Effects of Storage Conditions on Lipid Oxidation in Infant Formulas Based on Several Protein Sources

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ABSTRACT: The time course of several lipid oxidation parameters in infant formulas was the main focus of this study. Infant formulas based on different protein sources (milk protein, isolated soy protein, and hydrolyzed milk protein) were stored at different temperatures (32 and 55°C) in sealed containers in air for 1 yr. Levels of thiobarbituric acid-reactive substances and 7-ketocholesterol, and losses of essential fatty acids and tocopherols were measured to monitor lipid oxidation. Tocopherol losses and 7-ketocholesterol concentrations were better lipid oxidation parameters than the others. Their concentration and losses depended on type of infant formula, storage temperature, and time.
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Cholesterol and unsaturated fatty acids undergo oxidation during technological procedures and storage. Oxidative changes should be considered as a system of complex interactions among different components in foods. Lipid oxidation has received much scientific attention, due both to its undesirable implications in human health and to its contribution to a decrease in the nutritional value of foods (1,2). Special attention has been paid to the oxidation process in infant formulas, because they are the sole source of nutrients for the majority of non-breast-fed infants during the first months of life. Formula-fed infants are thus exposed to the acute and chronic effects of lipid oxidation products, which in turn influence protein digestibility and essential fatty acid (EFA) and vitamin stability.

Antioxidants inhibit lipid oxidation. The most important natural antioxidants present in foods are tocopherols, several amino acids and proteins, and ascorbic acid (2). The addition of antioxidants to foods containing fats and oils is desirable because they increase the shelf life of foods, allowing food to be transported and stored for long periods (3). Tocopherols are potent, lipid-soluble, chain-breaking antioxidants (4), and they are the major antioxidants present in infant formulas.

The main objective of this study was to evaluate the effect of different storage conditions on lipid oxidation of several protein-based infant formulas (milk-based, hydrolyzed milk protein-based, and soy protein-based infant formulas). Thiobarbituric acid-reactive substances (TBARS), 7-ketocholesterol concentrations, and losses of EFA and tocopherols were measured to monitor lipid oxidation.

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MATERIALS AND METHODS

Reagents. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from Fluka Chemie (Buchs, Switzerland). Tocopherols (α-, γ-, and δ-tocopherol), fatty acid standards, 5 α-cholestane, 7-ketocholesterol, and tetraethoxypropane (TEP) were from Sigma Chemical Co. (St. Louis, MO).

Samples. Milk-based (MIF), soy-based (SIF) and hydrolyzed protein-based (HIF) infant formulas were obtained from the Spanish market. All infant formulas contained different percentages of dairy fat: 14, 12, and 5% for MIF, SIF, and HIF, respectively. They were divided into 50-g batches and stored for 1 yr in the dark in sealed containers in air. Three replicate analysis were performed at 0, 1, 3, 6, 9, and 12 mon.

Determination of 7-ketocholesterol. Forty micrograms of the internal standard, 5 α-cholestane, was added to 1.000 ± 0.05 g of powder sample. Total lipids were extracted by the method of Folch *et al.* (5), and they were saponified following the method described previously (6). Total lipids were dissolved in 10 mL of 4% sodium hydroxide in methanol/benzene (3:2, vol/vol) solution, and held for 2 h at room temperature. Water (25 mL) water was added, mixed, and held until two layers were obtained. Ethyl ether (10 mL) was added to the mixture, mixed again for 30 s, and centrifuged at 1500 × *g* for 10 min at 5°C. After centrifugation, the lower layer was reextracted with 10 mL of ethyl ether. The upper layers were combined and washed with 5 mL of a 0.1 N sodium hydroxide solution, mixed, and centrifuged at 1000 × *g* for 15 min at 5°C. The upper layer was washed with water to neutral pH and evaporated under nitrogen stream. The residue was dissolved in a pyridine/HMDS/TMCS (5:2:1) solution and held 20 min at room temperature to form trimethylsilyl (TMS) ether derivative of the 7-ketocholesterol.

A Hewlett-Packard (Palo Alto, CA) gas–liquid chromatograph (GLC) Model HP-5890 with a flame-ionization detector was used. Chromatography was performed using a 30 m × 0.25 mm i.d. SPB-1 column with 0.25 μm film thickness (Su-

pelco Inc., Bellefonte, PA). The injector and detector temperatures were held at 300 and 310°C, respectively. The oven temperature was programmed as follows: initial temperature 250°C for 2 min, 2.5°C/min to 265°C, held 2 min, 1°C/min to 281°C and 2.5°C/min to 300°C. Other conditions were: carrier gas and make-up gas, nitrogen; column head pressure, 24 psi. Two microliters of 7-ketocholesterol as TMS derivative were injected onto the column with a split ratio of 30:1.

A mass spectrometer system (Varian Associates Inc., Harbor City, CA) consisting of a GLC, model STAR 3400 CS GLC, equipped with a Mass Selective Detector, SATURN- 4D, was employed to confirm 7-ketocholesterol identity detected in selected products. Gas chromatography–mass spectrometry (GC–MS) analysis was performed on the same capillary column and conditions used in routine GLC analysis. Spectra were obtained by electron impact ionization within a mass range of 100 to 600 *m/z*. Background subtraction and renormalization were performed. Peaks were identified by comparing their mass spectra with those of the pure compound.

