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ORIGINAL PAPER

Human Milk Fat Substitute from Butterfat: Production by Enzymatic Interesterification and Evaluation of Oxidative Stability

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Abstract Recent data have suggested that the fatty acid composition and molecular structure of fats in infant formulas should be as similar to human milk fat as possible to obtain optimal fat and calcium absorption from the infant formula. This work investigated the possibilities of using enzyme technology and butterfat as a material to produce a fat similar to human milk fat with respect to the above parameters. Moreover, the oxidative stability of the enzyme modified human milk fat substitute (HMFS) was compared to the fat blend used for the production of HMFS. Using a combination of enzyme technology, fractionation and batch deodorization and with butterfat in combination with soybean oil and rapeseed oil as raw materials it was possible to produce HMFS with a molecular structure and fatty acid composition that was very similar to that of human milk fat. The oxidative stability of the HMFS oil was lower than that of the reference oil with the same fatty acid composition. However, oxidation did not lead to a severe increase in rancidity scores during storage. Rather, the panel gave high intensity scores for other off-flavors such as burnt and bitter. Further optimization of the deodorization process is therefore necessary to remove these off-flavors.

Keywords Structured lipids · Enzyme modification · Peroxides · Volatiles · Sensory analysis

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Introduction

Fat in human milk is a major source of energy. The structure of human milk triacylglycerol is unique as 60–70% of palmitic acid (16:0) is located at the *sn*-2 position and 18:0, 18:1 and 18:2 are preferentially esterified in the *sn*-1/3 positions. Since pancreatic lipase selectively cleaves the fatty acids in the *sn*-1 and *sn*-3 positions of the triglyceride, palmitin will mainly be present as 2-monopalmitin together with mainly C18 free fatty acids. 2-Monopalmitin is efficiently absorbed while free palmitic acid forms poorly absorbed calcium soaps in the intestine resulting in reduced absorption of both calcium and fat. Thus, the position of C16:0 on the triglyceride is of considerable significance for the absorption of fat and minerals in infants [1]. Therefore, the fatty acid composition and distribution in triacylglycerols in infant formulas have recently gained much attention. Human milk fat substitutes (HMFS) have been developed to mimic human milk fat composition and structure from material basically based on palm oil and lard or from tripalmitin and vegetable oil blends [2–5].

Butterfat is a natural product from the dairy industry. Even though its composition and structure are significantly different from human milk fat (Table 1), butterfat has a lot in common with mother's milk fat in terms of content of

Table 1 Fatty acid compositions and *sn*-2 positional distributions of typical mother's milkfat and butterfat (% of total for the individual fatty acids)

	Human			Bovine		
	sn-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3
C4:0				9.8	5.7	84.6
C6:0				16.1	25.8	58.1
C8:0				16.7	42.6	40.7
C10:0	9.1	9.1	81.8	20.5	50.0	29.5
C12:0	13.7	22.1	64.2	24.6	47.6	27.8
C14:0	18.2	41.5	40.3	27.6	53.7	18.7
C16:0	20.0	72.3	7.7	45.6	41.6	12.8
C16:1	23.1	30.1	46.8	49.2	35.6	15.3
C18:0	73.9	16.3	9.9	58.6	25.5	15.9
C18:1	42.5	11.7	45.8	41.9	27.8	30.3
C18:2	33.3	22.1	44.5	32.4	67.6	0.0
C18:3	15.4	23.1	61.5			
C20:1	55.6	25.9	18.5			
C20:4	0.0	75.0	25.0			

Human milk fat data are from [6]. Butterfat data were obtained from the butterfat used in the present study

short chain fatty acids and minor fatty acids such as conjugated linoleic acid [6–8]. Studies on the use of butterfat as materials for HMFS production has not been widely conducted and no studies using immobilized lipase reactors for this purpose have to our knowledge been performed [9]. One early work by Christensen and Hølmer [10] reported the use of butterfat modification for infant formula applications. The work mainly focused on mimicking the fatty acid composition of human milk fat. The structure in terms of high distributions of saturated fatty acids at the *sn*-2 position was not pursued.

When developing new HMFS products, it is important not only to optimize the nutritional and physical properties, but also to ensure that the oxidative stability of the HMFS is acceptable.

The objective of this work was thus to investigate the possibilities of using butterfat as a material to produce a fat similar to human milk fat, using already available enzyme technology together with traditional fractionation technology. A second objective was to determine the oxidative stability of the HMFS product compared to the fat blend used for the production of HMFS. Human milk fat also contains arachidonic (20:4 n-6) and docosahexaenoic (22:6 n-3) acids, which are not present to any significant extent in butterfat. These oils are highly unsaturated and are therefore very susceptible to oxidation. If these oils are included in the butterfat—vegetable oil blend before enzyme modification they will most likely oxidize as also observed by Maduko et al. [5]. An alternative strategy to avoid this could be to add these oils to the final HMFS product. A third objective was therefore to evaluate the effect on lipid oxidation of addition of arachidonic and docosahexaenoic acids to HMFS immediately before the oxidation experiment.

