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Human Milk Fat Substitutes from Lard and Hemp Seed Oil Mixtures

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Abstract: This paper discusses our attempt to generate substitutes for human breast milk fat through the interesterification of mixtures composed of lard and hemp (Cannabis sativa) seed oil. The interesterification was run at 60 °C for 2, 4, and 6 h in the presence of Lipozyme RM IM preparation containing a lipase specific for the cleavage of sn-1,3 ester bonds in triacylglycerol molecules. The interesterification products were analyzed regarding their fatty acid composition and distribution in triacylglycerol molecules. In order to assess the quality of the generated substitutes, in the interesterification products the following were determined: acid value, peroxide number, and oxidative stability. The collected data were statistically processed using Tukey's test. Following the interesterification, the fats revealed an elevated percentage of free fatty acids and primary oxidation products and reduced oxidative stability compared to those of lard. The last of the above-mentioned phenomena could have been due to the incorporation of polyenic fatty acids into the external positions of triacyclglycerols of lard. The interesterification of lard and hemp seed oil allows scientists to acquire substitutes rich in essential fatty acids and similar to human breast milk fat with respect to the distribution of fatty acids in triacylglycerol molecules.

Keywords: human milk fat substitutes; structured lipids; interesterification; lipid oxidation; oxidative stability; omega-3 fatty acids; hemp seed oil; lard

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1. Introduction

Inter- and intraesterification are industrial transformation processes based on the redistribution of fatty acids between separate molecules of triacylglycerols (intermolecularly) or inside them (intramolecularly), respectively. Such reactions take place until thermodynamic equilibrium is reached, and they change the physico-chemical properties of mixtures of fats and oils. Compared to the original mixtures, the resulting products feature a similar degree of saturation and fatty acid profile. However, they are different in terms of triacylglycerol stereochemistry, which affects their physicochemical and nutritive properties [1–5]. The reaction of inter- or intraesterification may be carried out in the presence of chemical or enzymatic catalysts. An enzymatic interesterification is a simpler process than a chemical one as it involves a smaller number of stages, and the temperature used is relatively low (from 55 °C to 70 °C). Chemical interesterification usually takes

place at higher temperatures within the range of 70–120 °C. Enzymatic interesterification may be controlled and discontinued at every stage [6,7]. Its advantage over chemical interesterification stems from the fact that the discussed process becomes more specific in the presence of an enzyme, particularly if lipases of various specificity are used. As a result of using specific lipases, scientists may synthesize fats with the required physical and nutritional properties—a process unaccomplishable by means of chemical intra- or interesterification. Further, due to the relatively low temperatures used during the process, unstable fatty acids do not undergo oxidation, and the remaining components contained in the oil, such as vitamin E, do not degrade. Also, from a dietary point of view, no harmful trans fatty acid isomers are formed during the process [7–9]. However, not all trans fatty acid isomers are undesirable. Some of them, such as, for example, conjugated linoleic acids (CLA), which are present in dairy products and ruminant meats, may present benefits for health, e.g., protection against cancer and obesity [10].

Breast milk is only natural food for newborns, but due to occasional problems with lactation, people have always tried to find alternative solutions. Breast milk is a source of the nutritive elements needed for normal infant growth and development. Human milk contains 1–2% proteins, 6–8% carbohydrate, and 3–5% fat [11]. To add more benefits, it is characterized by an adequate temperature, antibacterial properties, and constant freshness, and it does not require any special preparation [12]. Human breast milk contains fats, i.e., substances of peculiar physiological significance for infants and younger children. Fats are nutritive agents necessary for the normal development of infants and small children. This is true mainly due to the fact that fats introduce polyunsaturated fatty acids (PUFAs) into small children's bodies, compounds required for normal development of the brain, nervous system, and cellular membranes. The PUFAs also constitute carriers for fat-soluble vitamins and milk-contained hormones [13]. A specific feature of breast milk fat is the content of long-chain polyunsaturated fatty acids (LCPUFAs). The predominant LCPUFAs contained in breast milk fat are eicosapentenoic acid (EPA), docosahexenoic acid (DHA), and arachidonic acid (ARA). These are the acids responsible for normal growth and bone mineralization and for proper development of the central nervous system. LCPUFAs are also precursors of prostaglandins and eicosanoids, which play regulatory roles. Furthermore, they are mediators of immunological reaction, vascular blood flow, and platelet aggregation, among other things [14]. Breast milk fat features not only a specific content of fatty acids but also their specific distribution in the molecules of triacylglycerols. According to various scientific studies, this very stereoisomeric specificity of breast milk triacylglycerols contributes to the enhancement of fat absorption from food, diminution of insoluble calcium salt formation, and prevention of the excretion of excessive calcium and magnesium from the body [15]. The chemical composition of breast milk is stable within some ranges. The content of selected chemical components, including fatty acids, changes depending on a number of factors, i.e., lactation phase, daytime, and mother's diet [16,17].

