases pressure until the coat breaks and separates from the rest of the seed [4]; thus, starch granules are forced to expand and to convert into a foam matrix [5].

Consumption of amaranth seeds has increased the interest of many researchers in this crop [6]. Amaranth oily extract is constituted mainly by fatty acids and, in minor concentrations, unsaponifiable matter such as squalene [1].

Squalene is a triterpene ($C_{30}H_{50}$) commonly found in plants and animal tissues also, with a role as intermediate in phytosterols and cholesterol biochemical pathways [7]. Several research works have reported its biochemical importance as antioxidant [8–10] and chemopreventive agent [11]. During the last century, shark liver oil was the largest source of squalene extraction [12]; however, nowadays vegetable sources are mostly exploited such as olive oil [13, 14] and oily seeds [12, 15]. Selective extraction from plants to obtain bioactive substances is achieved with appropriate solvents and methods [16]. Squalene is of high commercial importance; for example, 2500 tons with a commercial value of 93 million dollars were produced in 2013 [17] and highly demanded by the cosmetics, food, and pharmaceutical industries [7, 17].

Different techniques are used to obtain the oily fraction from amaranth seeds. The literature has compared three techniques, expeller pressing, organic solvent extraction, and supercritical fluid extraction (SCFE) showing that the best yield is achieved 69.5 g/kg by SCFE [18]. Other methods had been proved by Krulj et al. [19] who extracted amaranth oil from raw seeds using accelerated solvent extraction and achieved higher yield than supercritical fluid extraction 78.1 and 61.1 g/kg, respectively. Relatively low temperature extraction results in almost no-thermal degradation of labile compounds. One advantage about SCFE is that the extracts and matrices are solvent-free; vegetables matrices after extraction can be exploited for foodstuff purposes [20]. Among substances used as solvents in SCFE, carbon dioxide (CO_2) is commonly used by its known characteristics as an eco-friendly, inert, economic, selective, and nontoxic compound [21, 22]. SCFE as free solvent process allows the absence of squalene oxidation products and the presence of phenolic compounds in extract [23]. Usually, SCFE is proved under several pressure, temperature, flow, and time conditions; Yin et al. [24] used not only experimental conditions, but also mathematical simulation to predict the best extraction conditions.

Previous works have already performed SCFE from amaranth seeds previously ground to obtain amaranth oily fractions [22, 25, 26]. As mentioned above, the most common presentation to commercialize amaranth is a puffed seeds; milling seeds would limit its commercialization, increasing steps in extraction process and rising production cost; however, it is possible to obtain oily extracts from nonmilled raw seeds, although with relatively low yields [19, 22].

The aim of this work was to obtain squalene by SCFE from whole puffed amaranth seeds and to compare the squalene content obtained by Soxhlet extraction, as well as determining the possible morphological and chemical modifications in the seeds.

2. Methodology

2.1. Materials and Supplier Information. Carbon dioxide (CAS number 124-38-9) 99.995% pure was provided by Infra (Mexico). Squalene (CAS number 111-02-4) 99.0% pure was provided by SIGMA. Amaranth *(Amaranthus hypochondriacus)* raw and puffed seeds from the same batch were kindly donated by a manufacturer from Puebla, Mexico, harvested in November 2013. Puffing conditions were not provided by the supplier.

2.2. Chemical Proximal Characterization. Main components (water, proteins, lipids, and ashes) of puffed seeds were determined by standard methods; water content m.b. (g/100 g) by method 925.09; proteins by the Kjeldahl method 920.87; lipid content (g/100 g) with Soxhlet 920.39; ashes by 942.05 [27]. Finally, nonnitrogenous matter was calculated by difference; the obtained results were reported as the average of three repetitions ± standard deviation.

2.3. Seed Morphology. Amaranth, nonpuffed, and puffed seeds as well as seeds after extraction were observed under an optical microscope (Nikon Eclipse Ci) at 60x. Images for no less than 100 seeds were captured by a camera attached to the microscope tube with 1600 × 1200 pixels resolution and JPEG format. Images were processed using the ImageJ 1.50c software (Wayne Rasband, National Institutes of Health, USA) with automatic threshold and analyzed in binary format to determine size and shape parameters as follows: area: the total pixels of the projected area of each analyzed seed; perimeter: number of pixels constituting the contour of the projected area of the seed; circularity: defined as 4π multiplied by area and divided by perimeter squared; a perfect circle has circularity equal to 1; Feret's diameter: widest distance of two pixels at different object orientations; and aspect ratio: relation between maximum and minimum axes. The scale used was 0.5253 pixels/μm.

2.4. Expansion Ratio. Expansion ratio is the quotient from dividing the apparent bulk volume of nonpuffed and puffed seeds. Bulk volume was measured with a graduated cylinder using the same mass of seeds (60 g) according to Murakami et al. [28]. Results were reported as averages of three repetitions and their respective standard deviation.

2.5. Amaranth Oily Extraction

2.5.1. SCFE. SCFE was carried out through dynamic method using the Thar Technologies (SFE-500 model) extraction plant (see Figure 1) located at ESIQIE-IPN in Mexico City, Mexico. This extraction plant is composed of a high pressure-pump (BS) which supplies liquid CO_2, two heat exchangers, a cooler (ICE) located behind the high pressure-pump, and a heater (ICC) located at the mixer's (MX) exit. The MX function is to introduce a cosolvent (SC) and mix it with CO_2 if necessary. Two valves (RAPI and RMPI) avoid pressure backward in tubes. Temperature and pressure conditions influence the amount of extracted oily matter; hence, studied conditions ranged by 313–333 K and 10–30 MPa, respectively. Carbon dioxide was used as solvent at constant flow (0.18 Kg CO_2/h) for 8 h, and SC-CO_2 passed through the puffed seeds (60 g) in the extractor (E) vessel and separator vessel (S) at lower pressure, both with 500 mL capacity. Amaranth extracts were collected from the separator vessel, weighed, and analyzed.

2.5.2. Soxhlet Method. Organic solvent extraction using a Soxhlet apparatus was performed with 60 g of puffed amaranth seeds and an excess of hexane that condensed and recirculated throughout the Soxhlet distillation path for 8 h at 68°C and atmospheric pressure. To compare the amount obtained by both extraction methods, all the extracts were stored at 277 K under a nitrogen atmosphere to minimize thermal and oxidative degradation. Effectiveness contact area was maximized by using milled seeds as described by Wejnerowska et al. [22]. Hexane is typically the solvent used for large scale extractions due to its relatively low cost and high extraction efficiency [29].

