Composition and Oxidative Stability of a Structured Lipid from Amaranth Oil in a Milk-Based Infant Formula

ASHANTY M. PINA-RODRIGUEZ and CASIMIR C. AKOH

ABSTRACT: Amaranth oil can be enzymatically modified to match breast milk fat analog requirements. We have developed a structured lipid (SL) from amaranth oil that, in combination with milk fat, delivers recommended amounts of docosahexaenoic acid (DHA) with palmitic acid specifically esterified at the sn-2 position of the triacylglycerol (TAG) backbone. The aim of this study was to study the final fatty acid (FA) contribution and oxidation stability of an infant formula prepared using the structured lipid DCAO (DHA-containing customized amaranth oil). DCAO was included as complementary fat in a “prototype” infant formula, and prepared in parallel with a “control” infant formula under the same processing conditions. The same ingredients but different complementary fat sources were used. A blend of the most commonly used vegetable oils (palm olein, soybean, coconut, and high-oleic sunflower oils) for infant formula was used instead of DCAO in the “control” formula. Additionally, “prototype” and “control” infant formulas were compared to a “commercial” product in terms of FA composition. The oxidative stability index (OSI) of the extracted fats from “prototype,” “control,” and “commercial” infant formulas were evaluated and compared to the OSI of the substrate fat replacers used. DCAO was the least stable compared to other fat analogs. The use of commercial antioxidants in DCAO containing products should prevent oxidation and therefore increase their stability.

Keywords: amaranth oil, application of structured lipid, infant formula, OSI

Introduction

Several studies have been conducted to develop a suitable breast milk fat analog to meet the recommended nutritional characteristics, as well as being affordable for industrial production. Infant formulas intended for healthy term infants should mimic the composition of breast milk from healthy mother’s (Innis 1991). The approximate composition of breast milk has been reported by several researchers in the past to contain mainly 26.7% to 35.3% oleic, 18.3% to 25.9% palmitic, and 10.2% to 16.49% linoleic acids (Bitman and others 1983; Finley and others 1985; Fidler and others 2000; Weber and Mukherjee 2005). Similar to other animal fats, the sn-2 position of breast milk triacylglycerols (TAGs) is mainly occupied by saturated fatty acids. Palmitic acid is over 60% by weight esterified at the sn-2 position in breast milk fat. The regiospecificity of palmitic acid in breast milk reduces the formation of “calcium soaps” produced by the interaction between long chain saturated fatty acid and calcium (Tomarelli and others 1968; Dutson and others 1992; Martin and others 1993; López-López and others 2001). Research has shown that in breast milk fat, the large amount of palmitic acid esterified at the sn-2 position improves the infant’s absorption of fat and calcium, while preventing the formation of calcium soaps (Kennedy and others 1999; Jandacek 2008). The fat portion of infant formulas currently in the market is usually achieved with vegetable oils such as coconut, soybean, sunflower, and corn oils (Innis 1991). Other commercial products have been developed from palm and high-oleic sunflower oils to yield a structured lipid (SL) with a high portion of OPO (sn-1,3-dioleoyl-2-palmitoylglycerol) fraction (Chen and others 2004; Weber and Mukherjee 2005). Blends of these oils are readily used to balance the fatty acid (FA) content of the final product; hence composition of the formula is dependent on the type and portions of oils used. Some of the most recently researched breast milk fat analogs were SLs from the inter- or transesterification of substrates such as tripalmitin, hazelnut oil FAs, n-3 polyunsaturated FAs concentrates (such as marine oil blends), rapeseed oil FAs, soybean FAs, lard, coconut oil, safflower oil, palm oil, olive oil, and butter oil to resemble breast milk fat composition (Christensen and Holmer 1993; Mukherjee and Kiewitt 1998; Schmid and others 1999; Yang and others 2003; Sahin and others 2005, 2006; Karabulut and others 2007; Maduko and others 2007). Many of these products are intended to be used in combination with other vegetable oils or milk fat.

According to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), infant formulas should provide at least 4.4 g of total fat for each 100 kcal consumed, and at least 1.8 g of total protein and 9 g of total carbohydrates in the same portion (Koletzko and others 2005). Other organizations, such as the Life Science Research Office (LSRO) and the Food and Drugs Administration (FDA) have reported similar nutrient recommendations for infant formula in the United States (LSRO 1998).