Although breast milk is the most beneficial kind of food for feeding infants, breast-feeding is not always possible for economic or health-related reasons. Unfortunately, in both developing and industrialized countries, the number of breast-feeding women is getting lower and lower. Many infants are fed with animal milk—mainly cow's milk or baby-food products [12]. The elaboration of modern technologies for the production of modified milk designated for feeding infants, mimicking the original breast milk as natural food in a more and more sophisticated way, is very interesting for food manufacturers. Also, it constitutes a challenge for scientists conducting research studies on the profile of fatty acids in human breast milk and their essential role in the feeding of infants [18,19].

Lard is a fat that mimics breast milk fat with respect to the distribution of fatty acids in triacylglycerol molecules. Compared to breast milk fat, lard has a similar content of palmitic and oleic acids but features a lower percentage of essential fatty acids [19–21]. On the other hand, hemp oil can be used as a health-oriented product due to its high percentage of essential fatty acids. It is composed of 80% polyunsaturated fatty acids. The main fatty acid is linoleic acid, a chemical compound belonging to the family of ω-6

acids. The second most abundant fatty acid contained in hemp oil seeds, required for the normal functioning of the human body, is α-linolenic acid, a representative of the ω-3 family. Moreover, hemp oil contains approximately 1–5% γ-linolenic acid (GLA), which is rarely found in seed oils. Next, this particular oil is characterized by an optimum ω-6/ω-3 ratio (3:1). Such a property makes it a wholesome oil of a very high nutritive value [22,23].

The aim of our study was to acquire a substitute for human breast milk fat through interesterification of lard and hemp seed oil mixtures and to monitor the properties of the resulting substitutes via calorimetric and chromatographic methods. We used enzymatic interesterification with lipolytic enzymes as catalysts, because this method is considered one of the best choices for the modification of fat properties.

2. Materials and Methods

2.1. Materials

The materials used in this study included liquid pork (*Sus domestica*) lard (L) and hemp seed (*Cannabis sativa* L.) oil (HO), which were collected from the local firms Zakład Mięsny Wierzejki and PPHU Maszyny i Przetwórstwo Nasion Oleistych Ol'Vita. The samples were stored at sub-zero temperature (−20 °C) for further preparations in tightly closed packages.

2.2. Preparation of Human Milk Substitutes—Enzymatic Interesterification

The human milk substitute samples obtained from liquid lard and hemp oil with an 8:2 mass ratio were applied to enzymatic interesterification in the presence of Lipozyme RM IM preparation. Lipozyme RM IM, procured from Sigma Aldrich, is a food-grade granulated silica preparation of a microbial 1,3-specific lipase from *Rhizomucor miehei* (activity 150 IUN/g). The enzymatic modifications of the lard and hemp seed oil mixtures took place in a shaker equipped with a thermostat at 60 °C for 2 (Mix 60 °C_2 h), 4 (Mix 60 °C_4 h), or 6 (Mix 60 °C_6 h) hours. Prior to the beginning of the process, the mixture of fats was incubated at the required temperature for 10 min. The interesterification was triggered by the addition of a defined amount of enzymatic preparation (8% compared to the mass of the fatty mixture) to the system. The reaction was stopped by the separation of the enzyme from the fat sample through reduced-pressure filtration using a Büchner funnel. Then, the samples were stored at sub-zero temperature (−20 °C) until further analysis.