Materials and Methods

Materials

Butterfat was obtained from Arla Foods (Holstebro, Denmark). The fat had the following characteristics: water content 0.07 wt%, and free fatty acid content 0.13%. Rapeseed oil and soybean oil fatty acids were obtained from AAK (Aarhus, Denmark) with similar water content to butterfat. Lipozyme RM IM was obtained from Novozymes (Bagsvaerd, Denmark).

ARASCO (43.2% 20:4 n-6) and DHASCO (43.2% 22:6 n-3) algal oils were obtained from Martek (Boulder, CO). All chemicals and solvents for oxidation measurements were of analytical grade.

HMFS Production from Butterfat

Rapeseed oil and soybean oil fatty acids were mixed in a weight ratio of 7 to 3. Lipozyme RMIM-catalyzed acidolysis was conducted in a pilot-scale packed enzyme bed reactor with the similar setup as previously described [4]. The conditions for the process were as follows: reactor dimension 800 mm in length and 60 mm in diameter, packed bed, temperature 65 °C, water content in substrate 0.07 wt%, substrate weight ratio between butterfat and fatty acid mixture 1:2, and feeding rate 500 mL/h with gradient flow reduction of 10 mL/day. Approximately 150 kg products were produced in total in about 2 weeks. The mixture was stored at −30 °C until further purification.

The separation of the free fatty acids remaining after the reaction was conducted with short path distillation (KD6, UIC, Alzenau-Hoerstein, Germany) using a procedure described previously [11]. The distillation was conducted in two steps. The conditions for the first step were: condenser temperature 40 °C, heat exchanger 80 °C, evaporator temperature 90 °C, feeding rate 40 mL/min, roller speed 500 rpm, and vacuum 0.001 mbar. For the second step, the conditions were the same except that the evaporator temperature was 185 °C. The distilled product (approx. 47 kg) was stored at −30 °C until further processing. The distilled product was fractionated in a pilot crystallizer (De Smet, Zaventem, Belgium) using a wet method. The product was melted and loaded to the crystallizer with 2.5 volumes of dried acetone. The temperature was controlled at room temperature at the beginning and gradually (10 °C/h) reduced to 0 °C and then the temperature was maintained for 3 h. The stirring speed was

100 rpm. The solid fraction was then obtained through filtration with a belt filter. The solid fat was melted in a tank and temperature controlled at 40 °C under 60 mbar vacuum for 1 h. The fat (approx. 30 kg) was stored at −30 °C until further processing.

The fractionated fat was deodorized with a pilot batch deodorizer. The operation was similar to that previously described [12]. The conditions for the deodorization were: evaporation temperature 190 °C, vacuum 2.5 mbar, steam dosage approx. 1.5%, and time 2.5 h. After deodorization, the fat was cooled down to room temperature and stored at −30 °C until use for the oxidation experiment. The fat product after this stage had the following characteristics: diacylglycerol content 4.0%, free fatty acid 0.6%, and peroxide value 0.2 mequiv/kg. This fat was named HMFS.

GC Analysis of Fatty Acid Composition and *sn*-2 Positional Distribution

Fatty acid compositions of the butterfat and products were determined by gas chromatography (GC) (Hewlett-Packard 6890 Gas Chromatograph) after methylation with 2 M KOH in methanol using the same method as previously described [4]. The fatty acids at the *sn*-2 position of the fats were determined by Grignard degradation followed by separation using thin-layer chromatography (TLC) and GC analysis [13].

Experimental Design for Oxidation Stability Study

The oxidative stability of the HMFS was compared with a reference fat with the same fatty acid composition as the HMFS. The reference fat consisted of the same fats and oils used for the production of HMFS plus palm stearin (butterfat/rape seed oil (3:1) 588 g, rape seed oil 118 g, palm stearin 118 g and sunflower oil 176 g). The addition of palm stearin was necessary as the fractionation step during the HMFS production changed the total fatty acid composition of the HMFS compared with the original blend. Furthermore, the oxidative stability of the reference and HMFS oils to which 1% ARASCO plus DHASCO had been added was evaluated. The experimental design is shown in Table 2. Code 1 and 2 are replicates of the same sample and the same is true for code 4 and 5 and these codes were used for all analyses except sensory analysis where only one replicate was evaluated. The oils were stored for 21 days in darkness at room temperature under continuous stirring in an open beaker. Throughout the storage period there was access to oxygen. The oils were sampled at day 0, 5, 10, 14 and 21 and stored at −40 °C until analysis. During the oxidation experiment, the temperature was measured each 5 min. The temperature was between 25 and 31 °C and the average temperature was 28 °C.