We studied the interesterification of amaranth oil with ethyl palmitate to increase the content of palmitic acid at the sn-2 position (Pina-Rodriguez and Akoh 2009a). In our previous study, we found that the content of palmitic acid and other major FAs in amaranth oil might be suitable for infant formula (Pina-Rodriguez and Akoh 2009b). Furthermore, a SL called DCAO (DHA-containing customized amaranth oil) was prepared with increased content of palmitic acid at the sn-2 position, and with docosahexaenoic acid (DHA) esterified mainly at the sn-1,3 positions. Compared to other
Materials and Methods

Materials

Amaranth oil was purchased from Nu World Amaranth Inc. (Naperville, Ill., U.S.A.). DHASCO (DHA-containing single cell oil) was a gift from Martek Bioscience Corp. (Columbia, Md., U.S.A.). High-oleic sunflower (Frymax Sun Supreme) and palm olein oils were generously donated by Stratas Foods (Memphis, Tenn., U.S.A.) and Loders Croklaan (Glen Ellyn, Ill., U.S.A.), respectively. Evaporated whole milk (Glendale, Calif., U.S.A.), milk-based concentrated liquid Infant Formula (Glendale), coconut oil (Spectrum) and soybean oil (Publix brand) were purchased from local convenience stores in Athens, Ga., U.S.A. Alpha-lactalbumin enriched whey protein concentrate (WPC) was donated by Hilmar Ingredients specially designed for infant formula application to compensate for the protein difference. DCAO (SL) was used as a complementary fat in the prototype formula. In “control” formula, the complementary fat was obtained from a blend of vegetable oils commonly used for infant formula preparation (Nelson and Innis 1999). The oil blend was made of 62% palm olein, 25% soybean, 8% coconut, and 5% high-oleic sunflower oils. Coconut and palm olein oils were melted at 30 °C before blending with soybean and high-oleic sunflower oils. Ingredients were mixed at 50 to 60 °C using a high-speed benchtop homogenizer. Standardization was calculated from ingredients composition. Samples were passed through a Gaulin homogenizer (55.17 MPa maximum capacity) in 2 phases: I, 15 to 20 MPa; II, 5 MPa. Vial size samples were taken for microscopy analysis. After homogenization, formulas were collected in opaque plastic screw-cap containers (1 L), closed tightly, and quickly cooled. “Control” and “prototype” formulas were stored at 4 °C for 24 h to stabilize before any further analysis. The most recent sample obtained from the “commercial” formula was opened and bottled in a plastic container similar to the formulas made in the laboratory at 4 °C for 24 h before analysis. The containers that were...

Table 1 – Infant formula composition (1000 g liquid-ready to use infant formula).*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Composition (g)</th>
<th>Fat (g)</th>
<th>Protein (g)</th>
<th>Carbohydrates (g)</th>
<th>Energy (Kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>550</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evaporated whole milk</td>
<td>450</td>
<td>29.7</td>
<td>29.7</td>
<td>45</td>
<td>566.1</td>
</tr>
<tr>
<td>WPC*</td>
<td>0.4</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
<td>1.7</td>
</tr>
<tr>
<td>Complementary fat</td>
<td>6.3</td>
<td>6.3</td>
<td></td>
<td></td>
<td>56.7</td>
</tr>
<tr>
<td>Infant formula</td>
<td>36</td>
<td>30</td>
<td>45</td>
<td></td>
<td>624.5*</td>
</tr>
</tbody>
</table>

*Composition of “control” and/or “prototype” infant formula.
*Evaporated whole milk: 6.6% fat, 6.6% protein, 10% carbohydrates.
*WPC: 12% fat, 78% protein, 1.5% carbohydrates.
*Approximate energy contribution: 62 kcal/100 mL.
not used immediately after stabilization overnight were stored for longer periods in the dark at −80 °C for future analysis.

Microstructural confirmation
Homogenization and samples’ likeness were assessed by measuring fat globule size and distribution in infant formulas (“control,” “prototype,” and/or “commercial”). Microstructural observation of fat globules was conducted using a light microscope (Leica Microsystem Inc., Allendale, N.J., U.S.A.). Digital images were obtained using an attached AxioCam digital camera (Zess Inc., Göttingen, Germany). Stabilized samples (kept at 4 °C for 24 h) were spotted on microslides, covered with coverslips, and observed under a microscope using a 100× water immersion lens (Leica Microsystems Inc.).