2.3. Analysis

2.3.1. Determination of Fatty Acid Composition

The composition of fatty acids was determined by means of gas chromatography (GC), using a capillary column and a flame ionization detector (FID). In order to generate volatile derivatives of fatty acids, the studied samples of fats were applied to esterification with methanol, in compliance with standard PN-EN ISO 5509:2001 [24] and in accordance with the methods described in papers by Bryś et al. [25], resulting in fatty acid methyl esters (FAMEs). Prepared samples were introduced onto the column. The study was conducted using a YL6100 GC apparatus equipped with a BPX-70 capillary column (60 m long, 0.25 mm internal diameter, 0.25 µm film thickness) using nitrogen as a carrier gas. The FAME separation conditions were as follows: an initial temperature of 60 °C was maintained for 5 min; the increment of temperature rise was 10 °C/1 min within the range from 60 °C to 180 °C, then the increment of temperature rise was 3 °C/1 min within the range from 180 °C to 230 °C; the end temperature of 230 °C was maintained for 15 min; the temperatures of the detector and injector were 250 °C and 225 °C, respectively; and the total analysis time was 51 min. Fatty acids were identified based on retention time values compared with the standard (Supelco 37 Component FAME Mix).

2.3.2. Determination of Generated sn-2 Monoacylglycerols

The distribution of fatty acids in fats was also determined with regards to their positions—central or external positions of triacylglycerols—in accordance with methods described in papers by Bryś et al. [25].

2.3.3. Determination of Free Fatty Acid Content

The samples were prepared in accordance with the method described in ISO 660:2009 [26] and with methods described in papers by Bryś et al. [25]. Briefly, the acid values were determined via titration of fat samples dissolved in a mixture of diethyl ether and ethanol (1:1, v/v) with 0.1 M ethanolic potassium hydroxide solution. The free fatty acid content was computed using the value of the molar mass of oleic acid and the acid values for the studied samples.

2.3.4. Determination of the Peroxide Value

According to the procedure in ISO 3960:2007 [27] and methods described in papers by Bryś et al. [25], the fat samples were prepared. The peroxide values were determined via an iodometric technique with a visual endpoint detection.

2.3.5. DSC Measurements

Differential scanning calorimetry (DSC) was used to define the oxidative stability of the generated human breast milk fat substitutes. Experiments using pressure differential scanning calorimetry (PDSC) were carried out with the help of a DSC Q20 TA Instruments apparatus linked to a high-pressure chamber in accordance with methods described in papers by Bryś et al. [25]. Fat samples (3–4 mg) were placed in small aluminum vessels, in an oxygen atmosphere, and under pressure of 1.400 kPa. Measurements were taken isothermally at 120 °C. The oxidation induction time was determined from the PDSC curves.

2.4. Statistical Analysis

The statistical analysis was performed using Stargraphics Plus version 4.1 software. The collected data were statistically processed via one-way analysis of variance with the use of Tukey's test, and differences between the samples were considered to be significant at the level of $\alpha = 0.05$, in accordance with methods described in papers by Bryś et al. [25]. Values are represented as the mean and standard deviation.