Table 2 Experimental design

Code	Description	AA	DHA
1	Reference 1	–	–
2	Reference 2	–	–
3	Reference + PUFA	1%	1%
4	HMFS 1	–	–
5	HMFS 2	–	–
6	HMFS + PUFA	1%	1%

Analysis of Oxidation Parameters

Free Fatty Acids

The content of free fatty acids (FFA) was determined (in duplicate) by titration with NaOH using phenolphthalein as the indicator [14]. The amount of free fatty acids was calculated as % oleic acid.

Peroxide Value

Peroxide values (PV) were determined spectrophotometrically using the IDF method (Shantha and Decker [15]). The reaction is based on the ability of peroxides to oxidize FeII ions to FeIII ions, which react with thiocyanate to form a red colored complex. Analyses were performed in triplicate.

Anisidine Value

The anisidine value (AV) as an unspecific measure of carbonyl compounds was measured spectrophotometrically after reaction of the lipid with *p*-anisidine, which produces a yellow color [16]. Analyses were performed in duplicate.

Volatile Oxidation Products

Identification and determination of volatile secondary oxidation products was performed by dynamic headspace GC–MS. A 4-g sample of lipid was weighed into a pear-shaped glass flask. Headspace volatiles were collected in Tenax™ tubes (Perkin Elmer, Norwalk, CT, USA) [17]. Briefly, sampling of volatiles was performed at 75 °C for 30 min. Trapped volatiles were separated and quantified by 5890 IIA gas chromatograph (Hewlett-Packard, CA, USA) equipped with a DB 1701 column (length 30 m × I.D. 0.25 mm × 1.0 μm film thickness, J&W Scientific, CA, USA) coupled to a HP 5972A mass selective detector. The oven temperature program used was: 35 °C, 3 min isothermal; increased at 3 °C/min to 140 °C; increased at 5 °C/min to 170 °C; increased at 10 °C/min to 240 °C; 240 °C, 8 min isothermal. Calibration curves were made

with external standards to quantify the volatiles. External standards included in the calibration curve were 1-penten-3-ol, 1-penten-3-one, pentanal, *trans*-2-pentenal, *trans*-2-heptenal, octanal, *trans*,*trans*-2,4-heptadienal, 1-octen-3-ol and decenal. Results from the analyses were determined as ng/g oil. Analyses were performed in triplicate.

Induction Period

The oxidative stability of the different oils (15 g) was determined as the induction period at 90 °C in the Oxipres (MikroLab, Århus, Denmark) by recording the drop in the oxygen pressure in the reaction flasks as a result of oxygen consumption. The induction period was determined in duplicate as the crossing point of the tangents to the curve.

Sensory Analysis

The sensory analysis was performed by an expert panel composed of 4 people. Each assessor evaluated one oil at a time. After each oil, the assessors discussed their scores and agreed on an average score for each attribute. Statistical analysis of these data was therefore not possible. The oils were evaluated for odor and taste for the following attributes; rancid, fishy and other. The oils were evaluated on a intensity scale from 0 to 9 for increasing intensity.

Statistical Analysis

The results obtained in the different analyses were analyzed by two-way analysis of variance (ANOVA), and individual samples were compared on a 0.05 level of significance by the Bonferroni multiple comparison. This test allows comparison of selected pairs of columns (variables) for significant differences.

Results and Discussion

Production Strategy Evaluation

Enzyme technology has been developed over the last few decades to enable the production of structured lipids with specific molecular structures. Industrial production of similar products using enzyme technology has been performed for a decade by a number of companies. The production technology and conditional implementations have been widely used in the authors' laboratory and pilot plant. Fractionation technology is a traditional technology, which has been widely studied for butterfat fractionation. Detailed information can be found in handbooks [18]. In the present study, the central aim was to evaluate how to apply those technologies to make a HMFS product of a reasonable high quality from butterfat.

From Table 1, a general diagnosis can be made. Human milk fat contains higher amounts of unsaturated fatty acids than butterfat, but with higher amounts of saturated fatty acids at the *sn*-2 position, particularly palmitic acid. On the other hand, butterfat contains more saturated fatty acids; particularly the levels of saturated fatty acids other than palmitic acid are much lower at the *sn*-2 position. As for the first difference, enzyme technology can be used to incorporate more unsaturated fatty acids into the outer positions of the butterfat. As for the second difference, fractionation technology can be used to fractionate the solid part with the aim of having a higher content of palmitic acid at the *sn*-2 position.