2.6. Squalene Identification and Quantification. Squalene content in SCFE extracts was preliminary determined as

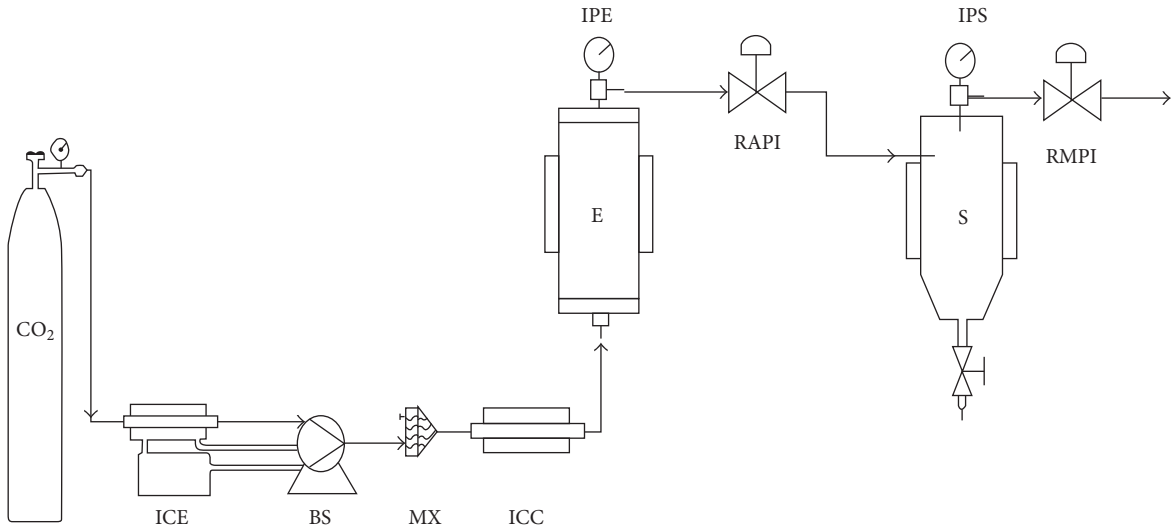

Figure 1: Schematic representation of the SCFE plant used.

described by Schneider [30], by means of high performance liquid chromatography (HPLC) at 208 nm, mobile phase composed of acetonitrile and acetone (60 : 40% volume). The HPLC apparatus has been described previously by Rojas-Ávila et al. [31]. Soxhlet and SCFE extracts were analyzed quantitatively by gas chromatography coupled to mass spectrometry (GC-MS); GS-MS conditions are outlined below. Squalene was quantified with a corresponding eight-point standard curve, coefficient of determination (r^2) of 0.98.

2.7. Fatty Acids Identification and Quantification. The obtained extracts were analyzed according to Aquino-Bolaños et al. [32] in a gas chromatograph (GC) Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) coupled to mass spectrometry (MS) system Agilent 5975C, split ratio 2 : 1. Sample separation was achieved using a column HP-88 ((88% cyanopropyl) aryl-polysiloxane, 100 m × 0.25 mm ID 0.20 μm, Agilent Technologies). Carrier gas used was helium at 6.6 mL/min flow. The temperature set in injector and transfer line was 250°C. Oven was heated through the following temperature rate: initial temperature was 50°C, increased by 2.5°C/min up to 85°C and then 10°C/min up to 170°C, hold for 20 min, and then increased by 10°C/min up to 250°C to allow fatty acid methyl esters separation (FAMEs) in a total run time of 87.5 min. Identification was conducted using the NIST 05 library. Fatty acids were quantified as relative area percent using squalene as internal standard.

3. Results and Discussion

3.1. Chemical Proximal Characterization. In the present work, the main components of *A. hypochondriacus* seeds (raw, puffed, and puffed-SCFE) were determined. Soxhlet-extracted seeds were not considered for further determination due to the elevated content of hexane after extraction and its potential toxicity in foodstuff formulations. Table 1 shows moisture, ashes, protein, lipid, and nonnitrogenous matter content in seeds. Minimum changes in composition due to the SCFE process may indicate the possibility of exploiting amaranth puffed seeds in food products after this type of extraction. Seed moisture content decreased due to the puffing process (heat treatment), which produced water evaporation [33]. SCFE diminished humidity even more, probably because seeds were kept during 8 h under constant temperature and CO_2 flow. Lipid content in puffed seeds may decrease because of partial loss of the peripheral embryo where most of lipids are contained; the same effect has been observed in *A. cruentus* and *A. caudatus* [34]. In addition, ash content is mainly constrained to the seed coat [35], and the coat may be lost during puffing, decreasing mineral content. It is important to point out that no protein and carbohydrates loss occurred due to SCFE. Under these conditions, seeds might be used for different foodstuff purposes after SCFE.

3.2. Morphology Changes. Morphology characteristics of raw, puffed, and SCFE after puffing seeds are shown in Table 2. Among the parameters evaluated, area of raw and puffed seeds presented an increment from 1.98 ± 0.7 to 5.92 ± 0.93 mm^2 due to structural changes which occurred in carbohydrates by puffing processes. *Amaranthus cruentus* raw seeds reported area of 3.26 to 3.60 mm^2 at 9.5% moisture content [36]. Perimeter seed also increased from 2.90 ± 0.07 to 4.72 ± 0.07 as a result of heat treatment. Feret's diameter increased from $0.99 \pm 7.3 \times 10^{-3}$ to 1.67 ± 0.02^{b} mm; 1.35 mm and 0.80 to 1.00 mm diameter corresponds to raw *Amaranthus cruentus* and *Amaranthus* sp. seeds, respectively [36, 37]. In raw amaranth, seed dimensions are influenced by water content according to Abalone et al. [38]. Morphology differences are also due to amaranth species as described by others [1]. SCFE does not seem to affect morphology parameters (area, perimeter, and Feret's diameter) and shape descriptors (circularity and aspect ratio) due to supercritical carbon dioxide SC-CO_2 noninvasive and easily removing properties [37]; thus, SCFE-puffed seeds could be used in

Table 1: Chemical proximal characterization of raw, puffed, and SCFE-extracted *A. hypochondriacus* seeds.

Parameter (g/100 g)	Raw seeds	Puffed seeds	SCFE extracted seeds
Moisture	10.50 ± 0.15^{a}	8.67 ± 0.04^{b}	7.11 ± 0.08^{c}
Ash*	3.55 ± 0.03^{a}	3.21 ± 0.05^{b}	3.19 ± 0.07^{b}
Protein*	13.68 ± 0.41^{a}	13.37 ± 0.16^{a}	13.43 ± 0.09^{a}
Lipid content*	10.32 ± 0.14^{a}	9.19 ± 0.31^{b}	8.57 ± 0.12^{c}
Nonnitrogenous matter**	72.45 ± 0.21^{a}	74.23 ± 0.19^{b}	74.81 ± 0.14^{c}

*Dry basis. **Obtained by difference. Different letters mean statistically significant difference at $2\alpha = 0.05$ level, evaluated by Tukey test.

Table 2: Morphology of raw, puffed, and SCFE-extracted *A. hypochondriacus* seeds.

Parameter	Raw seeds	Puffed seeds	SCFE-extracted seeds
Area (mm^2)	1.98 ± 0.7^{a}	5.92 ± 0.93^{b}	5.26 ± 0.7^{b}
Perimeter (mm)	2.90 ± 0.07^{a}	4.72 ± 0.07^{b}	4.46 ± 0.05^{b}
Circularity (dimensionless)	0.97 ± 0.08^{a}	1.00 ± 0.03^{a}	1.00 ± 0.03^{a}
Feret's diameter (mm)	$0.99 \pm 7.3\times10^{-3a}$	1.67 ± 0.02^{b}	1.57 ± 0.01^{b}
Aspect ratio (dimensionless)	1.06 ± 0.03^{a}	1.15 ± 0.15^{a}	1.14 ± 0.09^{a}

Different letters mean statistically significant difference at $2\alpha = 0.05$ level, evaluated by Tukey test.

food processing in the same way as nonextracted puffed seeds are used.