3. Results

3.1. Fatty Acid Profile and Their Distribution in Triacylglycerols (TAGs)

The studied lard contained over 80% palmitic acid in the sn-2 position of TAGs (Figure 1), while in the hemp seed oil, the share of this acid in the sn-2 position was only 40%. The acquired breast milk fat substitutes were characterized by a specific distribution of fatty acids in the TAG molecules that was different from those present in lard and hemp seed oil (Table 1, Figure 1). Having analyzed the percentage of unsaturated fatty acids in the sn-2 TAG position of the generated substitutes, we found that they occurred mainly in the external positions of TAGs. The share of oleic acid within the central position of TAGs in the resulting substitutes was between 12.5% and 19.6%, which accounts for the fact that it is contained mostly in sn-1,3 positions (the oleic acid content in sn-1,3 positions was between 32.8% and 39.5%, and that in the sn-2 position was between 12.5% and 19.6%). In contrast, a vast majority of saturated palmitic acid occupied the sn-2 position of TAGs in the acquired human breast milk substitutes. Depending on the process conditions, the share of this particular acid in the sn-2 TAG position was between 41.3% and 51.8% (the palmitic acid content in the sn-1,3 positions was between 13.8% and 21.7%, and that in the sn-2 position was between 41.3% and 51.8%). This triacylglycerol structure of the acquired substitutes is very close to that appearing in human milk fat (HMF).

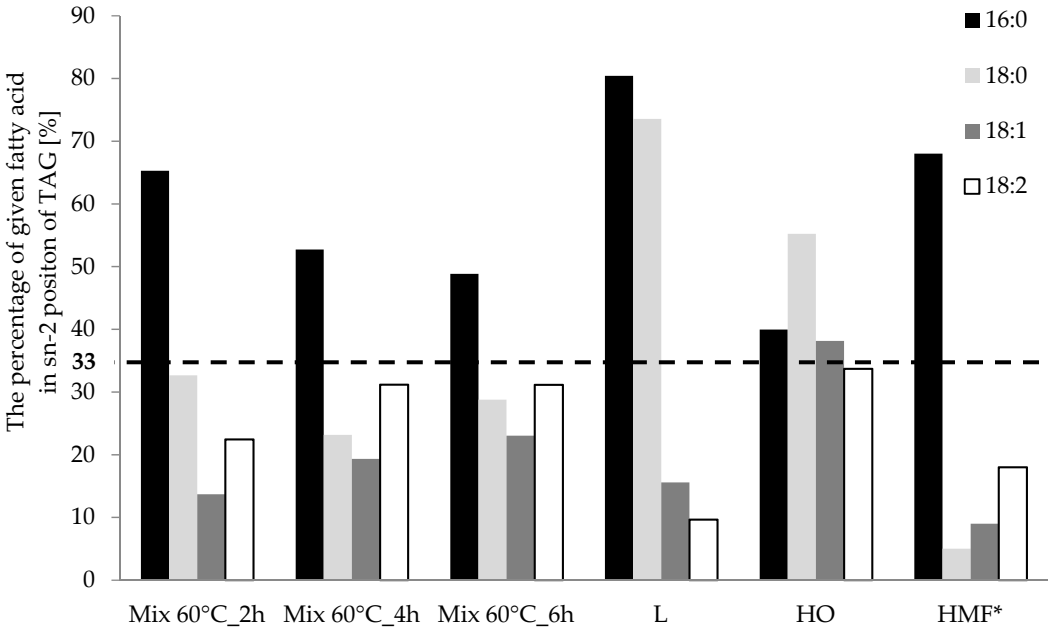

Figure 1. The percentage (wt%) of a given fatty acid in the sn-2 position of TAGs in lard (L), hemp seed oil (HO), human breast milk fat substitutes, and human milk fat. HMF * (human milk fat) sample values were derived from Lien [28].

Table 1. The fatty acid compositions of the sn-2 position and sn-1,3 positions of TAGs in human breast milk fat substitutes.

	Type of Sample	Fatty Acids (wt%)			
		16:0	**18:0**	**18:1**	**18:2**
sn-2	Mix 60 °C_2 h	51.8	13.1	12.5	11.4
	Mix 60 °C_4 h	47.1	8.5	16.5	15.1
	Mix 60 °C_6 h	41.3	11.7	19.6	16.1
sn-1,3	Mix 60 °C_2 h	13.8	13.5	39.5	19.8
	Mix 60 °C_4 h	21.1	14.1	34.4	16.6
	Mix 60 °C_6 h	21.7	14.5	32.8	17.8