To realize these aims, two process routes were evaluated on a laboratory scale. The first route was to start with fractionation of butterfat followed by enzymatic acidolysis to incorporate unsaturated fatty acids. The second route was the other way round, starting from enzymatic acidolysis followed by fractionation.

The process conditions for enzymatic reactions adopted were basically similar to earlier studies [4]. Soybean oil fatty acids were also used as before. To mimic the composition of Danish human milk fat with higher oleic acid content, rapeseed oil fatty acids was also blended at a level of 70% based on calculations. Fractionations were conducted in acetone for both routes. The fractionation conditions were primarily adopted from a previous study [18] with temperature optimizations considering both the product yield and the content of saturated fatty acid at the *sn*-2 position (detailed temperature optimization omitted). The conditions for butterfat fractionation finally used were 3 volume acetone, temperature 13 °C, and time 2 h. The yield of solid fraction was around 40% for several batches. The solid fraction was thus used for further enzymatic acidolysis with the mixture of rapeseed oil/soybean oil fatty acids using Lipozyme RM IM as catalyst after being placed in a vacuum to remove acetone. For the fractionation of enzymatically processed product after molecular distillation in the second route, the temperature was also tested from 20 to −10 °C. The final temperature decided on was 0 °C. The other selected conditions are described in the Method section. The yield was around 50% in laboratory batches.

The two products from the two routes under individually selected conditions are compared in Table 3. In general, no marked difference can be seen for total fatty acid compositions (Table 3). The second route, however, showed a higher palmitic acid content (56 vs. 47%) in the *sn*-2 position on the laboratory scale. Therefore, this method was used for the production of 30 kg HMFS for applications and oxidation studies. The detailed production methodology is described in the Method section and illustrated in Fig. 1. The detailed composition of the final

Table 3 Comparison of fatty acid composition in the products from two different routes and in the HMFS produced in pilot plant (mol%)

Wt %	Fractionation + acidolysis route		Acidolysis + fractionation route		HMFS from pilot plant	
	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2
C4:0	1.52	–	3.68	–	0.83	–
C6:0	–	–	–	–	0.59	–
C8:0	0.28	1.60	0.40	–	0.37	0.42
C10:0	0.95	3.33	1.01	1.75	1.00	1.54
C12:0	0.15	4.45	1.22	2.52	1.11	2.17
C14:0	4.55	18.15	4.50	14.84	4.20	9.59
C14:1	0.42	–	0.30	0.62	–	–
C15:0	0.68	1.59	0.34	1.52	–	–
C16:0	27.15	47.26	25.66	56.12	25.21	46.49
C16:1	0.58	1.16	0.71	1.43	0.90	1.66
C18:0	5.92	8.90	5.30	6.85	5.91	4.72
C18:1,t	1.73	–	1.53	1.42	0.91	1.10
C18:1 n–12	–	–	–	–	0.78	0.94
C18:1 n–9	35.51	10.75	31.48	9.52	31.01	17.86
C18:1 n–7	–	–	–	–	1.57	0.71
C18:2 n–6	15.92	0.75	18.80	1.05	18.51	5.45
C18:3 n–3	–	–	–	–	2.43	0.75
CLA C9-T11	–	–	–	–	0.22	0.20
Others	6.64	2.07	5.07	2.36	–	–

product is seen in Table 3 (HMFS from pilot plant). Obviously the palmitic acid content at the *sn*-2 position, after all the processing steps in the pilot process as shown in Fig. 1, was less in comparison to the laboratory process (46% in pilot plant vs. 56% in laboratory experiment). Further studies are needed to optimize the pilot process to obtain the same high 16:0 level in the *sn*-2 position as in the laboratory process.

Evaluation of Oxidative Stability of the Oils

Fatty Acid Profiles and Free Fatty Acid (FFA)

The fatty acid compositions of the reference and the HMFS oils were very similar and remained unchanged during 21 days of storage (data not shown). As expected the oils with PUFA added had a higher amount ($\sim$1.1%) of DHA and ARA compared to the reference and HMFS oils without PUFA added.

The free fatty acid (FFA) contents in the oils were between 0.08–0.74% and remained unchanged during 21 days of storage indicating that no lipid hydrolysis took place during storage (data not shown). The FFA % was highest in the HMFS oils (0.67–0.74%) compared to the reference oils (0.08–0.11%). This indicated that free fatty acids had not been completely removed during the purification process.