The expansion ratio is related to seed size change and puffing efficiency; this parameter was 6.09 ± 0.27, which refers to an increase in volume seed due to puffing. At similar water content and 225°C, expansion ratio was about 9.6 when a fluidized bed system was used for puffing. Traditional puffing process usually reports low efficiency and inhomogeneity [39]. The increase in contact surface influences extraction, as previously reported [40–42].

3.3. Amaranth Oily Extract. *A. hypochondriacus* oily extracts were obtained by SCFE and Soxhlet methods from puffed seeds. Soxhlet yield of total oily matter was 10.32% and 9.19% for raw and puffed seeds, respectively; the highest content of oily matter from amaranth by SCFE was attained at 333 K/30 MPa (1.79%) as shown in Figure 2. Higher content of oily matter has been obtained under higher pressure and temperature conditions; this behavior is commonly reported as a result of temperature increase and competition factors related to solvent density and solute-vapor pressure [26, 43]. Although the oily mass extracted was determined, squalene content in extracts defined the best extraction conditions. Lower operation conditions (temperature and pressure) would be unstable to supercritical fluids and difficult to maintain by SCFE plant, whereas higher temperature conditions could compromise squalene stability as described by Psomiadou and Tsimidou [44]; finally, higher pressure will not be achieved due to pressure-pump capabilities at the established flow.

3.4. Squalene Identification and Quantification. The content of squalene in extracts obtained by SCFE was determined by HPLC; the results are shown in Figure 3. It can be observed that at 313 K/20 MPa and 333 K/15 MPa there was no

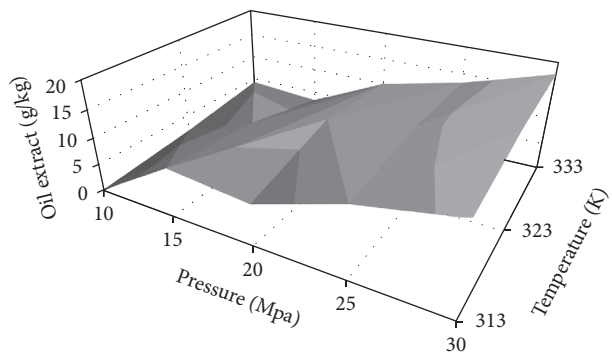

Figure 2: Effect of temperature and pressure on total oily amaranth extract [g/kg] obtained by SCFE from amaranth puffed seeds.

difference in squalene content in extracts obtained, although yield was higher at 313 K/20 MPa. Soxhlet extraction was applied to raw and puffed seeds and squalene content of extracts was determined by GC-MS, as shown in Figure 4. Squalene content in Soxhlet oily extracts was 44.2 ± 2.27 and 31.3 ± 1.0 squalene g/kg of oily extract from whole raw and puffed seeds respectively, whereas SCFE extraction from whole puffed seeds yielded 460 ± 28.1 g/kg squalene in the oily extract. Krulj et al. [19] obtained an average oily extract yield of 58.4 g/kg and 3.5 g/kg of squalene by applying SCFE in milled raw seeds. Our results indicate that SCFE extracts had a higher squalene content than Soxhlet-extracts when puffed seeds are used, suggesting that puffing not only gives better physical, functional, and mechanical properties [36] but also makes more available oil and particularly squalene for extraction.

Other authors have obtained the amaranth oily extract from seeds using *A. cruentus* [25] and obtained 148.8 g/kg of squalene in the oily extract at 313 K/15 MPa/180 min.

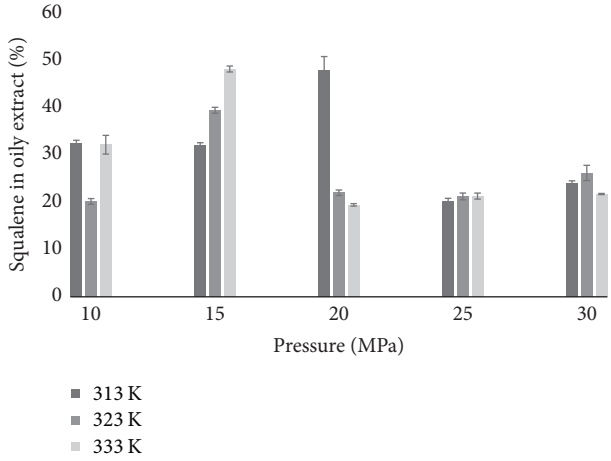

Figure 3: Squalene content in amaranth oily extracts (%) obtained at different operating conditions by SCFE from puffed seeds.

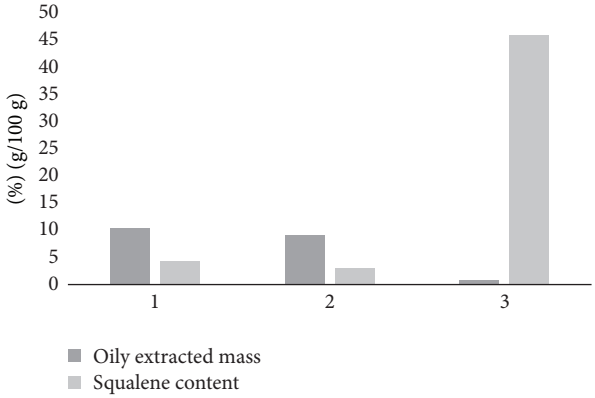

Figure 4: Oily extracted mass in seeds and squalene content in oily extracts (g/100 g). (1) Raw seeds hexane extract; (2) puffed seeds hexane extract; (3) puffed seeds SCFE extract.

Czaplicki et al. [18] attained 69.5 g/kg of squalene at 323 K/31 MPa/16 h. Wejnerowska et al. [22] obtained up to 600 g/kg of squalene at 403 K/20 MPa/30 min. Increasing temperature favored squalene purity and reduced yield simultaneously. He et al. [33] reached 153 g/kg of squalene at 323 K/20 MPa/2 h. Finally, Westerman et al. [26] using ground *Amaranthus* spp. seeds at 323 K/30 MPa/21 h extracted up to 65.7 g/kg squalene. The squalene content in the extract obtained in this work was 460 ± 28.1 g/kg as described above, at lower pressure and temperature compared to reference works using whole puffed seeds; this might represent an important advantage at industrial scale, avoiding the solvent removal stage and reducing costs since the whole seeds can be reused for further food purposes. Differences in squalene content in SCFE may be related to amaranth species used; longer extraction time could increase squalene extraction [22].

