JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Changes in the Lipid Composition of Powdered Infant Formulas during Long-Term Storage

LUIS M. RODRÍGUEZ-ALCALÁ,[†] MARÍA C. GARCÍA-MARTÍNEZ,[†] FÁTIMA CACHÓN,[‡] SUSANA MARMESAT,[§] LEOCADIO ALONSO,[‡] GLORIA MÁRQUEZ-RUIZ,[†] AND JAVIER FONTECHA*,[†]

Instituto del Frío (CSIC), 28040 Madrid, Spain; Instituto de Productos Lácteos de Asturias (CSIC), 33300 Villaviciosa, Asturias, Spain; and Instituto de la Grasa (CSIC), 41012 Sevilla Spain

Changes in the lipid composition of two standard infant formulas induced by 4 years of storage were determined. Lipids were thoroughly analyzed using different gas−liquid and liquid−liquid chromatographic techniques. Oleic acid and linoleic acid, which accounted for almost the total monounsaturated and polyunsaturated fatty acids, respectively, showed slight but significant decreases ($P < 0.05$) during the 4 years of storage (from 41.52 to 39.83% for oleic acid and from 17.35 to 15.99% for linoleic acid). Total trans fatty acid isomers showed low initial level (0.22% of total fatty acids), and such level remained unchanged during the storage period. Nonvolatile oxidation compounds including oxidized, dimeric, and polymeric triglycerides did not significantly increase during the storage period, although a significant loss of tocopherols was found in the surface oil fraction (10−15%). In general, the results obtained indicate that, although small losses of oleic and linolenic acid as well as tocopherols were found, the 4 year storage period did not lead to relevant changes in the lipid fraction of infant formulas.

KEYWORDS: Infant formula; storage; PUFA; trans fatty acids; oxidation compounds

INTRODUCTION

Data accumulated from scientific and clinical studies on long-chain polyunsaturated fatty acids (LCPUFAs) in human milk and as additives to infant formulas suggest that LCPUFAs are essential for energy and growth, organ differentiation and function, and cellular metabolism of preterm and term infants (*1*). Among LCPUFAs, docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are found in human milk. DHA and AA, which are formed from essential fatty acids (EFA), linoleic acid (LA, 18:2n-6), and α-linolenic acid (ALA, 18:3n-3) are rapidly accrued in the central nervous system and retina during the last trimester of pregnancy and the first postnatal year (*2*−*5*).

During the commercial preparation of most infant formulas, the butterfat of whole cow's milk is replaced by a blend of vegetable oils, added to bring the fatty acid composition, especially of polyunsaturated fatty acids, closer to that in human milk (*6*). The formulated raw material mix is blended, pasteurized, homogenized, concentrated, and spray-dried or sterilized, and many resources are used by manufacturers to ensure that products are of good quality and show a long shelf life.

Adequate LCPUFAs contents are achieved mainly by a number of highly unsaturated dietary lipid sources currently available, such as fish oils and oils obtained from fungal and algal organisms. Increases in LCPUFAs contents and parallel mineral fortification as well as technological treatments and long-term storage periods may lead to oxidative modifications with subsequent loss of essential fatty acids, trans fatty acid (TFA) formation, release of volatile compounds responsible for rancidity, and formation of nonvolatile oxidation compounds that may be detrimental to health. Therefore, the oxidation stability of powder formulas and their shelf life clearly depend in great part on the LCPUFAs content, storage temperature, and storage time (*7*).

With respect to TFA, a review of the literature shows little information on their influence in infants (*8*) and on the composition and levels in infant formulas (*9*). On the other hand, changes in lipid oxidation parameters during storage of infant formulas have been reported in a few studies, including monitoring of primary or secondary oxidation products determined by classical indices (*10*−*13*), or stability tests (*14*), as well as monitoring of tocopherol losses (*10*, *15*, *16*). Still, results obtained are often confusing, in part due to the use of methods applicable to only particular stages of the oxidation process.

The aim of the present study was to analyze the effects of long-term storage on infant formulas available on the market and to follow lipid changes, with special focus on the content

* Author to whom correspondence should be addressed (telephone 34 915445607; fax 34 915493627; e-mail jfontecha@if.csic.es.
[†] Instituto del Frío (CSIC).
[‡] Instituto de Productos Lácteos de Asturias (CSIC).
[§] Instituto de la Grasa (CSIC).

10.1021/jf0708591 CCC: $37.00
Published on Web 07/14/2007

Table 1. Composition of Infant Formulas (Values Were Obtained from the Company and Based on Label Claims)

	baby 1 formula[a]		baby 2 formula[b]	
	100 g powder	100 mL	100 g powder	100 mL
energy (kcal)	522	68	500	70
protein (g)	10.2	1.3	15	2.1
carbohydrate (g)	55.2	7.2	53.7	7.5
fat (g)	29.0	3.8	25.0	3.5

[a] Adapted formula. [b] Follow-on formula. Provided by Hero España, S.A.