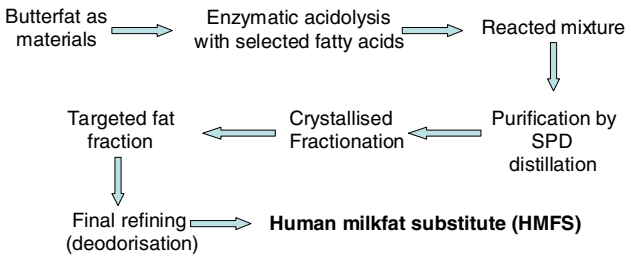

Fig. 1 Process scheme for the pilot production of HMFS. SPD *short path distillation*

Tocopherol Content

Tocopherols were only detected in the reference oils with or without PUFA added. Only α- and γ-tocoperols were present in these oils (Table 4). The absence of tocopherols in the HMFS oils indicated that tocopherols were lost during the purification (pilot batch deodorization plus short path distillation) process of the enzyme modified oil. Previous studies had shown that substantial amounts of tocopherols are lost during purification of enzyme modified lipids if short path distillation is used as the purification method [4, 11]. However, one of these studies showed that when batch deodorization was used the loss of tocopherol was much lower [11]. The complete loss of tocopherol during production and purification of the HMFS oil is

Table 4 Tocopherol content [mg/kg] in the reference oils

Tocopherols (α/γ) [mg/kg]	Day 0	Day 21
Reference	$176.6^{(a,x)} \pm 2.8/81.8^{(c,z)} \pm 2.2$	$166.4^{(b,x)} \pm 3.4/78.1^{(c,z)} \pm 1.5$
Reference + PUFA	$168.4^{(a,y)} \pm 0.5/81.7^{(c,z)} \pm 1.8$	$164.2^{(a,x)} \pm 4.5/83.5^{(c,w)} \pm 2.1$
HMFS	nd	nd
HMFS + PUFA	nd	nd

nd not detected

The letters in the parentheses: The first letter (a,b,c) denotes whether there was a significant difference in the concentration during storage in a specific sample. The second letter (x, y, z, w) denotes whether concentrations in the different samples at a specific storage day were significantly different. The same letters indicate no significant differences

therefore somewhat surprising and may suggest that tocopherols had been partly lost during the enzyme modification or fractionation steps and was further removed during the two purification steps. Haman and Shahidi [19] suggested that the formation of tocopheryl esters during the interesterification reaction might also be responsible for the loss of endogenous tocopherols present in the oils [19].

Addition of PUFA to the reference or HMFS oils did not increase the tocopherol levels, indicating that the tocopherol levels in the PUFA oils were too low to have any effect on the total level of tocopherol when the PUFA oils were added at a level of 1% each.

The tocopherol concentrations did not change significantly in the reference oil with PUFA during 21 days of storage (Table 4). However, in the reference oil without PUFA the α-tocopherol content was significantly reduced from day 0 to day 21 indicating that α-tocopherol had been consumed due to oxidation. Moreover, there was a significant difference between the reference and reference with PUFA added at day 0 with a lower level of α-tocopherol in the reference + PUFA sample. γ-tocopherol levels did not decrease during storage. Taken together the results indicated a preferential consumption of α-tocopherol during storage. This is in accordance with previous findings [20].

Peroxide Value (PV)

Lipid hydroperoxides are primary oxidation products formed during the oxidation process and their concentration (PV) was therefore measured during storage. PVs increased significantly during storage for the HMFS oils (Fig. 2). In contrast, only a slight increase in the PV was observed for the reference oils. Nevertheless, there was a significant increase in the PV from day 0 to day 21 in the reference oil without PUFA added. This was not the case for the reference oil with PUFA.

From Fig. 2 it is obvious, that the PV was different in the oils even at day 0, with HMFS oils having a lower PV compared to the reference oils. This could suggest that the purification process of the HMFS had removed peroxides. However, from day 5 and onwards, the PV was significantly

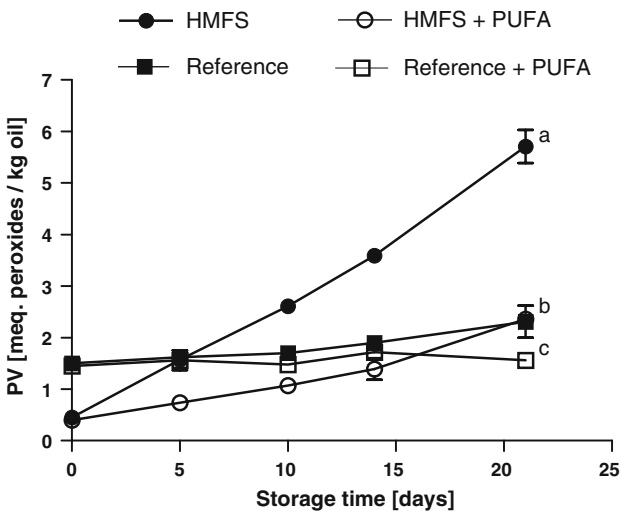

Fig. 2 Development of lipid hydroperoxides in the different oils during storage at room temperature (21 days). The *bars* indicate the standard deviation and the letters at day 21 indicate if there is a significantly different concentration in the different samples at that day (same letter no significance)

higher in the HMFS without PUFA added compared with the HMFS with PUFA added. The different reference oils were only significantly different at storage day 21, where reference oil with PUFA added had the lowest concentration of peroxides. Taken together these data indicated that HMFS oils were less oxidatively stable than the reference oils, but that the addition of PUFA reduced the PV.