3.5. Fatty Acids Identification and Quantification. Amaranth seeds fatty acids composition was determined through GC-MS. The obtained results are shown in Table 3. Some of the identified unsaturated fatty acids were oleic, linoleic, linolenic, and arachidonic, whereas saturated acids were myristic, palmitic, margaric, and stearic. Oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids are commonly present in some vegetal oils [45] and are known for their health benefits [46]; they are also used as raw material for bioproducts elaboration [47]. Arachidonic acid (C20:4) functions as signaling molecule regulating inflammation and acting on homeostasis [48, 49]. Myristic (C14:0), palmitic (C16:0), margaric (17:0), and stearic (C18:0) acids are generally found in unprocessed fats [50], easily to crystallize at room temperature [51], and demanded by oleochemical industry [52] such as palm oil, due to their composition; therefore, an oily extract with similar composition may be demanded as well [53].

Fatty acids content in crude extract obtained from raw ground *Amaranthus caudatus* seeds by SCFE (313 K/20 MPa) contained palmitic acid 19.89%, stearic acid 2.55%, oleic acid 25.47%, and linoleic acid 25.47% [54]. The SCFE extract in the present work contains more palmitic (28.71 ± 0.35%), stearic (6.05 ± 0.07%), and oleic acids (28.68 ± 0.35%) and less linoleic acid (18.95 ± 0.23%); this could be obtained by differences in flow rate (0.12 Kg CO_2/h) and extraction time (14 min) and Amaranth species used.

SCFE-amaranth extract contained more unsaturated than saturated fatty acids and lower concentrations of oleic and palmitic acids than olive and palm oils [55, 56]; it also had higher content of linoleic, linolenic, and arachidonic acids than olive and palm oils [55, 56]. SCFE extract compared to palm oil had similar palmitic/oleic ratio; however, its content of unsaturated fatty acids may be advantageous and healthier. Distribution of saturated and unsaturated fatty acids (palmitic and oleic acid, 28.71% and 28.78%, resp.) agrees with the experimental localization of squalene in cell membrane as reported by Hauß et al. [57] who showed that squalene is immersed in the lipid bilayer, where a balance between saturated and unsaturated fatty acids exists.

Squalene content in olive oil was reported in the range of 110 to 839 mg/100 g [14] and 2–4 g/100 g (wt%) in palm fatty acid distillate [53, 58, 59]. Amaranth extract obtained in this work reached, then, an acceptable purity as described above.

Table 3: Fatty acids content (mg/ml) from *A. hypochondriacus* extracts determined by GC-MS.

Fatty acid	Raw seeds Soxhlet extract	Puffed seeds Soxhlet extract	Puffed seeds SCFE extract
Myristic acid	$0.09 \pm 1.4e^{-3a}$	$0.08 \pm 5e^{-3b}$	$0.11 \pm 4.4e^{-3c}$
Palmitic acid	9.56 ± 0.12^{a}	$9.27 \pm 8.3e^{-3b}$	10.71 ± 0.35^{c}
Margaric acid	NI	NI	0.54 ± 0.02
Stearic acid	$0.56 \pm 9e^{-3a}$	$2.39 \pm 2e^{-3b}$	2.25 ± 0.07^{c}
Oleic acid	10.84 ± 0.14^{a}	$9.34 \pm 4e^{-3b}$	10.67 ± 0.35^{c}
Linoleic acid	11.00 ± 0.14	$7.03 \pm 5.2e^{-3}$	7.05 ± 0.23
Linolenic acid	$0.47 \pm 3.9e^{-3a}$	$0.10 \pm 2.5e^{-3b}$	0.41 ± 0.01^{c}
Arachidonic acid	$0.16 \pm 2.3e^{-3a}$	$0.65 \pm 3.6e^{-3b}$	0.58 ± 0.01^{c}

NI = not identified. Different letters mean statistically significant difference at $2\alpha = 0.05$ level, evaluated by Tukey test.

This work showed that SCFE extract from puffed amaranth seeds contains squalene accompanied by some unsaturated fatty acids. *A. hypochondriacus* puffed seeds after SCFE mostly preserved their nutritional components, such as proteins and carbohydrates, and presented a remnant lipid content, including some fatty acids important for human diet. Flour from defatted amaranth has been already used to supplement bread flour without modifications in bread quality by increasing protein and especially lysine content [60]. Further investigation should explore SCFE with mixtures with different cosolvents in order to increase yield, producing squalene extracts even more enriched from puffed amaranth seeds. Due to minimal changes in chemical composition and morphometric characteristics, it is reasonable to consider the further use of SCFE-extracted puffed amaranth seeds in food industry. Growing cosmetic and food industries surely will cause the squalene market to rise in the future which increases the marine life concerns, claiming new research on squalene extraction from vegetable sources [17].

4. Conclusion

SCFE was satisfactorily applied to obtain a greater amount of oily extract rich in squalene from whole puffed amaranth seeds (460 ± 28.1 g/kg) than what is obtained through hexane extraction; furthermore, seeds main composition remains after SCFE. High squalene extract was achieved through SCFE with CO_2 at 313 K/20 MPa. The Soxhlet method might be inconvenient due to solvent removal final stage; in SCFE, such stage may be avoided. Puffing of amaranth seeds affects their nutritional content; however, the effect of SCFE on nutrient content and morphology of puffed seeds is negligible, so that SCFE-extracted puffed seeds can be reused as regular puffed seeds in food processing. SCFE oily extract contains fatty acids such as palmitic, oleic, and linoleic acids in convenient quantity for human consumption.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Research Article

Formulation of Zero-Trans Crystalized Fats Produced from Palm Stearin and High Oleic Safflower Oil Blends

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High intake of *trans* fat is associated with several chronic diseases such as cardiovascular disease and cancer. Fat blends, produced by direct blending process of palm stearin (PS) with high oleic safflower oil (HOSO) in different concentrations, were investigated. The effects of the PS addition (50, 70, or 90%) and the rate of agitation (RA) (1000, 2000, or 3000 rpm) on physical properties, fatty acid profile (FAP), *trans* fatty acids (TFA), crystal structure, and consistency were researched. The blend containing 50% of each sort of oil (50% PS/50% HOSO) showed that melting point and features were similar to the control shortening. The saturated fatty acids (SFA) were higher followed by monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Significant differences in the content of palmitic and oleic acids among blends were observed. The 50% PS/50% HOSO blend contained higher oleic acid (42.9%) whereas the 90% PS/10% HOSO was higher in palmitic acid (56.9%). The blending of PS/HOSO promoted the β crystal polymorphic forms. The direct blending process of equal amounts of PS and HOSO was an adequate strategy to formulate a new zero-trans crystallized vegetable fats with characteristics similar to commercial counterparts with well-balanced fats rich in both omega 3 and omega 6 fatty acids.

1. Introduction

Bakery shortenings prepared by hydrogenation process of vegetable oils contain levels between 20 and 40% of *trans* fatty acids (TFA) [1]. TFA have negative health implications especially in terms of the ratio of high-density to low-density lipoproteins, which is correlated with risk of cardiovascular diseases [2]. Several published reports have indicated that TFA have also adverse effects on serum cholesterol, triglyceride levels, and coronary heart diseases [3, 4]. The harmful effects of TFA on the ratio of total cholesterol to HDL cholesterol are reported to be twice compared to saturated FA [5]. Kromhout et al. [6] concluded that a gram-for-gram basis TFA has been associated with an increase of 15 times of greater incidence of coronary heart diseases than SFA. TFA have also been related with risk of breast and colon cancer, diabetes, obesity, and allergies [2].