Table 2. Ingredients of Infant Formulas (Values Were Obtained from the Company and Based on Label Claims)[a]

ingredient	baby 1 formula	baby 2 formula
skim milk	+	+
demineralized milk whey	+	+
vegetable oils	+	+
lactose	+	+
minerals	+	+
lecithin	+	+
vitamins (A, C, E, B_1, B_2, B_6, B_{12}, D_3, K, niacin, pantothenic acid, folic acid)	+	+
choline	+	+
taurine	+	+
inositol	+	+
carnitine	+	+
amino acids (arginine, histidine, tryptophan)	+	
maltodextrins		+

[a] + indicates the presence of the ingredient.

of PUFAs and TFA, as well as the concentration of oxidation compounds, to determine if such storage could have adverse effects.

MATERIALS AND METHODS

Samples. Two milk-based adapted infant formulas: initiation formula (baby 1) and follow-on formula (baby 2) were provided by a local industry (Hero España, S.A.). Baby 1 formula was designed to meet the nutritional needs of infants from birth, and baby 2 formula was designed for the development and growth of infants up to the age of 4 months. Composition of the formulas is showed in **Table 1**. Four batches of each formula at four storage periods during the shelf life of this product (1−4 years) (32 samples in total) were evaluated in duplicate. First-year samples corresponded to samples received from the company after manufacture and analyzed during the first year of storage. The samples, packed in 900 g sealed metal containers blanketed with inert atmosphere, were stored at ambient temperature (25 ± 3 °C) during 4 years.

Both baby 1 and baby 2 infant formulas were prepared following the same technological process, as follows: milk was skimmed, pasteurized (72 °C/15 s), and concentrated (falling film evaporator at 85, 66, and 58 °C during 5 min). Then pasteurized whey (72 °C/15 s), lipids (a blend of vegetable oils), lactose, and minerals were added, and the formulas were mixed and sterilized (HTST 100 °C/22 s). Finally, the rest of the ingredients (see **Table 2**) were added and spray-dried by atomization (air input at 175−185 °C; air output at 90−94 °C).

Lipid Extraction. For analysis of fatty acid methyl esters (FAMEs) and TFA, lipids were extracted following a procedure described by an International Standard Method for milk powder, ISO-IDF (*17*). Briefly, it consists of the addition of an ammoniacal ethanolic solution to a test portion followed by lipid extraction using diethyl ether and hexane. Then, the upper layer is removed, and the solvent is completely evaporated. The lipid extracts obtained were stored in amber glass vials, exposed to a stream of nitrogen and frozen at −20 °C until analysis. For analyses of oxidized compounds and tocopherols, the fraction of free oil was additionally extracted. The free oil fraction, also known as the nonencapsulated oil fraction, was determined according to the method of Sankarikutty et al. (*18*). Thus, 200 mL of light petroleum ether (60−80 °C) was added to 4 g of powder sample. Stirring was applied at room temperature for 15 min. After filtration through a filter paper, the solvent was evaporated in a rotary evaporator, and the extracted oil was dried to constant weight using a stream of nitrogen.

Preparation of FAME. FAMEs were prepared by base-catalyzed methanolysis of the extracted lipids using 2 N KOH in methanol as described by International Standard ISO-IDF (*19*).

Standards. For GC-FID analysis, anhydrous milk fat with a certified fatty acid composition (reference material BCR-164, EU Commission, Brussels, Belgium, purchased from Fedelco Inc., Madrid, Spain) was used to determine the FAME response factors. For quantitative purposes glyceryl tritridecanoate (Sigma Chemical Co., St. Louis, MO) was also used as internal standard.

Tentative identification of *trans*-C18:2 and *trans*-C18:3 isomers was done by comparing the equivalent chain-length values of FAME obtained with those of reference oils: partially isomerized linseed oil FAME, refined rapeseed oil (BCR 686), partially hydrogenated sunflower seed oil (BCR-688), and a blend of palm oil and partially hydrogenated sunflower seed oil (BCR-687), which had served as test material in the research project SMT4-CT97-2144 of the European Union. Besides this test material, FAME pure isomers (C18:1: *cis*-9; *cis*-13; *trans*-9; *trans*-11; *trans*-13) and polyunsaturated fatty acid mixtures (C18:2 mixture: *trans*-9 *trans*-12 + *cis*-9 *trans*-12 + *trans*-9 *cis*-12 + *cis*-9 *cis*-12; C18:3 mixture: *trans*-9 *trans*-12 *trans*-15 + *trans*-9 *trans*-12 *cis*-15 + *trans*-9 *cis*-12 *trans*-15 + *cis*-9 *trans*-12 *trans*-15 + *cis*-9 *cis*-12 *trans*-15 + *cis*-9 *trans*-12 *cis*-15 + *trans*-9 *cis*-12 *cis*-15 + *cis*-9 *cis*-12 *cis*-15) supplied by Supelco (Bellefonte, PA) were also used as standards. Octanoic acid methyl ester and elaidic acid methyl ester, supplied by Sigma Chemical Co. (St. Louis, MO), were used as internal standards for TLC analysis.