Fat extraction
The original Bligh and Dyer method was used to extract fat from “control,” “prototype,” and “commercial” formulas (Bligh and Dyer 1959; Iverson and others 2001). Briefly, 100 g of infant formula (“control,” “prototype,” and “commercial”) were homogenized with 100 mL chloroform and 200 mL methanol. Then the solution was rehomogenized with 100 mL chloroform, and 100 mL weak salt solution (0.88% NaCl) was added. After homogenization, the solution was filtered through a porcelain Buchner funnel sealed with filter paper (nr 1) (Whatman, Florham Park, N.J., U.S.A.), and under vacuum. The precipitate remaining on the filter paper was rehomogenized in 100 mL chloroform and again filtered under vacuum with the previous filtered fraction. The final biphasic system was allowed to separate into 2 layers, and the lower phase (chloroform layer) collected. Excess solvent was removed using a Büchi rotovap (Flawil, Switzerland) at 40 °C. Oil was recovered in dark vials, labeled and stored at −18 °C under nitrogen for future analysis.

Determination of fatty acid profiles
DCAO, vegetable oil blend, and the extracted fat of infant formulas were converted to FAME following the AOAC Official Method 996.01, Section E (1998) with minor modifications. One hundred milligrams of oil were weighed into a Teflon-lined test tube, 1 mL C17:0 in hexane (20 mg/mL) was added as an internal standard and dried with nitrogen to remove solvent. Subsequently, 2 mL 0.5N NaOH in methanol were added followed by incubation for 5 min at 100 °C to saponify the lipid. After incubation, 2 mL of 14% boron trifluoride (BF3) in methanol were added. The sample was vortexed for 1 min and incubated again for 5 min at 100 °C to allow methyla-
mation. To stop the reaction and extract the FAMES, 2 mL hexane and 2 mL NaCl saturated solution were added to the sample, vortexed for exactly 2 min at room temperature and centrifuged for 5 min at 1000 rpm to separate the organic and aqueous phases. The upper organic layer was filtered twice through an anhydrous sodium sul-
fate column, separated and filtered twice through an anhydrous sodium sulfate column. The extracted solution was flushed with nitrogen to evaporate solvent until one third of the volume was left. The concentrated extract was spotted on silica gel G TLC plates and de-
veloped with hexane, diethyl ether, and formic acid (60 : 40 : 1.6,
v/v/v), 2-Oleylglycerol was spotted in parallel as an identification standard for 2-monocacylglycerol (2-MAG). Plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The band corresponding to 2-MAG was scraped off and converted to FAME as previously described. Fifty microliters C17:0 in hexane (20 mg/mL) were used as an internal standard. FAs esteri-
fied at the sn-2 position were quantified by GC and the amounts at sn-1,3 were calculated (Nawar 1996).

GC analysis
FAMES were analyzed using an Agilent Technology (Santa Clara, Calif., U.S.A.) 6890N gas chromatograph (GC) equipped with a flame ionization detector (Fidler and others 2000). Separation was achieved with a SP-2560 column, 100 m × 0.25 mm i.d., 0.20 μm film. Injection (1 μL) was performed at a split ratio of 5 : 1. The car-
ier gas was helium at constant pressure mode and 1.1 mL/min flow rate. The injection and detection temperatures were 250 and 260 °C, respectively. The sample was held at 150 °C for 3 min, then ramped up to 215 °C at 10 °C/min, and held isothermally for 40 min. FAME relative content was calculated by integration using an online computer. Averages of duplicate analyses were reported.

Oxidative stability index (OSI)
OSI of the extracted fat from the “prototype,” “control,” and “commercial” infant formulas, DCAO, vegetable oil blend, and extracted milk fat were determined with an Oil Stability Instrument (Ommion, Rockland, Mass., U.S.A.) at 110 °C according to the AOCS Official Method Cd 12b-92 (1997).

Statistical analysis
All extractions and analyses were performed in duplicate. Average, standard deviations, and least significant difference (LSD) were calculated using Minitab 15 statistical software (Minitab Inc., State College, Pa., U.S.A.) and reported.

Results and Discussion
The developed formulas contribute about 62 kcal/100 mL re-
sulting in 3.6% fat, 3% protein, and 4.5% carbohydrates, using whole milk and whey-protein concentrate (WPC) to make up for the protein difference. The process for preparing the “prototype” and “control” formulas in our laboratory was previously described in the methods section, and the final macronutrients and energy contributions are presented in Table 1.

Samples were visualized under microscopy to assess the homogenization equivalence between “control,” “prototype,” and “commercial” infant formulas (Figure 1). The purpose of the homogenization step is to break down the fat globule into small droplets, which will make the emulsion more stable (Zink 2003). Most of the fat globule reduction takes place in the 1st stage of homogenization (150 to 200 bar). However, the smaller fat globules have a tendency to cluster. Therefore, a 2nd homogenization stage (50 bar) is recommended to separate those clusters into individual droplets (Goff 1995). After homogenization, the average size of a milk fat globule was 0.4 μm (2 μm maximum) with a density of 12 fat globules per μm3 (Goff 1995). Several studies have shown a significant difference in structure between breast milk fat globules (MFG) and infant formula fat globules (FG). Specific structure of
Infant formula using structured lipid...