The World Health Organization (WHO) and Food and Agricultural Organization (FAO) recommend that the daily intake of TFA should not exceed 4% in foods and consequently, some countries impose maximum legal limits of these fatty acids [7]. Recently, the FDA mandated that partially hydrogenated oils, the primary dietary source of *trans* fats in processed foods, be removed from products entirely by 2018 [8]. Therefore, efforts have been made to replace hydrogenated oils by other fat sources with lower or zero-*trans* fats [9] without sacrificing the functional properties [10].

Chemical interesterification (CI), enzyme interesterification (EI), and direct blending (high-melting fraction with vegetable oils) are viable alternatives to the hydrogenation process. These processes are aimed to impart the desired functionality without generating TFA [10–12]. Direct blending has several advantages compared to either CI or EI [13, 14], the most relevant being that no chemical processes are involved, and is consistent with the consumer trend toward consumption of natural products [13]. Additionally, the knowledgeable selection of fat blends and the proper crystallization process is critical for the production of plastic shortenings without hydrogenation [14].

PS is an excellent substitute for animal fats for the production of shortenings without cholesterol [15] mainly because it is natural and cheaper. This high-melting fraction is obtained after fractionation of palm oil which is the second most produced oil worldwide. However, because of its high-melting profile ranging from 44 to 56°C, PS cannot be used directly for production of shortening as it causes low plasticity to the products [16]. Thus, it might be appropriately blended with liquid oils in order to modify the overall physical characteristics of the mixture to enhance functionality and the quality required for trans-free shortening preparation [14, 17, 18]. Furthermore, PS has the ability of promoting the polymorphic form of fat crystal β', which is desirable in shortenings. This particular polymorphic form has a large surface area, fine arrangement (packing of small crystals), and greater oil-holding capacity [14, 19].

Moreover, several studies have reported the feasibility of formulating zero-or low-*trans* shortenings using PS blended with other oils that after processing generate crystallized fats with similar organoleptic and functional properties compared to commercial shortenings [10, 14, 17, 18]. Mayamol et al. [14] blended PS and rice bran oil (50 : 50) to prepare *trans*-free bakery shortenings whereas other investigators [12, 20, 21] devised comparable bakery shortenings with similar blends without significantly affecting the physicochemical and textural properties of products. The vegetable oils from sunflower, palm, rapeseed, camellia, canola, rice bran, sesame, and soybean have been previously utilized in formulation of zero-or low-*trans* crystallized fats. However, sunflower oil, like other polyunsaturated oils, is unstable and its use as cooking oil limited because of its high degree of unsaturation or iodine value (linoleic acid content greater than 75%) which makes it more prone to both oxidative and thermal deteriorations [22].

Various oils with different fatty acid profiles have been developed by plant breeding, including low-linolenic soybean, high oleic sunflower, low-linolenic canola, high oleic canola, and high oleic safflower [23, 24]. All these modified natural oils have improved frying stability compared to unmodified counterparts [25]. *Carthamus tinctorius* seeds are rich in edible oil (high oleic safflower oil), with similar content to olive. This oil is typically composed of oleic (74%), linoleic (16–25%), and linolenic (1–6%) acids [26]. This is relevant because oleic acid consumption is considered cardioprotective according to studies of the Mediterranean diet rich in olive oil [27]. HOSO is used for its high resistance to oxidation and heat degradation enabling its use in many baking applications including high temperature frying. The *trans* free HOSO can diminish saturated fat levels, which is ideal in order to generate clean food labels to food manufacturers and for health-conscious consumers [26, 28].

When developing a new shortening, it is essential to optimize the physicochemical properties with an acceptable level of oxidative stability. During storage, the lipid oxidation is a major cause of deterioration, leading to the development of rancid off-odors and flavors [29]. Hence, to obtain a healthy product, oxidative stability is critical in practical use of shortenings. Therefore, the objective of this study was to develop zero-trans crystalized fat formulations produced from blending palm stearin and high oleic safflower oil without sacrificing functional properties and acceptability in terms of oxidative stability.

2. Materials and Methods

2.1. Materials. The palm stearin (PS) was purchased from RBD (Industrializadora Oleofinos SA de CV) whereas the high oleic safflower oil from OLEICO (Coral Internacional, SA de CV). The hydrogenated commercial shortening was obtained from SARITA (Proteinas y Oleicos SA de CV). Standards of FAME, linoleic acid methyl ester isomer mix, and linolenic acid methyl ester isomer mix were purchased from Sigma, St. Louis, MO. All other chemicals used were analytical grade and purchased from JT Baker Deventer, Netherlands.

2.2. Process of Production of Zero-Trans Fat. The PS was melted in a temperature controlled water bath set at 60°C. The melted PS and HOSO were blended in a mixer (vertical Robot Coupe PVM, model RS110V) in mass ratios (PS/HOSO) of 50 : 50, 70 : 30, or 90 : 10. Resulting blends were mixed for 10 min at 1000, 2000, or 3000 rpm at a constant temperature of 70°C. The homogeneous fat blend was poured in 500 mL closed plastic containers and tempered and equilibrated at 30 ± 1°C for 5 d according to the method previously described by Mayamol et al. [14] with slight modifications. The factors involved in this new modification were the sources of fat (palm stearin and high oleic safflower oil) and speed of agitation.

2.2.1. Physical Analyses. The different blends of PS/HOSO were analyzed in terms of color (Lovibond red) (method, Cc 13e-92), refractive index (method Cc 7-25), capillary melting point (method Cc-1-25), relative density (method Cc 10a-25), acidity (method Ca 5a-40), peroxide value (method Cd 8-53), moisture and volatile matter (method Ca 2d-25), and iodine value (method Cd 1c- 85) according to official AOCS [30] procedures.

2.2.2. Fatty Acid Profile. The fatty acid profile (FAP) of blends (PS/HOSO) was determined by gas chromatography as described by Medina-Juárez et al. [31]. The identification and quantification of the methyl esters were determined by the method Ce 1-62 of the AOCS [30]. The gas chromatograph (VARIAN 3400, Mexico City, Mexico) was equipped with a flame-ionization detector (FID) and an integrator (Model

1020, Perkin Elmer, Mexico City, Mexico). A capillary column with 100% biscyanopropyl polysiloxane as the stationary phase SP-2560 (100 m × 0.25 mm i.d. × 0.2 μm; Supelco, Inc., Bellefonte, PA 16823-0048 USA) was used. The oven temperature was set at 140–210°C for 4°C/min, followed by 210–215°C for 1°C/min, and finally 215–220°C for 0.5°C/min. Nitrogen was used as carrier gas with a flow of 20 cm/s. The temperature of the injector and detector was 250°C. The identification and quantification of the peaks were performed by comparison with the retention times and areas of the corresponding standards (Sigma Chemical Co., St. Louis, MO). *Trans* isomers were identified from the linoleic and linolenic acids methyl ester isomer mixes. Results were expressed as weight percentage (wt%) of fat.