Silver Argentation Thin Layer Chromatography (Ag^+-TLC). FAMEs were fractionated according to the number and geometry of double bonds by TLC. Briefly, the TLC glass plates (Merck, Darmstadt, Germany) were incubated with 20% aqueous solution of silver nitrate (Panreac, Barcelona, Spain) for 16 h, partially air-dried, and activated at 120 °C for 30 min. A 45 μL solution of FAME (100 mg/mL) was applied in a narrow band, and the plates were developed twice in a saturated chamber containing hexane and diethyl ether (9:1, v/v), leaving 15 cm of migration. At the end of chromatographic runs, the plates were air-dried and sprayed with a 0.20% ethanol solution of 2′,7′-diclorofluorescein (Merck), and the bands were visualized under UV light. The bands were scrapped off and the compounds eluted with diethyl ether and then analyzed by GC as described below.

GC-FID Analysis. FAMEs were analyzed on a Perkin-Elmer chromatograph (Autosystem model, Beaconsfield, U.K.) with a FID detector. Fatty acids were separated using CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, Chrompack, Middelburg, The Netherlands). The column was held at 100 °C for 1 min after injection, temperature-programmed at 7 °C/min to 170 °C, held there for 55 min, and then temperature-programmed at 10 °C/min to 230 °C and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 30 psi and at a split ratio of 1:20. The injector temperature was set at 250 °C, and the detector temperature was set at 270 °C. Injection volume was 0.5 μL.

Individual trans isomers were analyzed on the same system and column but under other chromatographic conditions: the initial temperature of 100 °C was maintained for 3 min, then raised to 160 °C at a rate of 7 °C/min and held for 62 min, then raised to 220 °C at a rate of 2 °C/min and held for 20 min until the end of the analysis. The split ratio was 1:50, and hydrogen was the carrier gas with a head pressure of 15 psig. The injector and detector temperatures were 250 °C.

Silver Ion HPLC (Ag^+-HPLC). Ag^+-HPLC separation of conjugated linoleic acid (CLA) methyl esters was carried out using an HPLC (Shimadzu Vp Series, Duisburg, Germany) equipped with a UV detector operating at 233 nm. FAMEs were separated using a ChromSpher 5 Lipid analytical column (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size; Varian-Chrompack International, Middelburg, The Neth-

Table 3. Fatty Acid Composition (Weight Percent on Fatty Acid Methyl Esters) in Infant Formulas during 4 Years of Storage[a]

	years of storage							
	1		2		3		4	
fatty acid[b]	mean	SEM	mean	SEM	mean	SEM	mean	SEM
C4:0	0.07a	0.007	0.07b	0.004	0.06b	0.004	0.08b	0.007
C6:0	0.19a	0.007	0.22b	0.004	0.22b	0.004	0.23b	0.007
C8:0	1.84a	0.032	2.12b	0.035	2.10b	0.014	2.08b	0.035
C10:0	1.32a	0.014	1.51b	0.021	1.49b	0.007	1.50b	0.025
C12:0	10.21a	0.088	11.33b	0.060	11.41b	0.021	11.09b	0.184
C14:0	4.02a	0.021	4.41b	0.025	4.43b	0.018	4.41b	0.085
C15:0	0.04a	0.004	0.04a	0.004	0.04a	0.004	0.05a	0.004
C16:0	16.78a	0.180	16.79a	0.095	16.61a	0.113	16.97a	0.223
C16:1	0.10a	0.004	0.09a	0.004	0.09a	0.004	0.12b	0.004
C17:0	0.05a	0.004	0.05a	0.004	0.05a	0.004	0.06a	0.004
C18:0	3.56a	0.035	3.63a	0.021	3.66a	0.018	3.63a	0.028
trans C18:1 (*t*4 to *t*12)	0.13a	0.014	0.13a	0.007	0.19b	0.011	0.20b	0.018
C18:1 *cis*9	41.52a	0.159	40.50b	0.092	40.10b	0.177	39.83b	0.216
C18:2 *cis*9, *cis*12	17.35a	0.283	16.66ab	0.219	16.91ab	0.145	15.99b	0.170
C20:0	0.27a	0.004	0.26a	0.004	0.26a	0.004	0.26a	0.004
C20:1 *cis*9	0.13a	0.007	0.10a	0.007	0.12a	0.014	0.12a	0.011
C20:1 *cis*11	0.14a	0.007	0.11a	0.007	0.12a	0.007	0.14a	0.018
C18:3n-3	1.49a	0.025	1.16b	0.018	1.15b	0.032	1.05b	0.039
C18:2 conjugated	0.03a	0.004	0.03a	0.004	0.03a	0.004	0.03a	0.004
C20:4n-6	0.30a	0.004	0.32b	0.004	0.33b	0.004	0.29a	0.004
C20:5n-3	0.12a	0.004	0.13a	0.004	0.13a	0.004	0.13a	0.004
∑ SFA	38.3a	0.329	40.4a	0.311	40.3a	0.318	40.6a	0.311
∑ MUFA	42.0a	0.159	40.9b	0.163	40.6b	0.163	40.4b	0.187
∑ PUFA	19.3a	0.286	18.3ab	0.223	18.6a	0.163	17.5b	0.198
∑ n-6	17.7a	0.290	17.0a	0.276	17.3a	0.283	16.3a	0.290
∑ n-3	1.8a	0.028	1.5b	0.028	1.5b	0.028	1.3b	0.028
ratio LA/ALA	11.6		14.3		14.7		15.3	

[a] Data are expressed as means and standard error of the mean (SEM), $n = 8$. Means within the same row with different letters differ ($P < 0.05$). [b] *c* = *cis*; *t* = *trans*; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; LA = linoelic acid; ALA= α-linolenic acid.

erlands). The mobile phase, daily prepared, was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 mL/min. The flow was initiated 0.5 h prior to the sample injection, and the injection volume was 10 μL. Pure and mixed CLA FAME isomers from Nu-Chek Prep. Inc. (Elysian, MN) were used as standards.