In order to enrich the lard TAGs in polyunsaturated fatty acids, the interesterification process was initiated. PUFAs originating from hemp seed oil were built into the lard TAG structure in its course. Given the results regarding the content of fatty acids in the analyzed HMF substitutes (Figure 2), we observed that, irrespective of the process duration time, the interesterification products featured similar profiles of fatty acids. Among all the researched modified fats, saturated fatty acids (SFAs) prevailed. Their content ranged from 43.2% to 46.6%. Furthermore, these fats contained considerable amounts of monounsaturated fatty acids (MUFAs). Jointly, they constituted from 32.4% to 33.5% of all the fatty acids. In addition, the interesterification products turned out to be an abundant source of polyunsaturated fatty acids, ranging between 21.3% and 22.8%.

3.2. Free Fatty Acid Content and Oxidative Stability

The content of free fatty acids was calculated based on determined values of the acid number (Figure 3). Their content in lard was 0.3%, while in hemp seed oil, it was 1.0%. The interesterification process led to a remarkable increase in the content of free fatty acids in relation to initial chemical feeds. The percentage of free fatty acids in the interesterification products ranged from 14.9% to 25.9%.

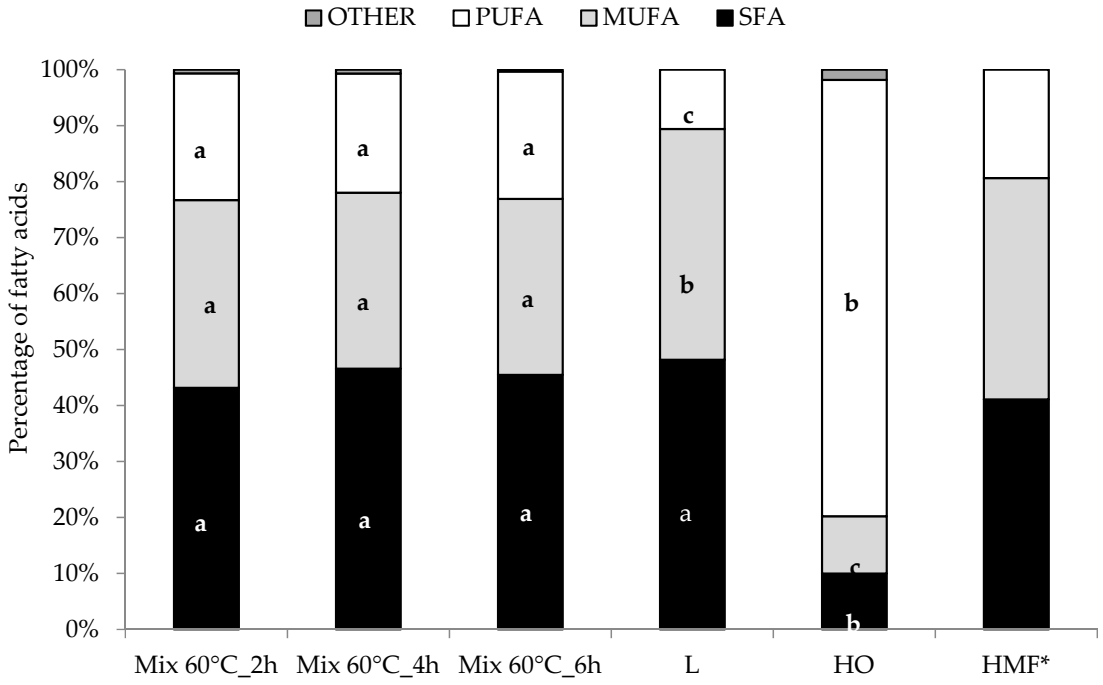

Figure 2. Percentage (wt%) content of fatty acids (SFA—saturated fatty acid, MUFA—monounsaturated fatty acid, PUFA—polyunsaturated fatty acid) in lard (L), hemp seed oil (HO), human breast milk fat substitutes, and human milk fat. * HMF (human milk fat) sample values were derived from López-López et al. [29]. Different letters indicate that the samples are significantly different at $p < 0.05$ separately for each group of fatty acids.