2.2.3. Polymorphism. The polymorphic forms of fat in the blends were determined by X-ray diffraction (XRD) according to the methodology described by Mayamol et al. [10], using a Broker Model D8 ADVANCE X-ray diffractometer emitting Cu α radiation. Data were collected at room temperature from 5 to 45 2θ. X-ray data were processed by a computer programmed to calculate absorption intensity-background, intensity, and peak width in degrees for each crystalline form. The relative contents of α, β, and β' crystals were acquired. The β form was calculated from the intensity of the short spacing at 4.6, 3.8, and 3.7 Å whereas the β' polymorph was calculated from the intensities of the short spacing of 3.8 and 4.2 Å.

2.2.4. Consistency. The consistency of the crystallized blends was tested with a cone penetrometer (KOEHLER Model Instrument Company INC) in samples tempered at three different temperatures (12°C, 20°C, or 30°C) according to method Cc 16-60 AOCS [30]. The crystallized fats were tempered in bioclimatic chambers set at 12, 20, or 30°C.

2.3. Statistical Analysis. A completely randomized experiment was performed. PS addition (50, 70, or 90%) and the rate of agitation (RA) (1000, 2000, or 3000 rpm) were the factors considered. Analysis of variance (ANOVA) and comparison of means by Tukey (significance level of 95%) were performed. Data was reported as means and standard deviations. Analyses were performed using the JMP 5.0.1 statistics software (SAS Institute, Cary, NC, USA).

3. Results and Discussion

3.1. Physicals Properties. The results of the color, refraction index, melting point, density, acidity, moisture, and peroxide and iodine values of palm stearin, high oleic safflower oil, PS/HOSO crystallized blends mixed at different agitation rates, and HCS are depicted in Tables 1 and 2. As expected, the various physical properties were significantly affected ($p < 0.05$) by the different ratios of PS and HOSO. Hydrogenated fats should have a red color value of 1.5 [32]. Among the experimental blends, the color of the 90% PS/10% HOSO mix had higher reddish (3.0) scores ($p < 0.05$) compared to the control HCS (2.0). The observed differences in color can be attributed to PS addition, HOSO addition, and fat type (vegetable or animal sources). The PS had a higher red color score of 3.5. This could be attributed to the presence of tocopherols and β-carotenes that are responsible for imparting orange, reddish, and yellow colorations [14, 33]. Furthermore, the HOSO contained high concentrations of total tocopherols (178 ppm) which contained 85.82% α-tocopherol [28].

Various blends containing PS and vegetable oils were screened based on iodine value (IV) and melting point [10, 17]. IV is an important parameter used in the hydrogenation industry because its value is closely related to melting point and oxidative stability. Shortenings with low-iodine value have lower melting points and are less susceptible to oxidation or are more stable [17]. The IV of HOSO was 90 (Table 1). After blending with the hard fraction of the palm oil, there was a decrease in IV. As expected, the lowest value was observed in the blend containing 90% palm stearin. There was a gradual increase in the melting point from 46°C to 50°C when the PS concentration in the PS/HOSO blends increased (Table 2). Hydrogenated fats should have a melting point between 35°C and 45°C [26] and the PS showed a melting point of 52°C (Table 1). The melting point of the blends containing PS of 90% was higher compared to the 50% blend (Table 2). There were no significant differences ($p > 0.05$) in the acidity, peroxide value, and moisture of the blends. The PS did not affect significantly ($p > 0.05$) the physical properties of the experimental blends.

3.2. Fatty Acids Profile. The fatty acid profiles of PS/HOSO blends (50 : 50, 70 : 30, and 90 : 10) processed at agitation rates of 3000 rpm and HCS are presented in Table 3. As expected, the FAP was significantly affected ($p < 0.05$) by addition of different ratios of PS and HOSO. The PS presented higher content of palmitic and oleic acids (49.81% and 20.56%, resp.) whereas the HOSO contained comparatively higher levels of oleic and linoleic acids (77.50% and 12.01%, resp.) (Table 1). The FAP of the PS/HOSO blends indicated that the components in order of abundance were palmitic (36.8–56.9%), oleic (27.1–42.9%), linoleic (6.1–12.3%), stearic (4.4–4.9%), and palmitoleic acids (0.3–0.52%). In PS/HOSO blends, the amounts of total saturated ΣSFA (palmitic and stearic) were higher compared with monounsaturated fatty acids (ΣMUFA) (oleic and palmitoleic) and ΣPUFA (linoleic and linolenic). Similar results were reported by Mayamol et al. [10], who assayed the fatty acid profiles of binary blends of PS and rice bran oil, finding as main components palmitic (37.4–43.2%), oleic (34.3–36.3%), linoleic (16.5–20.6%), stearic (2.9–3.0%), and linolenic (0.31–0.51%) acids. Recently, Latip et al. [21] reported in a binary mixture of 50% PS/50% sunflower oil a higher composition of palmitic (36.3%), linoleic (31.7%), oleic (25.2), and stearic (4.7%) acids.

As the concentration of PS increased in the PS/HOSO blends the content of SFA also increased, particularly palmitic acid. Among all blends, the one consisting of 50% PS/50% HOSO contained the lowest levels of ΣSFA (43.29%) and highest levels of both ΣMUFA (43.20%) and ΣPUFA (12.38%). On the other hand, the 90% PS/10% HOSO blend contained the highest levels of ΣSFA (63.64%) and lowest levels of ΣMUFA (27.67%) and ΣPUFA (6.17%), respectively. Addition

Table 1: Physical properties, fatty acid compositions, and consistencies of palm stearin and high oleic safflower oil[1].

Characteristics	Palm stearin	High oleic safflower oil
Colour (Lovibond red)	3.50	1.00
Refractive index (50/25°C)	1.45	1.463
Melting point (°C)	52.00	—
Relative density (60/25°C; g/ml)	0.8728	0.919
Acidity (Oleic%)	0.0375	0.21
Peroxides (Meq O_2/Kg)	0.20	0.50
Moisture and volatile matter (%)	0.22	0.05
Iodine value (gr. I_2/100 gr)	32.23	90.00
Fatty acids composition (%)[†]		
Myristic (14:0)	1.83	[*]ND
Palmitic (16:0)	55.33	6.09
Palmitoleic (16:1 n-7)	0.83	ND
Stearic (18:0)	9.72	2.64
Oleic (18:1 n-9)	22.84	77.50
Linoleic (18:2 n-6)	8.75	12.01
Linolenic (18:3 n-3)	0.66	0.25
Fatty acids *trans*	[*]ND	[*]ND
Polymorphic forms	$\beta \approx \beta'$	β
Consistency (mm/10 g) to:		
12°C	70.00	—
20°C	117.00	—
30°C	209.00	—

[1]Values are the mean of three replicates; [†]weight percentage; [*]ND: not detectable.

Table 2: Physical properties of zero-trans crystallized fats produced from palm stearin-high oleic safflower oil blends compared to hydrogenated commercial shortening[1,2].