Quantitation of Oxidation Compounds. Quantitative analysis of total nonvolatile oxidation compounds was carried out by separation of polar compounds by solid-phase extraction (SPE) and subsequent analysis by high-performance size-exclusion chromatography (HPSEC) according to the method of Márquez-Ruiz et al. (*20*).

Separation of polar compounds by SPE. A volume of 2 mL of a hexane solution containing 50 mg of extracted lipids and 1 mg of monostearin (Sigma), used as an internal standard, was separated into two fractions by SPE. A first fraction, comprising the unoxidized triglycerides, was eluted with 15 mL of hexane/diethyl ether (90:10, v/v). The second fraction was eluted with 25 mL of diethyl ether and comprises the total nonvolatile oxidation compounds, the internal standard, hydrolytic alteration compounds, that is, diglycerides (DG) and free fatty acids (FFA), and polar unsaponifiable matter. Thus, the oxidation compounds are separated as compounds with higher polarity than that of the nonoxidized triglyceride molecules. After evaporation of the solvent in a rotary evaporator, the polar fraction was dissolved with 1 mL of diethyl ether. The efficiency of the separation was checked by TLC using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) for development of plates and exposure to iodine vapor to reveal the spots.

Analysis by HPSEC. The fraction of polar compounds was analyzed in an HPSEC chromatograph equipped with a Rheodyne injector with a 10 μL sample loop, a Waters 510 pump (Waters, Milford, MA), and a Waters refractive index detector. The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm × 0.77 cm i.d.) packed with porous, highly cross-linked styrene−divinylbenzene copolymers (film thickness = 10 μm) (Agilent Technologies, Palo Alto, CA) connected in series, with tetrahydrofuran (1 mL/min) as the mobile phase. The peaks resolved by HPSEC correspond to triglyceride dimers (TGD), oxidized triglyceride monomers (oxTGM), DG, monostearin, and finally peaks corresponding to FFA of various chain lengths and the polar unsaponifiable matter.

Determination of Tocopherols. Tocopherols were determined by normal-phase HPLC with fluorescence detection according to IUPAC Standard Method 2.411 (*21*).

Statistical Analysis. For statistical analysis, one-way analysis of variance (ANOVA) as well as multiple-comparisons procedure for each formula type and storage time was used. We conducted statistical analysis utilizing the SPSS package for Windows version 11 (SPSS, Chicago, IL). The level of statistical significance was set at 5% for all analyses.

RESULTS AND DISCUSSION

According to the information supplied by the manufacturer's (listed in **Tables 1** and **2**) infant formulas baby 1 and baby 2 contained the same blend of vegetable oils and were subjected to identical technological process. In fact, the statistical study applied to them confirmed no significant differences between both types of infant formulas with regard to contents of the lipid compounds analyzed in this study for the same storage year. This suggests that differences in other ingredients between the two infant formulas did not affect lipid composition during storage under the conditions used. As a consequence, all of the lipid analysis results obtained from the samples within the same year of storage were joined for statistical analyses.

Fatty Acid Profile. Mean values and standard deviations for fatty acid composition during 4 years of storage are shown in **Table 3**. The major fatty acid present in the infant formulas in quantitative terms was oleic acid (C18:1*cis*9) with around 40% of total FAME, suggesting that rich oleic acid oils, such as the olive oil or high-oleic sunflower oil, could be part of the mixture of vegetable oils used. Likewise, the oleic acid content reported for human milk is in the range of 24−40% (*2*). The level of saturated fatty acids (SFA) in infant formulas was around 40% (also within the range found in human milk of 35−50%), and approximately half of this level was palmitic acid (C16:0). The

Table 4. Trans Monoene Isomer Composition (Milligrams per 100 g of Oil) in Infant Formulas during 4 Years of Storage[a]

	years of storage							
	1		2		3		4	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
C16:1 *trans*	6.91a	0.424	6.20a	0.499	6.56a	0.283	7.36a	0.187
C18:1 *trans*								
trans 4–5	3.88a	0.728	3.39a	0.594	3.19a	0.357	4.05a	0.813
trans 6–8	12.75a	1.538	14.63a	1.294	16.33a	1.895	16.29a	1.146
trans 9	27.37a	1.676	31.20a	1.075	36.11a	1.605	33.22a	1.821
trans 10	38.03a	1.828	38.95a	2.839	43.20a	1.191	42.80a	2.005
trans 11	46.82a	2.386	53.07a	4.221	52.16a	2.669	54.21a	1.446
trans 12	19.07a	1.782	19.89a	1.128	15.91a	1.092	19.55a	0.969
trans 13–14	21.88a	0.933	22.36a	0.760	22.63a	1.255	25.72a	2.669
trans 15	6.41a	0.506	6.16a	0.481	5.88a	0.707	6.45a	0.640
trans 16	7.43a	0.488	6.99a	0.481	7.49a	0.824	7.21a	0.590
C18:1 total *trans* (%)	0.18a	0.007	0.20a	0.007	0.21a	0.007	0.21a	0.007