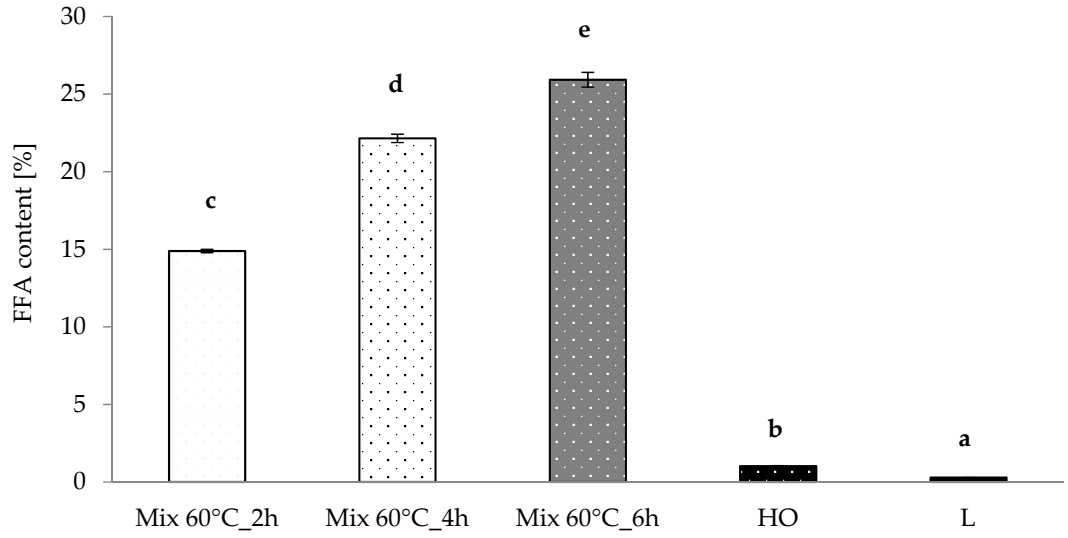

Figure 3. Free fatty acid (FFA) contents (wt%) in lard (L), hemp seed oil (HO), and human breast milk fat substitutes. Different letters indicate that the samples are significantly different at $p < 0.05$.

In order to determine fat oxidative stability, the content of primary oxidation products was measured using titration, and the induction time was calculated based on an accelerated test for oxidation with the help of DSC. The content of primary oxidation products is defined by the peroxide value (Figure 4).