Concentration PS/HOSO[3] (%)	Rate of agitation (rpm)	Color (red)	RI[4]	Melting point (°C)	Density (g/mL)	Acidity (Oleic %)	Peroxides (Meq O_2/Kg)	Moisture (°C)	Iodine value (gr. I_2/100 gr)
50 : 50	1000	2.7^{c}	1.4610^{a}	46^{c}	0.908^{d}	0.015^{a}	0.60^{a}	0.5^{a}	52.45^{a}
	2000	2.7^{c}	1.4610^{a}	46^{c}	0.909^{c}	0.015^{a}	0.60^{a}	0.5^{a}	52.45^{a}
	3000	2.7^{c}	1.4610^{a}	46^{c}	0.908^{d}	0.015^{a}	0.60^{a}	0.5^{a}	52.45^{a}
70 : 30	1000	2.9^{b}	1.4595^{b}	48^{b}	0.931^{b}	0.015^{a}	0.60^{a}	0.5^{a}	46.45^{b}
	2000	2.9^{b}	1.4595^{b}	48^{b}	0.932^{a}	0.015^{a}	0.60^{a}	0.5^{a}	46.45^{b}
	3000	2.9^{b}	1.4595^{b}	48^{b}	0.931^{b}	0.015^{a}	0.60^{a}	0.5^{a}	46.45^{b}
90 : 10	1000	3.0^{a}	1.4580^{d}	50^{a}	0.877^{e}	0.015^{a}	0.60^{a}	0.5^{a}	34.03^{d}
	2000	3.0^{a}	1.4580^{d}	50^{a}	0.877^{e}	0.015^{a}	0.60^{a}	0.5^{a}	34.03^{d}
	3000	3.0^{a}	1.4580^{d}	50^{a}	0.877^{e}	0.015^{a}	0.60^{a}	0.5^{a}	34.03^{d}
Hydrogenated commercial shortening		2.0^{d}	1.4590^{c}	45^{d}	0.810^{f}	0.015^{a}	0.55^{b}	0.4^{b}	43.00^{c}

[1]Different letter within each column indicates highly significant differences ($p < 0.05$). [2]Values are the mean of three replicates; [3]PS/HOSO = palm stearin-high oleic safflower oil blends; [4]RI = refractive index (50/25°C).

of 90% of PS to blend increased levels of palmitic acid up to 19.8% and decreased the amounts of oleic acid, down to 14.7%. All PS/HOSO experimental blends did not contain assayable levels of TFA. The differences observed among blends could be attributed to the typical fatty acid compositions of PS [10, 17, 34] and HOSO. The observed changes in fatty acids profile clearly affected the functional characteristics of binary mixtures [21].

The HCS had higher levels of ΣSFA (40.91%), ΣMUFA (38.01%), TFA (17.02%), and only 4.01% of ΣPUFA (Table 3). Commonly, the HCS contain from 20 to 40% TFA [1] which have beneficial effects on the texture and appearance of foods

Table 3: Fatty acid compositions of zero-*trans* crystallized fats produced from palm stearin-high oleic safflower oil blends compared to commercial hydrogenated shortening[1,2].

Fatty acids composition (wt%)[†]	Shortening			
	Hydrogenated commercial shortening	PS/HOSO[3] (50 : 50)	PS/HOSO (70 : 30)	PS/HOSO (90 : 10)
Myristic (14:0)	*ND	1.59[e]	1.96[e]	2.32[e]
Palmitic (16:0)	22.02[b]	36.80[b]	47.84[a]	56.90[a]
Stearic (18:0)	18.89[c]	4.90[d]	4.74[d]	4.42[d]
ΣSFA[4]	40.91	43.29	54.54	63.64
Palmitoleic (16:1 n-7)	*ND	0.30[f]	0.34[f]	0.52[f]
Oleic (18:1 n-9)	38.01[a]	42.90[a]	37.77[b]	27.15[b]
ΣMUFA[5]	38.01	43.20	38.11	27.67
Linoleic (18:2 n-6)	4.01[e]	12.38[c]	7.06[c]	6.17[c]
Linolenic (18:3 n-3)	*ND	*ND	*ND	*ND
ΣPUFA[6]	4.01	12.38	7.06	6.17
Trans fatty acids	17.02[d]	*ND	*ND	*ND

[1]Different letter within each column indicates highly significant differences ($p < 0.05$). [2]Values are the mean of three replicates; [3]PS/HOSO = palm stearin-high oleic safflower oil blends; [4]SFA = saturated fatty acids; [5]MUFA = monounsaturated fatty acids; [6]PUFA = polyunsaturated fatty acids; [†]weight percentage; *ND: not detectable.

Table 4: X-ray diffraction patterns after stabilization at 25°C for 24 h of zero-trans crystallized fats produced from palm stearin-high oleic safflower oil blends compared to commercial hydrogenated shortening[1,2].

Concentration PS/HOSO[3] (%)	Rate agitation (rpm)	Short-spacing (Å)[4]						
		4.5	4.4	4.1	3.9	3.8	3.7	PF[5]
50 : 50	1000	4.48[a]				3.81[b]	3.74[c]	β
	2000	4.48[a]				3.81[b]	3.74[c]	β
	3000	4.48[a]				3.81[b]	3.74[c]	β
70 : 30	1000	4.54[a]				3.86[b]	3.73[c]	β
	2000	4.54[a]				3.86[b]	3.73[c]	β
	3000	4.54[a]				3.86[b]	3.73[c]	β
90 : 10	1000	4.46[a]				3.82[b]	3.66[c]	β
	2000	4.46[a]				3.82[b]	3.66[c]	β
	3000	4.46[a]				3.82[b]	3.66[c]	β
Hydrogenated commercial shortening		—	4.46[a]	4.10[b]			3.70[c]	β'

[1]Different letters indicate significant differences in the same row ($p < 0.05$). [2]Values are the mean of three replicates; [3]PS/HOSO = palm stearin-safflower high oleic oil blends; [4]Å = angstrom; [5]PF = polymorphic forms.

products but adverse health implications. It is known that TFA increases low-density lipoprotein (LDL) serum levels. If the blood keeps excess levels of LDL-cholesterol there is an increased risk of cardiovascular diseases [2–5].

The concentration of palmitic acid was highest in all blends of PS/HOSO. This particular saturated fatty acid has no adverse effect on serum lipoprotein profiles [35]. The most suitable blend, considering all physical properties analyzed, was the one containing 50% PS/50% HOSO. This particular blend had a fairly balanced FA composition (36.80% palmitic, 42.90% oleic, and 12.38% linoleic acids), did not contain *trans* fatty acid isomers, and had adequate functionality as a natural vegetable shortening. Therefore, it is suitable as food ingredient for the development of new functional foods with clean labels.

3.3. Polymorphism. The patterns of XDR and polymorphic forms of fat crystals from PS and PS/HOSO blends obtained at different agitation rates and HCS are depicted in Table 4. The polymorphic forms of fat crystals are of upmost importance because they are closely related to the functional properties of shortenings [14]. Regardless of the ratio of PS/HOSO and agitation rates, all blends tempered at 25°C possessed the β-crystal polymorphic forms. All PS/HOSO blends exhibited short-spacing which lied between 4.54, 3.86, and 3.73 Å. These spaces are distinctive and characteristic patterns for β crystals. In our study, the tempering at 30 ± 1°C affected the β' crystal formation for all samples. However, these blends presented a smooth consistency throughout 5 days' storage time. These PS/HOSO blends promoted the most stable β polymorphic form, but the least desirable in terms of crystal size. The PS presented β' polymorphic form [36]. However, in our study PS exhibited a tendency to the forms $\beta \approx \beta'$ (Table 1). The results obtained from the experimental blends (PS/HOSO) presented similarity to those reported by Nor Aini et al. [37]; Jeyarani and Reddy

[13]; Mayamol et al. [14]; and Berger and Idris [38] who formulated blends of palm stearin varying the type of other complementing vegetable oils (rice bran, sesame, or soybean oil). These authors also elaborated tertiary blends palm oil-palm stearin-palm kernel olein (40 : 30 : 30) obtaining in finished products the presence of β polymorphic forms [37].