[a] Data are expressed as mean and standard error of the mean (SEM), $n = 8$. Means within the same row with different letters differ ($P < 0.05$).

content in medium-chain fatty acids (MCFA C10:0−C14:0) in the infant formulas studied was much higher than that in human milk, especially due to the high content of C12:0 (11% in infant formulas vs 3−8% in human milk). Linoleic acid (LA, C18:2 *cis*9-*cis*12) was the most abundant fatty acid of the PUFA fraction, with values of about 16% (90% of PUFA) and within the range found in human milk (8−18%). Formulas containing large amounts of corn oil have considerably larger contents of LA (>30%), although such high amounts are not recommended for infant formulas (*22*). The content of ALA (C18:3 *cis*9-*cis*12-*cis*15) accounted for approximately 1%. The LA/ALA ratio of the studied formula ranged from 12 to 15, which falls almost within the ratio in human milk and that established by several nutritional recommendations for infant formulas (between 5 and 15). The amounts of very long chain PUFAs were 0.3% of AA and 0.1% of EPA (C20:5) (all in the range found in human milk), although DHA (C22:6) was not detected in the samples studied (present at around 0.3% in human milk).

During the storage period, a gradual decrease in the content of MUFA and PUFA (represented mainly by oleic acid, C18:1*cis*9 and linoleic acid, C18:2 *cis*9-*cis*12, respectively) was observed (**Table 3**). The reduction was especially significant during the first year of storage for oleic and linolenic acid (C18:3 *cis*9-*cis*12-*cis*15), whereas linoleic acid decreased significantly only during the fourth year of storage of the infant formulas studied. Assuming the stability of saturated fatty acids, the normalization of the other fatty acids on the C16:0 amount reveals the absolute losses of 2% of MUFA (mainly oleic acid) and 2% of PUFA (1.5% of LA and 0.5% of ALA). These results are normally attributed to peroxide development during the storage of infant formulas enriched in PUFA (*13*). In this context, data on oxidation compounds and tocopherol levels obtained in this study are discussed below.

As expected, the total CLA content was low (0.3% of total FA), mainly due to the absence of milk fat in these infant formulas, and in agreement with the results of McGuire et al. (*23*). Nevertheless, due to the existing interest in the occurrence of CLA isomers and the lack of available data about its presence in infant formulas, the distribution of these isomers was studied. There are about 20 different CLA isomers in natural milk fat as shown by Ag^+-HPLC separation (*24*). The CLA isomers distribution (in relative proportions of total CLA) accounted for 38% of C18:2 *trans*,*trans* isomers (the major isomers were C18:2 *trans*10,*trans*12 and C18:2 *trans*9,*trans*11 CLA.), 16% of C18:2 *cis*,*cis* isomers, and 46% of C18:2 *cis*,*trans* plus *trans*,*cis* isomers. The most biologically important isomers described are C18:2 *cis*9,*trans*11 (rumenic acid) and C18:2 *trans*10,*cis*12, which accounted for 22 and 8.5% respectively, in the infant formulas analyzed. This isomer distribution did not match with milk fat, where rumenic acid is the most abundant isomer (around 80%) but was in the same range as that reported by Jung and Jung (*25*) for soybean oil determined by the same method.

On the other hand, Juaneda et al. (*26*) reported that the CLA level was positively influenced by deodorization temperature of sunflower oil (total CLA increased 0.2% at 180 °C and 1.3% at 220 °C). These authors also reported that the main CLA isomers found in fresh or heated oils were the C18:2 *trans*,*trans*, mainly 9, 11, and 10, 12, isomers. In this work, it is remarkable that neither the CLA content nor the CLA isomers distribution showed significant differences during the storage period of the studied infant formulas.

Trans Fatty Acid Isomers Content. Determination of total TFA content as well as the amount of individual TFA isomers in infant formulas during the storage period is important due to their possible adverse effects on the health of newborns and infants. The preliminary isolation of trans isomers by Ag^+-TLC is essential due to the complexity of their analysis by direct GC because of the coelution of *trans*-octadecanoic isomers (*t*-C18:1) with oleic acid. Also, the C16:1 *trans* isomer coelutes with C17:0 in milk fat (*27*). Normally, the vegetable oils used in infant formulas are not hydrogenated, and therefore the presence of TFAs is mainly a consequence of the deodorization conditions of the refining process performed at temperatures >200 °C (*28*).