Anisidine Value (AV)

Table 5 shows the AV at day 0 and day 21. The AV increased slightly during storage in all samples, but the increase was only significant for the HMFS oils Moreover, the HMFS oils had a significantly higher AV than the reference oil both at day 0 and at day 21. Addition of PUFA to the two kinds of oils did not have a significant influence on the measured AV (Table 5).

Comparison of the PV and AV results at day 21 for the HMFS oils shows that the HMFS without PUFA had the

Table 5 The anisidine value in the different oils measured at day 0 and day 21

AV	Day 0	Day 21
Reference	$1.71^{(a,x)} \pm 0.20$	$1.81^{(a,x)} \pm 0.12$
Reference + PUFA	$2.49^{(a,x)} \pm 0.18$	$2.91^{(a,y)} \pm 0.59$
HMFS	$12.20^{(a,y)} \pm 0.97$	$13.46^{(b,z)} \pm 0.41$
HMFS + PUFA	$12.48^{(a,y)} \pm 0.20$	$13.16^{(a,z)} \pm 0.05$

nd not detected. The letters in the parentheses: The first letter (a,b) denotes whether there was a significant difference in the concentration during storage in a specific sample. The second letter (x, y, z) denotes whether concentrations in the different samples at a specific storage day were significantly different. The same letters indicate no significant differences

highest PV and AV and the reference oils had the lowest PV and AV, whereas the HMFS oil with PUFA added had the lowest PV and an AV comparable to the HMFS oil without PUFA. These findings indicate that the low PV in the HMFS oil with PUFA was a result of decomposition of the peroxides to volatile oxidation production, which are reflected in the high AV in this oil.

Volatile Oxidation Products

The following volatile oxidation products were quantified: 1-penten-3-one, 1-penten-3-ol, 2,4-heptadienal, 1-octen-3-ol, 2-heptenal, 2-pentenal, pentanal, octanal and decanal. The concentrations of these volatiles increased significantly during storage in all samples as exemplified by 1-penten-3-one and 2,4-heptadienal in Fig. 3a and b, respectively. Except for octanal and decanal, the HMFS oils had higher concentrations of the quantified volatiles than the reference oils irrespective of the addition of PUFA. However, the effect of PUFA addition was different for the different volatiles. For 1-penten-3-one and 1-penten-3-ol, the oxidative stability increased as follows: HMFS with PUFA < HMFS < reference with PUFA < reference (Fig. 3a). In contrast, the concentration of 2,4-heptadienal, 1-octen-3-ol, 2-heptenal, 2-pentenal and pentanal was higher in the oils without PUFA added than with PUFA added, but higher concentrations for the HMFS oils than for the reference oils were however also observed for these volatiles (Fig. 3b). In a previous study with HMFS based on lard, 2,4-heptadienal, 1-octen-3-ol, 2-heptenal, 2-pentenal and pentanal were also found to increase significantly during storage [4].

As mentioned above the picture was different for the development of octanal and decanal. Thus, the concentration was much higher in the reference oils than in the HMFS oils, but no clear effect of PUFA addition to the oils was observed. Octanal and decanal are decomposition products of n-6 PUFA, whereas several of the compounds, which were found in highest concentrations in the HMFS