MFG provides unique nutritional properties that FG does not have (Armand and others 1996). In general, the homogenization process of infant formula significantly reduces the droplet size of fat particles to increase the stability of the emulsion. Several researchers agree that the approximate diameter of MFG is about 4 μm, while the average diameter of FG is usually <1 μm (Rüegg and Blanc 1981; Simonin and others 1984; Armand and others 1996; Michalski and others 2005, 2008). It has been shown that the differences in fat particle size between MFG and FG result in a better fat absorption and metabolism (Armand and others 1996). There are also significant differences in the interface of TAGs from MFG than FG in subsequent hydrolysis resulting in a better fat absorption and metabolism (Armand and others 1996).

According to our results, as shown in Figure 1, evaporated milk (a) fat globules vary in diameter; however, the largest globule observed was about 0.01 μm dia. The average fat globule size was similar in all 3 formulas. “Commercial” formula (b) showed a higher density of fat globules compared to any of the infant formulas prepared in this study. After analyzing these results, we concluded that the process was effective in reducing fat globule size; we also concluded that “control” (c) and “prototype” (d) formulas were equivalent in terms of their corresponding complementary fat source. Further studies are suggested to determine the nutritional effect of smaller FG.

Infant formulas were analyzed 24 h after preparation. FA profile and positional analysis were determined. The results are shown in Table 2. Several important compositional differences can be observed among the samples. Both infant formulas prepared in our laboratory (“control” and “prototype”) contained superior amounts of palmitic acid compared to “commercial” formula. Palmitic acid regiospecificity at the sn-2 position was more evident in “prototype” infant formula (33%) than in its “commercial” analog (7.3%). The sn-2 position of “commercial” infant formula TAGs was mainly occupied by oleic acid (41.6%). The higher content of palmitic acid in “prototype” infant formula was at the expense of oleic and linoleic acids as expected from previous observations (Pina-Rodriguez and Akoh 2009b). Both formulas prepared in our laboratory resulted in significantly lower content of lauric acid (C12:0) than the “commercial” formula. Since only the nutritional effects of palmitic acid in infant nutrition had been extensively studied, it is generally considered that other saturated FA could lead to hypercholesterolemic effects and care must be taken when increasing saturated fat content at the sn-2 position (Pai and Yeh 1997). Therefore, we believe the reduced content of lauric acid in the “prototype” formula could be beneficial, and such observation would require further research. Even though DCAO contained a higher level of linoleic acid (18:2n-6), when combined with milk fat it reaches a beneficial polyunsaturated FA balance (oleic > linoleic acids) similar to the “control” and “commercial” infant formulas. Although “commercial” infant formula is fortified with arachidonic acid (ARA) and DHA, we did not detect DHA in the fat extracted from the “commercial” infant formula, but ARA and eicosapentaenoic acid (EPA) in a 2 : 1 ratio. Our final “prototype” formula contained 0.2% ARA (natural from milk fat) and 0.2% DHA (incorporated by enzymatic interesterification in amaranth oil); it also contained a larger amount of palmitic acid specifically esterified at the sn-2 position. The FA composition of the “prototype” formula represents an improved contribution of palmitic acid at the sn-2 position than commercial formulas, which suggests better fat absorption and a reduced production of calcium soaps. At the same time, the ARA and DHA balanced composition will contribute to proper infant nutrition.

The OSI of the extracted fat from the infant formulas and their fat substrates were evaluated (Figure 2). The “commercial” infant formula showed higher OSI, while “prototype” infant formula
Table 2—Fatty acid composition of complementary fat sources and extracted fat from infant formulas.