TFAs values detected in the infant formulas studied during 4 years of storage are presented in **Table 4**. Among TFAs, *t*-C18:1 was the main isomer present, whereas *trans*-octadecadienoic (*t*-C18:2) and *trans*-octadecatrienoic acids (*t*-C18:3) were not detected. Isomers of *trans*-C18:1 were well identified and may be used as quality markers for vegetable oils incorporated in infant formulas. The three major *t*-C18:1 isomers, C18:1*t*-11 (vaccenic acid), C18:1*t*-10, and C18:1*t*-9 (elaidic acid), accounted for more than half of the total TFAs, and storage time of up to 4 years did not result in any significant increase. Overall, the total *trans*-C18:1 content was in the range of 0.20% of total fatty acids in infant formulas, 10-fold lower as compared to values reported in human milk (*29*, *30*), and did not change significantly during storage.

Formation of Oxidation Compounds and Loss of Tocopherols. In this study, two complementary approaches have been used to monitor oxidative changes during long-term storage,

Table 5. Oxidized Triacylglycerol Monomers (oxTGM), Triglycerol Dimers (TGD), Diacylglycerols (DG), and Free Fatty Acids (FFA) (Weight Percent on Extracted Lipids) in Total and Free Oil Fractions of Infant Formulas during 4 Years of Storage[a]

		years of storage							
		1		2		3		4	
		mean	SEM	mean	SEM	mean	SEM	mean	SEM
oxTGM	total oil	1.3	0.27	1.1	0.06	1.1	0.06	1.2	0.23
	free oil fraction	1.5	0.08	1.2	0.21	1.1	0.05	1.5	0.23
TGD	total oil	0.5	0.02	0.5	0.03	0.5	0.03	0.5	0.06
	free oil fraction	0.4	0.05	0.5	0.12	0.3	0.11	0.5	0.06
DG	total oil	2.1	0.17	2.0	0.07	2.0	0.15	2.1	0.09
	free oil fraction	2.2	0.26	2.2	0.41	2.2	0.10	2.4	0.09
FFA	total oil	0.6	0.04	0.7	0.02	0.7	0.10	0.6	0.02
	free oil fraction	0.5	0.04	0.6	0.02	0.5	0.03	0.5	0.02

[a] Data are expressed as mean and standard error of the mean (SEM), $n = 8$.

Table 6. Tocopherols (Toc) (Milligrams per Kilogram of Extracted Lipids) in Total and Free Oil Fractions of Infant Formulas during 4 Years of Storage[a]

		years of storage							
		1		2		3		4	
		mean	SEM	mean	SEM	mean	SEM	mean	SEM
total Toc	total oil	293a	15.6	331a	18.3	317a	14.8	382a	10.3
	free oil fraction	246b	14.3	281b	12.2	282b	7.3	332b	17.6
α-Toc	total oil	151a	9.8	173a	11.3	156a	11.1	188a	7.4
	free oil fraction	109b	14.6	138b	8.7	135a	6.2	160a	11.9
β-Toc	total oil	9a	1.7	11a	2.3	8a	1.4	10a	1.2
	free oil fraction	11a	2.1	13a	1.7	11a	1.9	12a	1.2
γ-Toc	total oil	102a	5.5	97a	15.5	116a	5.8	142a	4.1
	free oil fraction	83b	6.6	96a	4.0	94a	9.6	123b	4.7
δ-Toc	total oil	30a	1.6	51a	13.1	37a	2.1	40a	1.5
	free oil fraction	42a	7.4	34a	2.8	42a	8.4	37a	1.8

[a] Data are expressed as mean and standard error of the mean (SEM), $n = 8$. Values in each column for total oils and free oil fractions in samples of the same time period with different letters are significantly different ($P < 0.05$).

that is, quantitation of nonvolatile oxidation compounds, which include primary and secondary products, and determination of tocopherols, the major antioxidants present in these samples. Additionally, not only were the total oils extracted analyzed but also the minor lipid fraction normally called free or unencapsulated fraction. Isolation of this lipid fraction is achieved by simply washing with hexane (see Materials and Methods). Even though it constituted only 3−7% of total oil in these samples, its analysis is of great relevance because the free oil fraction may be more susceptible to oxidation than the oil fraction embedded in the matrix (encapsulated oil), and rancidity of the free oil fraction is easily perceived by consumers (*31*, *32*).

Table 5 shows data of oxidized triglyceride monomers (oxTGM), triglyceride dimers (TGD), diglycerides (DG), and free fatty acids (FFA) in samples stored for up to 4 years. The group of oxTGM includes the primary oxidation compounds formed (hydroperoxides) and secondary oxidation compounds (alcohols, ketones, epoxides, etc.) in monomeric TG structures, whereas TGD formation marks the start of the advanced oxidation stage (*20*, *33*, *34*). No significant differences were found in oxidation compounds (oxTGM and TGD) or in hydrolysis products (DG and FFA), either in total oils or in free oil fractions, during the storage period. The values reported are within those normally found in refined vegetable oils.