The HCS showed short-spacing between 4.42, 4.14, and 3.72 Å, characteristics of a pattern for β' crystal (Table 4) associated with most vegetable shortenings. It is important to mention that animal fats contain β forms [39, 40]. Fats with β' form are preferred for plastic shortenings as the crystals tend to be smaller, more uniform, and smoother, whereas the β form imparts sandiness and graininess [10, 41]. It is reported that a palmitic acid content above 44% in fats crystallizes in β' polymorphic forms desirable in margarines and shortenings [11]. However, in our study, high percentages of palmitic acid contents were observed in the PS/HOSO blends (37.8 to 57.7%) and therefore this particular fatty acid limited the formation of high levels of β' crystals. The use of PS to produce the zero-*trans* shortenings and the proposed strategy of adding the HOSO to the blends were essential to favor the formation of β polymorph crystals. The blend PS/HOSO could be used in bakery products such as cookies, wheat flour tortillas, and others.

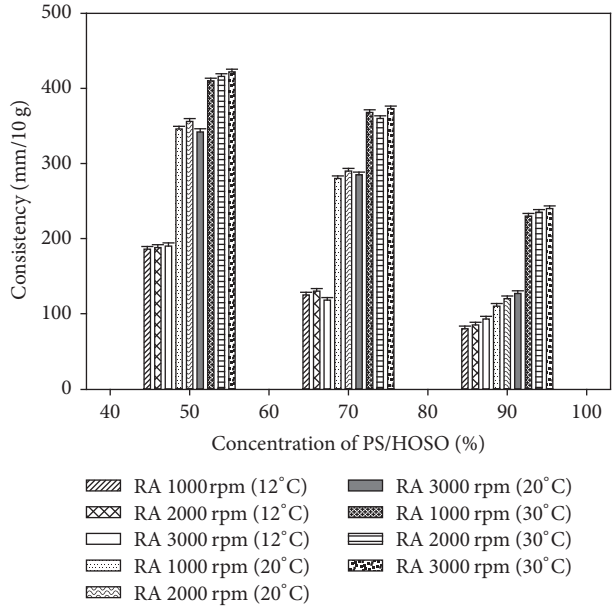

Figure 1: Effect of PS/HOSO (palm stearin and high oleic safflower oil) blends and rate of agitation (ra) on the consistency of zero-trans crystallized fats equilibrated at three storage temperatures.

3.4. Consistency. The PS and HOSO ratios, speed of agitation, and storage temperatures (12°C, 20°C, or 30°C) affected significantly ($p < 0.05$) the consistency of the blends (Figure 1). The consistency decreased sharply with a decrease in storage temperature for all blends. When PS was added in higher amounts, the consistency decreased for all tested temperatures. Some experimental blends showed comparable consistencies compared with HCS (150–300 mm/10 g) in spite of their modified fatty acid composition (high in unsaturated fatty acids with zero-TFA) (Table 3). The blends containing 50%, 70%, or 90% of PS/HOSO had consistencies similar to hydrogenated commercial shortening. Additionally, at lower temperatures (12°C), all PS/HOSO blends showed a consistency similar to commercial bakery fats, while, at higher temperature (20°C), the 70% and 90% PS/HOSO blends exhibited consistencies similar to HCS. The consistency of 150–300 mm/10 g at the temperature of 30°C is advantageous for use in cake manufacturing as it can retain the air incorporated during whipping and baking, and besides it is advantageous for better creaming performance [32, 42]. The 90% PS/10% HOSO blend stored at 30°C had a consistency similar to HCS, but it was not suitable as plastic fat, because of its relatively high-melting point (50°C) and low IV (34.03) (Table 2). A shortening with these features imparts a waxy mouthfeel [14]. The HCS contained emulsifiers that allow crystal growth and/or polymorphic transitions which improved the consistency necessary for commercial bakery fats [43]. Furthermore, according to Mayamol et al. [14] crystallized fats manufactured from high amounts of palm stearin and low amounts of vegetable oil (90 : 10) tend to gradually produce grainy textured fats mainly because of their high amounts of β crystal polymorphic forms. This undesirable texture is also attributed to the high-melting point of the palm stearin. In several instances, this crystal transformation can produce oil exudation from the fat crystals with the partial coalescence of the aqueous phase and separation. Moreover, blends with high concentrations of palm stearin take longer to form fat crystals [38].

The PS is composed of high-melting triacylglycerides, which resulted in a hard consistency (240 mm/10 g) at 30°C. The mixing improved miscibility between the PS and HOSO. These PS/HOSO blends therefore are not operable over a wide range of temperatures. The blends equilibrated at low temperatures (12°C) presented a hard consistency; however, counterparts equilibrated at 30°C exhibited a desirable softer texture. Nevertheless, blends stored at 30°C presented oil exudation and phase separation.

The consistency of the blend produced with 50% PS/50% HOSO was comparable to the PS and canola oil mixture used for a production of wheat doughs for baking [20]. The direct blending is the preferred method for formulation of zero-trans crystallized fats because it enhances the miscibility between vegetable oils and saturated fats [14]. Hence this method could assist in eliminating the posthardening phenomena, which was very notorious in PS-based fats [10]. Blends of PS with other oils (rice bran oil, canola oil, sesame oil, and soybean oil) and fats provided good plasticity and consistency [10, 12, 14]. Results herein demonstrated that the combination by direct blending of PS with HOSO could provide an array of new fat products with the desired consistency.

4. Conclusions

The direct blending process proposed herein indicated that the 50% PS/50% HOSO blend was ideal for producing zero-*trans* crystallized fats because this particular blend showed

a melting point similar to commercial bakery shortening. This specific blend system free of TFA contained the highest amounts of PUFA and MUFA. These acids are positively related to various health benefits and nutritional properties. Several of the experimental blends showed comparable consistency to hydrogenated shortenings. Thus, zero-*trans* crystallized plastic fats suitable for use in bakery items can be manufactured utilizing high oleic safflower oil and palm stearin. This strategy has the advantages of producing vegetable fats with enhanced functionality and well-balanced fats rich in both omega 3 and omega 6 fatty acids.

Additional Points

Practical Applications. Hydrogenated fats (*trans* fats) are widely used in the food industry and are highly consumed worldwide. The consumption of *trans* fats is related to adverse effects on serum cholesterol, triglycerides, and cardiovascular diseases. The formulation of zero-*trans* fats from direct mixing of vegetable sources is an alternative for the food industry. This strategy is essential to produce *trans* free fats that have similar characteristics to hydrogenated counterparts, with the advantage of increasing omega 3 and 6 fatty acids.

Conflicts of Interest

No conflicts of interest exist.

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