<table>
<thead>
<tr>
<th>Fatty acid (wt %)</th>
<th>Total (wt %)</th>
<th>sn-2 Position (wt %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oil blend*</td>
<td>DCAO*</td>
</tr>
<tr>
<td>8 : 0</td>
<td>0.7 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>10 : 0</td>
<td>0.5 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>11 : 0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>12 : 0</td>
<td>4.1 ± 0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>13 : 0</td>
<td>0.0</td>
<td>--</td>
</tr>
<tr>
<td>14 : 0</td>
<td>2.3 ± 0</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td>14 : 1n-5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15 : 0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>16 : 0</td>
<td>28.5 ± 0</td>
<td>33.5 ± 0</td>
</tr>
<tr>
<td>16 : 1</td>
<td>0.1 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>18 : 0</td>
<td>4.5 ± 0</td>
<td>2.8 ± 0</td>
</tr>
<tr>
<td>18 : 1n-9</td>
<td>35.2 ± 0</td>
<td>23 ± 0</td>
</tr>
<tr>
<td>18 : 2n-6</td>
<td>20.9 ± 0</td>
<td>36.9 ± 0</td>
</tr>
<tr>
<td>18 : 3n-6</td>
<td>0.1 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>18 : 3n-3</td>
<td>2.2 ± 0</td>
<td>0.7 ± 0</td>
</tr>
<tr>
<td>20 : 0</td>
<td>0.4 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>20 : 1</td>
<td>0.3 ± 0</td>
<td>0.3 ± 0</td>
</tr>
<tr>
<td>20 : 2</td>
<td>--</td>
<td>0.3 ± 0</td>
</tr>
<tr>
<td>21 : 0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>22 : 0</td>
<td>0.2 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>20 : 3n-6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20 : 4n-6</td>
<td>0.1 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>22 : 6n-3</td>
<td>--</td>
<td>1.9 ± 0</td>
</tr>
</tbody>
</table>

*aOil blend composition: 62% palm olein oil, 25% soybean oil, 8% coconut oil, and 5% high-oleic sunflower oil.
*bDCAO composition (Pina-Rodriguez and Akoh 2009b).
*cPrototype* formula made with DCAO as described previously.
*dCommercial* formula was a concentrated liquid presentation of a commercial brand. Values are average of duplicate analysis ± SD; means with the same letter in the same row are not significantly different (P < 0.05).
Infant formula using structured lipid . . .

resulted in an OSI of 3.18 h, even below the OSI of the fat substrates used for the "commercial" and "control" infant formulas. DCAO only showed an OSI of 1 h. The fat portion of "prototype" infant formula was composed of milk fat and DCAO as complementary fat. According to our current results and previous research, the interesterification process and the purification by short-path distillation of DCAO significantly reduced the content of natural antioxidants (tocopherols) (Pina-Rodriguez and Akoh 2009b). The OSI results that showed that "prototype" formula was more susceptible to oxidation can be explained by the restructuring of DCAO (inclusion of DHA), the removal of natural antioxidants during synthesis, and the lack of extra tocopherol addition before being used in the formula. Even though milk fat's OSI was acceptable, the significantly low OSI of DCAO adversely affected the OSI in "prototype" infant formula compared to the other 2 formulations.

A more efficient interface composition could help in preventing lipid oxidation. Infant formulas contain up to twice more protein than breast milk, and up to 7-fold more vitamin E to prevent the oxidation of polyunsaturated FAs. Formula processing should also be considered as possible variables to control to prevent FAs oxidation. As mentioned previously, the homogenization of infant formula produces smaller fat particles to stabilize the emulsion. However, it also produces a more extensive surface area than in breast milk. Some researchers have shown that the larger fat interface can promote lipolysis and lipid oxidation (Michalski and others 2008). A significant increase in oxidation products was observed from dissolved powder compared to liquid infant formulas. The authors suggested that the readiness for use of the lipid present contributions to a lower lipolysis and oxidation, and when powders get in contact with water the lipid fraction becomes more accessible to oxygen and catalysis prior to being ready for consumption (Michalski and others 2008).

Conclusions

We developed a SL (DCAO) from amaranth oil, which in the final formulation of milk-based infant formula yielded the recommended DHA content for proper infant development, and higher palmitic acid esterified at the sn-2 position for better fat absorption. The characteristics (physical and chemical) determined in previous studies (Pina-Rodriguez and Akoh 2009a, 2009b) support the feasibility of using DCAO as a partial fat source or a complement for milk-based infant formula. Finally, DCAO was incorporated into a standardized formula, and compared to "control" and "commercial" infant formulas. An appropriate FAs balance for infant nutrition was confirmed from the FA composition of our "prototype" infant formula containing DCAO from amaranth oil. The significantly low OSI of DCAO adversely affected the oxidative stability of the lipid fraction in the "prototype" formulation. However, the use of proper antioxidants (tocopherols) compensation would easily enhance the stability of DCAO and therefore its functionality in the final infant formula product. Further research is recommended to assess DCAO stability in milk-based infant formula in the presence of antioxidants.

Figure 2 — Oxidative stability index (OSI) of lipid fractions from infant formulas and their lipid substrates. Values with the same letter are not significantly different (P < 0.05). DCAO = DHA-containing customized amaranth oil; IF = infant formula.

References


Infant formula using structured lipid . . .