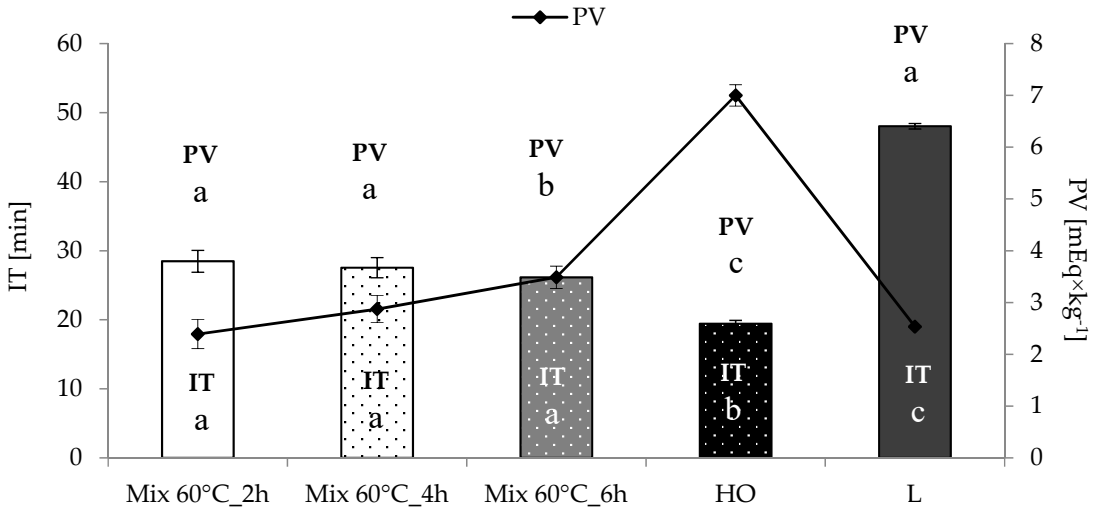

Figure 4. Oxidation induction times (ITs) and peroxide values (PVs) of lard (L), hemp seed oil (HO), and human breast milk fat substitutes. Different letters indicate that the samples are significantly different at $p < 0.05$ separately for PV and IT.

The HMF substitutes were characterized by smaller peroxide values, within the range of 2.4 mEq O_2/kg up to 3.5 mEq O_2/kg, compared to the hemp seed oil, for which this parameter equaled 7.0 mEq O_2/kg. However, lard featured a low peroxide value of 2.5 mEq O_2 kg^{-1}. Substitutes acquired via interesterification carried out for 6 h had higher peroxide values in comparison with substitutes acquired via interesterification for 2 or 4 h. Moreover, Figure 4 presents the oxidation induction times for the interesterification products and their original feeds. In studies on the stability of fats, a rule is taken into account that the longer the induction time, the higher the oxidative stability of the fat [30]. The determined induction times for the interesterification products, ranging from 26.2 min to 28.5 min, similarly to the peroxide value, indicate their lower oxidative stability than that of lard, for which the induction time was 48 min. Nonetheless, they were more stable than the hemp seed oil, for which the induction time was 19.4 min.

4. Discussion

Fat in human breast milk occurs mainly in the form of triacylglycerols that make up over 98% of all the milk fats. The discussed TAGs have a unique composition and structure, as palmitic acid is contained mainly in the sn-2 position (approximately 40–60%), whereas positions sn-1 and sn-3 are occupied by unsaturated fatty acids. In the sn-1,3 positions of TAGs, the most abundant are oleic and linoleic acids. To the contrary, in the majority of plant fats, the situation is reversed, i.e., palmitic acid occupies mainly positions sn-1 and sn-3 of TAGs, while unsaturated fatty acids take the central position [12,31]. From a dietary point view, the distribution of acyls in the TAG molecules plays a key role. The type of a fatty acid and its location in the TAG molecule determine the physical properties of a given fat and its digestion and absorption [32]. The structure is responsible for normal fat absorption from food. It also prevents from the formation of insoluble calcium salts. This is because pancreatic lipase is an enzyme selectively hydrolyzing fatty acids in the sn-1,3 positions, with the production of free fatty acids and 2-monoacylglycerols. Palmitic acid in the monoacylglycerol form is more efficiently assimilated than free palmitic acid, as the former can bind calcium and magnesium ions, among others, with the formation of insoluble soaps excreted with feces. Hence, feeding an infant with fat of different TAG spatial structures from the one present in human breast milk gives rise to increased losses of calcium, magnesium, and the fat itself from the child's body [13,15,28,29,33–36]. The best modified mixture mimicking human breast milk fat, in terms of the fatty acid composition, was the product of two hours of interesterification at the temperature of 60 °C.

The composition of HMF features the best health-oriented and nutritional properties for infants and small children. The fat contains mainly palmitic acid (17.0–24.4%),