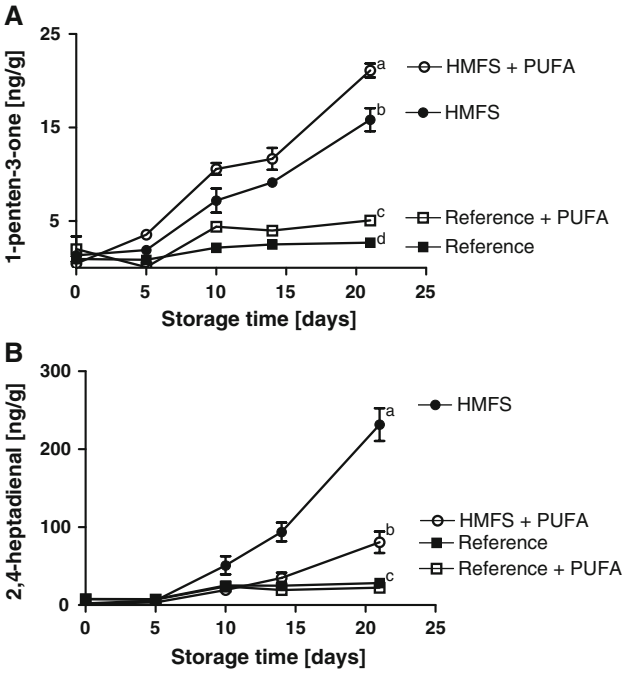

Fig. 3 Development of selected volatile oxidation products in the different oils during storage (21 days). **a** 1-penten-3-one, **b** 2,4-heptadienal. *The bars* indicate the standard deviation and the letters at day 21 indicate if there is a significantly different concentration in the different samples at that day (same letter no significance)

oils (1-penten-3-one, 1-penten-3-ol, 1-octen-3-ol, heptadienal and pentenal) are decomposition products from n-3 PUFA. For the HMFS oils without PUFA the only n-3 fatty acid was 18:3 n-3. Hence, the increased formation of the above mentioned volatiles in the HMFS oil was most likely due to lipid oxidation of 18:3 n-3 and this indicates that this fatty acid was more susceptible to oxidation in the HMFS oil than in the reference oil. As already mentioned the HMFS oil without PUFA had lower PV and a higher AV at day 0 than the reference oil without PUFA. Therefore, it may also be expected that the HMFS oil would have higher levels of 1-penten-3-one, 1-penten-3-ol, 1-octen-3-ol, heptadienal and pentenal at day 0. However, this was not the case. No obvious explanation for these apparently contradictory findings can be given. Hence, the increased formation of 1-penten-3-one, 1-penten-3-ol, 1-octen-3-ol, heptadienal and pentenal in the HMFS oil during storage is therefore most likely both due to the fact that the HMFS oil did not contain any antioxidative tocopherols and that some oxidation had ocurred during the production of the HMFS oils.

The higher levels of octanal and decanal in the reference oils compared with the HMFS oils are not easy to explain, but decanal also developed differently from other volatiles in a previous study on HMFS based on lard [4]. In that study, decanal concentrations increased in the initial part of

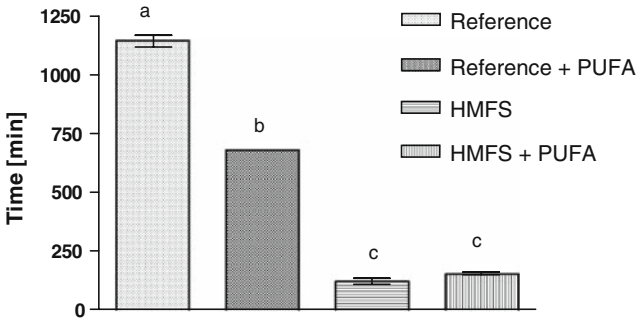

Fig. 4 Induction times evaluated by the accelerated stability test. The *bars* indicate the standard deviation and the letters on the top of the bars indicate if there is a significantly different concentration in the different samples (same letter, no significance)

the storage period followed by a decrease. From the available data it is not possible to conclude whether the unexpected behavior of octanal and decanal in these studies is due to an analytical problem or a phenomenon related to the particular structure of HMFS oils.

Accelerated Stability Test (Oxipres)

Results from the accelerated stability test are shown in Fig. 4. The oxidative stability was significantly higher for the reference oils compared to the two HMFS oils, since the time for drop in oxygen pressure (induction time) was longer than for the HMFS oils. The accelerated stability test thus confirmed the observations from PV, AV and volatiles analyses. The finding that HMFS oxidized faster than an unmodified oil blend was also in accordance with the study by Maduko et al. [5]. In the present study, the accelerated test showed that addition of PUFA to the reference oil significantly reduced its induction time. Surprisingly, PUFA addition slightly increased the induction time in the HMFS oils. This finding is not in agreement with the data for 1-penten-3-one and 1-penten-3-ol, which suggested that PUFA addition reduced the oxidative stability of the HMFS oil. In contrast, the data for PV, AV, 2,4-heptadienal, 1-octen-3-ol, 2-heptenal, 2-pentenal and pentanal also indicated that PUFA addition increased the oxidative stability of the HMFS. These contradictory data may suggest that the PUFA oils contained antioxidative compounds that had different effects on the different volatiles measured. Other studies have also observed different effects of the same antioxidants on different oxidation parameters [21].