Table 6 shows data of total tocopherols and specific values for α, β, γ, and δ isomers. Infant formulas contain tocopherols derived from the vegetable oils used and from their specific addition during their manufacture. In this study, α-tocopherol and γ-tocopherol constituted the major fraction of the total tocopherol content. European legislation requires a minimum content of 0.5 α-tocopherol equivalents per 100 kcal in infant formulas (*35*). In this study, no significant differences due to storage time were found, and the total vitamin E activity exceeded by far the minimum content established by European law. However, values of total tocopherols were significantly lower in free oil fractions than in total oils independent of the storage time. Accordingly, the data obtained indicate that losses of tocopherols in this fraction were not due to storage conditions but to the manufacturing processes involving thermal conditions (e.g., sterilization and atomization processes) because differences between total and free oil were of the same order independent of the storage time. Although the free oil fraction was still well protected by remaining antioxidants in all samples, this finding clearly suggests that free oil is more susceptible to oxidation than encapsulated oil. These results are first reported here because tocopherols have been only analyzed in the total oil extracted from powdered infant formulas so far (*10*, *15*, *16*).

In conclusion, significant although very small decreases of MUFA (mainly oleic acid) and PUFAs (AA and ALA) were found during the first year of storage of the infant formulas studied. Parallel small losses of other PUFAs, such as LA, were significant only after 3 years of storage. However, formation of oxidation compounds or trans fatty acids was not detected; losses in total tocopherol levels were not significant, which occurs prior to significant oxidation. Therefore, we can conclude that long-term storage did not lead to greatly appreciable lipid changes in the infant formulas studied.

ACKNOWLEDGMENT

We thank Hero España S.A. (Murcia, Spain) for providing the samples of infant formulas and for the kind help of Isabel Vasallo.