oleic acid (20.8–33.5%), and polyunsaturated fatty acids (13.2–22.7%) [37]. Among the polyunsaturated fatty acids, this fat contains such acids as linoleic, α-linolenic, arachidonic, eicosapentenoic, and docosahexenoic [29]. Compared to HMF, the studied lard featured a similar content of palmitic acid (approximately 27.7%), but a higher percentage of oleic acid and a lower percentage of polyunsaturated acids, i.e., 38.0% and 10.6%, respectively. The composition of fatty acids in the hemp seed oil was completely dissimilar from those in both lard and HMF. The hemp seed oil contained much more polyunsaturated fatty acids (approximately 78.0%) than the other two tested mixtures. However, the content of palmitic acid (6.2%) and oleic acid (9.6%) was lower in the hemp seed oil compared to the remaining two studied products. From among all the SFAs, palmitic acid dominated in the TAGs of the generated substitutes, a situation similar to that in HMF. Its content in the acquired substitutes was within the range of 26.7–29.8%. The analysis of the obtained interesterification products revealed that oleic acid was the prevailing monounsaturated fatty acid in these products. Its content was approximate to the one present in HMF and ranged from 28.4% to 30.5%. From among the PUFAs identified in the synthesized substitutes, linoleic acid played a major role. A similar situation was recorded in the case of HMF. The interesterification products contained between 16.1% and 17.0% of linoleic acid, belonging to the family of ω-6 acids. The TAGs of the resulting substitutes also contained other essential fatty acids, such as α-linolenic acid (0.6–0.7%, ω-3 family) and γ-linolenic acid (approximately 1%, ω-6 family). Although the synthesized interesterification products did not contain beneficial DHA, EPA, and ARA, they contained their precursors. DHA, EPA, and ARA may be formed in the human body in the course of metabolic changes from fatty acids such as α-linolenic acid, γ-linolenic acid, and linoleic acid [38].

TAGs are the main product of the interesterification reactions. In enzymatic modification of fats, due to hydrolysis, some incomplete acylglycerols and free fatty acids arise in parallel with the TAG fraction. A high share of free fatty acids in the interesterification products is unfavorable, and its reduction is desired. This is achievable by the diminution of water activity, e.g., by the application of reduced pressure, multiple uses of the same catalyst, or the maintenance of a continuous process [39].

Fats and oils are susceptible to oxidation processes, with the generation of reactive species, hydroperoxides, and polymers [40]. Such products exert a negative effect on the organoleptic traits of fats and may be harmful to public health. Therefore, foodstuffs containing oxidized fats are of worse quality [40,41]. The most important factors affecting the rate of oxidation are the composition of fatty acids and the presence of pro-oxidants and antioxidants [42]. The most susceptible to oxidation are fats containing polyunsaturated fatty acids [41,42]. Classical analyses are often used to determine oxidative stability, but these methods are time consuming [43]. Taking the aforementioned into account, DSC measurements are nowadays considered to be a very useful accelerated technique to determine the quality of fat samples [44,45]. In the majority of studies, a reduction in the oxidative stability of interesterification fat mixtures has been reported. This was particularly true in the case of such mixtures rich in unsaturated fatty acids, when compared with feeds like lard or cow milk fat [18,19,25,36,40,46]. The decrease in the oxidative stability of interesterification products in relation to that of lard might have resulted from the incorporation of polyenic fatty acids in the marginal TAG positions of lard. In turn, this might facilitate the access of oxygen to the acids and their easier oxidation [47].

5. Conclusions

One of the most important properties of fat is its oxidative stability. The oxidative stability of the human milk fat substitutes developed in this study was determined using the calorimetric method and by measuring the peroxide value. The results of the obtained research indicate a deterioration in the oxidative stability of the obtained interesterification products compared to that of the raw materials. The resulting human milk fat substitutes are characterized by a shorter oxidation induction time and higher peroxide values than those of lard. One of the reasons for the lower oxidative stability of the obtained human

milk fat substitutes compared to that of lard might be the incorporation of polyunsaturated fatty acids into the structure of the triacylglycerols of lard.

The interesterification caused a considerable increase in the free fatty acid content in comparison with those in the source feeds. A high percentage of free fatty acids in the interesterified products is disadvantageous and should be minimized. Palmitic acid was contained mainly in the sn-2 position of the triacylglycerols of the obtained interesterified products, whereas positions sn-1 and sn-3 were occupied by unsaturated fatty acids. Such a structure is responsible for normal fat absorption from food, and it prevents the formation of insoluble calcium salts. From a dietary point of view, the lard and hemp seed oil interesterification products contained rewarding fatty acids such as linoleic acid, α-linolenic acid, and γ-linolenic acid.

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