Sensory Evaluation

Table 6 shows the average scores given by the 4 panellists. Furthermore, Table 7 summarizes the attributes, which the assessors used to describe the odor and taste of the different oils under the descriptor "other".

Table 6 The average scores for odor and flavor for the different oils (Scale 0–9)

	Odor			Flavor		
	Rancid	Fishy	Other	Rancid	Fishy	Other
Day 0						
Reference	0	0	3½	1	0	4
Reference + PUFA	0	1	4	3	0	3
HMFS	1	0	1½	1	0	3
HMFS + PUFA	0	0	1	1	0	3
Day 21						
Reference	0	0	3	0	0	3
Reference + PUFA	½	0	3	½	0	2
HMFS	1	0	1½	½	0	4
HMFS + PUFA	1	0	2	1½	0	3

Table 7 Attributes describing for the odor and flavor of the different oils

	Odor	Flavor
Reference	Soap, perfume	Soap, citrus, cheese, perfume
Reference + PUFA	Cheese, butter, perfume, citrus	Perfume, citrus, butter
HMFS	Hay, burnt	Bitter, citrus, burnt
HMFS + PUFA	Hay, burnt, citrus, green, butter, cheese, bitter	Citrus, bitter, burnt, species

Table 6 indicates that the intensities of rancid odor and taste generally were low in all oils. For the rancid taste the intensity seemed to decrease during storage of the reference oils, whereas it remained more or less stable for the HMFS oils. There was no obvious explanation to the decrease in the rancid taste. Only small differences in the rancid odor and taste between the reference and the HMFS oils could be observed with the HMFS oils tending to be more rancid than the reference oils. No effect of PUFA addition on rancid off-flavors were observed. Interestingly, the panel was neither able to detect any fishy odor or flavor in the oils with PUFA oils added after storage for 21 days, despite the high content of DHA in the DHASCO oil. This suggested that the DHASCO oils added to the oils did not give rise to formation of "fishy" volatiles to an extent where they could be detected by the sensory panel. Previous studies have suggested that 2,4,7-decatrienal, 2,6-nonadienal, 3,6-nonadienal and 4-heptenal may contribute significantly to fishy off-flavors in fish oils [22, 23]. These volatiles could not be detected in the oils in the present experiment and this may explain why no fishy off-flavor could be detected.

The other off-flavors in the reference oils were primarily described as soap, perfume and cheese, whereas they were described as hay, bitter, citrus and burnt for the HMFS oils (Table 7). These odors were more dominating than the rancid odor and flavor. Hence, although the chemical data indicated that the HMFS oils oxidized during storage, the sensory data indicated that oxidation was a less pronounced problem than the formation of other off-flavors. It is, however, also possible that the off-flavors evaluated under "other" masked the rancid off-flavors formed due to oxidation. The different off-flavors reported in Table 7 may be due to free fatty acids or volatile oxidation products in the two oils. It is likely that the relatively high level of free fatty acids present in the HMFS oils contributed significantly to the off-flavors identified in this product. This problem may be solved by optimizing the deodorization process as described by Rønne et al. [12].

Conclusions

Using a combination of enzyme technology, fractionation and batch deodorization and with butterfat in combination with soybean oil and rapeseed oil as raw materials it was possible to produce HMFS with a molecular structure and fatty acid composition very similar to those of human milk fat. The production on a laboratory scale resulted in the most optimal product with respect to fatty acid composition and molecular structure and further optimization of the pilot scale conditions is necessary to obtain a HMFS product with the optimal composition. The processing steps are certainly not simple including enzymatic acidolysis to change the fatty acid composition, short path distillation to remove free fatty acids, solvent fractionation to obtain the right fraction, and solvent removal by distillation followed by deodorization to obtain edible products. With limited information from industry, similar process steps have been used for the industrial production of commercial products with similar profiles [2]. A similar economical situation can be therefore be expected for the current process. Certainly the first approach proposed will lead to a reduced workload in the enzymatic reaction step as well as the short path distillation step as the fractionation step had reduced the material size for reactions and distillations, while the second approach had the better performance and was used for the production. Obviously a balance has to be considered in terms of product quality and process cost. The oxidative stability of the HMFS oil was lower than that of the reference oil with the same fatty acid composition. The lower oxidative stability of the HMFS oil was due to loss of tocopherol during the production and purification process and to oxidation during production. However, oxidation did not lead to severe increase in rancidity scores during storage as evaluated by a sensory panel. Rather, the panel gave high intensity scores for other off-flavors such as burnt and bitter, which were most likely due to free fatty acids present in the oil. Further optimization of the deodorization process is therefore necessary to remove these off-flavors. Moreover, it is possible that oxidation may be prevented by addition of antioxidants such as tocopherols as also suggested by Maduko et al. [5].

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