LITERATURE CITED

(1) Innis, S. M. Essential fatty acids in growth and development. *Prog. Lipid Res.* **1991**, *30*, 39−103.
(2) Jensen, R. G.; Ferris, A. M.; Lammi-Keefe, C. J.; Henderson, R. A. Lipids of bovine and human milks: a comparison. *J. Dairy Sci.* **1990**, *73*, 223−240.
(3) Martinez, M. Tissue levels of polyunsaturated fatty acids during early human development. *J. Pediatr.* **1992**, *120* (4 II Suppl.).
(4) Agostoni, C.; Giovannini, M. Cognitive and visual development: influence of differences in breast and formula fed infants. *Nutr. Health* **2001**, *15*, 183−188.
(5) Gil, A.; Ramirez, M.; Gil, M. Role of long-chain polyunsaturated fatty acids in infant nutrition. *Eur. J. Clin Nutr.* **2003**, *57* (Suppl. 1).
(6) Fleith, M.; Clandinin, M. T. Dietary PUFA for preterm and term infants: review of clinical studies. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 205−229.
(7) Romeu-Nadal, M.; Chavez-Servin, J. L.; Castellote, A. I.; Rivero, M.; Lopez-Sabater, M. C. Oxidation stability of the lipid fraction in milk powder formulas. *Food Chem.* **2007**, *100*, 756−763.
(8) Larque, E.; Zamora, S.; Gil, A. Dietary trans fatty acids in early life: a review. *Early Hum. Dev.* **2001**, *65*, S31−S41.
(9) Dionisi, F.; Golay, P. A.; Fay, L. B. Influence of milk fat presence on the determination of *trans* fatty acids in fats used for infant formulae. *Anal. Chim. Acta* **2002**, *465*, 395−407.
(10) Angulo, A. J.; Romera, J. M.; Ramírez, M.; Gil, A. Effects of storage conditions on lipid oxidation in infant formulas based on several protein sources. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1603−1607.
(11) Giammarioli, S.; Lammardo, A. M.; Sanzini, E.; Bellomonte, G. Preliminary study on lipid oxidation in infant formulas during storage. *Riv. Sci. Aliment.* **1997**, *26*, 80−88.
(12) Manglano, P.; Lagarda, M. J.; Silvestre, M. D.; Gonzalo, C. V.; Farré, C. R. Stability of the lipid fraction of milk-based infant formulas during storage. *Eur. J. Lipid Sci. Technol.* **2005**, *107*, 815−823.
(13) Thomkinson, D. K.; Mathur, B. N. Stability of fatty acids and nutritional adequacy of PUFA rich infant formula during storage. *Indian J. Dairy Sci.* **1990**, *43*, 203−206.
(14) de la Presa-Owens, S.; Lopez-Sabater, M. C.; Rivero-Urgell, M. Shelf-life prediction of an infant formula using an accelerated stability test (Rancimat). *J. Agric. Food Chem.* **1995**, *43*, 2879−2882.
(15) Albala-Hurtado, S.; Veciana-Nogues, M. T.; Vidal-Carou, M. C.; Marine-Font, A. Stability of vitamins A, E and B complex in infant milks claimed to have equal final composition in liquid and powdered form. *J. Food Sci.* **2000**, *65*, 1052−1055.
(16) Miquel, E.; Alegria, A.; Barbera, R.; Farre, R.; Clemente, G. Stability of tocopherols in adapted milk-based infant formulas during storage. *Int. Dairy J.* **2004**, *14*, 1003−1011.
(17) ISO. I. S. milk and milk products−extraction methods for lipids and liposoluble compounds. *ISO 14156*; International Dairy Federation: Brussels, Belgium, 2001; p 172.
(18) Sankarikutty, B.; Sreekumar, M. M.; Narayanan, C. S. A. G. M. Studies on microencapsulation of cardamon oil by spray-drying technique. *J. Food Sci. Technol.* **1988**, *25*, 352−356.
(19) ISO. I. S. milk fat−preparation of fatty acid methyl esters. *ISO 15884*; International Dairy Federation: Brussels, Belgium, 2002; p 182.
(20) Márquez-Ruiz, G.; Dobarganes, C. *Analysis of Nonvolatile Oxidation Compounds by High-Performance Size-Exclusion Chromatography*; AOCS Press: Champaing, IL, 2005; pp 40−69.
(21) IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th ed.; International Union of Pure and Applied Chemistry, Blackwell Scientific: Oxford, U.K., 1992.
(22) Koletzko, B.; Baker, S.; Cleghorn, G.; Neto, U. F.; Gopalan, S.; Hernell, O.; Hock, Q. S.; Jirapinyo, P.; Lonnerdal, B.; Pencharz, P.; Pzyrembel, H.; Ramirez-Mayans, J.; Shamir, R.; Turck, D.; Yamashiro, Y.; Zong-Yi, D. Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. *J. Pediatr. Gastroenterol. Nutr.* **2005**, *41*, 584−599.
(23) McGuire, M. K.; Park, Y.; Behre, R. A.; Harrison, L. Y.; Shultz, T. D.; McGuire, M. A. Conjugated linoleic acid concentrations of human milk and infant formula. *Nutr. Res.* **1997**, *17*, 1277−1283.
(24) Sehat, N.; Yurawecz, M. P.; Roach, J. A. G.; Mossoba, M. M.; Kramer, J. K. G.; Ku, Y. Silver-ion high-performance liquid chromatographic separation and identification of conjugated linoleic acid isomers. *Lipids* **1998**, *33*, 217−221.
(25) Jung, M. Y.; Jung, M. O. Identification of conjugated linoleic acids in hydrogenated soybean oil by silver ion-impregnated HPLC and gas chromatography−ion impacted mass spectrometry of their 4,4-dimethyloxazoline derivatives. *J. Agric. Food Chem.* **2002**, *50*, 6188−6193.
(26) Juaneda, P.; de la Perriere, S. B.; Sebedio, J. L.; Gregoire, S. Influence of heat and refining on formation of CLA isomers in sunflower oil. *J. Am. Oil Chem. Soc.* **2003**, *80*, 937−940.
(27) Alonso, L.; Fraga, M. J.; Juarez, M. Determination of trans fatty acids and fatty acid profiles in margarines marketed in Spain. *J. Am. Oil Chem. Soc.* **2000**, *77*, 131−136.
(28) Chardigny, J. M.; Wolff, R. L.; Mager, E.; Bayard, C. C.; Sebedio, J. L.; Martine, L.; Ratnayake, W. M. N. Fatty acid composition of French infant formulas with emphasis on the content and detailed profile of trans fatty acids. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1595−1601.
(29) Hayat, L.; Al-Sughayer, M. A.; Afzal, M. Fatty acid composition of human milk in Kuwaiti mothers. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.: Biochem. Mol. Biol.* **1999**, *124*, 261−267.
(30) Martin, C. A.; Carapelli, R.; Visantainer, J. V.; Matsushita, M.; de Souza, N. E. Trans fatty acid content of Brazilian biscuits. *Food Chem.* **2005**, *93*, 445−448.
(31) Márquez-Ruiz, G. V., J.; Dobarganes, M. C. Oxidation in dried microencapsulated oils. In *Lipid Oxidation Pathways*; Kamal-Eldin, A., Ed.; AOCS Press: Champaign, IL, 2003; pp 245−264.
(32) Velasco, J.; Marmesat, S.; Dobarganes, C.; Marquez-Ruiz, G. Heterogeneous aspects of lipid oxidation in dried microencapsulated oils. *J. Agric. Food Chem.* **2006**, *54*, 1722−1729.
(33) Marquez-Ruiz, G.; Martin-Polvillo, M.; Dobarganes, C. Effect of temperature and addition of alfa-tocopherol on the oxidation of trilinolein model systems. *Lipids* **2003**, *38*, 233−240.
(34) Márquez-Ruiz, G.; Jorge, N.; Martín-Polvillo, M.; Dobarganes, M. C. Rapid, quantitative determination of polar compounds in fats and oils by solid-phase extraction and size-exclusion chromatography using monostearin as internal standard. *J. Chromatogr., A* **1996**, *749*, 55−60.
(35) ECC. Commission Directive 91/321/EC of 14 May 1991 on infant formulae and follow-on formula. *Off. J. Eur. Communities* **1991**, *175*, 35−49.

Received for review March 23, 2007. Revised manuscript received June 1, 2007. Accepted June 8, 2007. This study was carried out with funds from the Spanish Ministry of Science and Technology (Project AGL-2003-01712) and from the Comunidad Autónoma de Madrid (Project S-0505/AGR-0153).

JF0708591