Determination of EFA. EFA were determined following the method described by Lepage and Roy (7), adding 350 µg of tripentadecanoine as internal standard to 50 mg of infant formula. A GLC Hewlett-Packard Model HP-5890 with a flame-ionization detector was used to resolve and quantify fatty acids as methyl esters. Chromatography was performed using a 60 m × 0.3 mm i.d. SP-2330 column with 0.2 µm film thickness (Supelco Inc.). The injector and detector were both maintained at 275°C, respectively. Temperature programming was: initial temperature 80°C, 15°C/min to 165°C, 3°C/min to 211°C, held 10 min. Other conditions were: carrier gas and make-up gas, nitrogen; column head pressure, 17 psi. One microliter of fatty acid methyl esters was injected onto the column at a split ratio of 29:1.

Determination of tocopherols. Tocopherols were extracted by the method of Folch *et al.* (5). The organic phase was evaporated under a nitrogen stream and the lipid fraction was redissolved in hexane. The liquid chromatographic system (Waters, Milford, MA) consisted of a double piston pump, autosampler, fluorescence detector, and 10 µm normal-phase µ-Porasil column (250 mm × 4.9 mm i.d.) (Waters). The fluorescence detector was set at an excitation wavelength of 295 nm and emission of 325 nm.

Determination of TBARS. Infant formulas diluted to 10% (wt/vol) in water and 1% thiobarbituric acid solution in 5% trichloroacetic acid in water were mixed 1:1 (vol/vol). The mixture was shaken and held for 1 h at 80°C. After cooling, it was centrifuged at 1500 × *g* for 10 min at 5°C, and TBARS were quantified by fluorimetry with an excitation wavelength of 515 nm and emission wavelength of 535 nm. TEP was used as standard of malondialdehyde (MDA). Temperature and time reaction were set at 80°C and 1 h, respectively, to diminish sugar reactivity toward thiobarbituric acid and to complete the TBARS reaction.

Statistical analysis of experimental data. Linear regression and analysis of variance of regression coefficients over groups were carried out using the 1R program of BMDP statistical software (Los Angeles, CA).

RESULTS

Initial values of the quantified lipid oxidation parameters. The initial values for the quantified lipid oxidation parameters, 7-ketocholesterol, TBARS, EFA, and tocopherol concentration are shown in Table 1.

Effects of storage conditions on 7-ketocholesterol generation. Figures 1 to 6 show the time course of the 7-ketocholesterol concentration during 12 mon of storage of infant formulas at 32 and 55°C, packed in air. There was an influence of both temperature ($P < 0.0001$) and storage time ($P < 0.001$) on 7-ketocholesterol generation in stored infant formulas. At 32°C, 7-ketocholesterol generation was modest. In MIF and HIF it was similar, at rates of 0.24 and 0.16 ppm/mon, respectively. SIF suffered a significantly higher ($P < 0.05$) 7-ketocholesterol generation, at a rate of 0.60 ppm/mon. At 55°C, 7-ketocholesterol generation was different among infant formulas ($P < 0.001$). The constant rates, in ppm/mon, were 2.58 for SIF, 1.01 for MIF, and 0.98 for HIF.

Effects of storage conditions on TBARS generation. Figures 1 to 6 show the time course of the TBARS concentration during 12 mon of storage of infant formulas at 32 and 55°C, packed in air. Storage temperature exhibited no influence on TBARS generation in MIF, at a similar TBARS generation rate at 32 and 55°C, 0.38 and 0.44 µmol/mon, respectively. In SIF, there was an influence of storage temperature ($P < 0.01$), although TBARS generation rate was higher at 32 than at 55°C, 1.23 and 0.53, respectively. TBARS generation rate in HIF was influenced by temperature ($P < 0.001$), at a rate of 0.24 and 1.31 µmol/mon, at 32 and 55°C, respectively.

Effects of storage conditions on tocopherol losses. Figures 1 to 6 show the time course of the tocopherol losses during 12 mon of storage of infant formulas at 32 and 55°C, packed in

TABLE 1
Initial Values of Some Lipid Oxidation Parameters in Stored Infant Formulas[a]

	7KETO (ppm)	TBARS (mmol/100 g)	αT (mg/100 g)	γT (mg/100 g)	δT (mg/100 g)	tT (mg/100 g)	EFA (g/100 g)
MIF	7.1	1.5	1.16	1.12	0.72	3	2.92
SIF	12.7	2.8	2.37	1.55	0.59	4.5	2.21
HIF	10	1.5	3.03	2.17	1.47	6.7	3.26

[a]Results as mean of three replicates. Abbreviations are expressed as follow: 7KETO, 7-keto-cholesterol; TBARS, thiobarbituric acid-reactive substances; αT, α-tocopherol; γT, γ-tocopherol; δT, γ-tocopherol; tT, sum of αT, γT, and δT; EFA, essential fatty acids; MIF, milk-based infant formula; SIF, soy-based infant formula; HIF, hydrolyzed-portion-based infant formula.

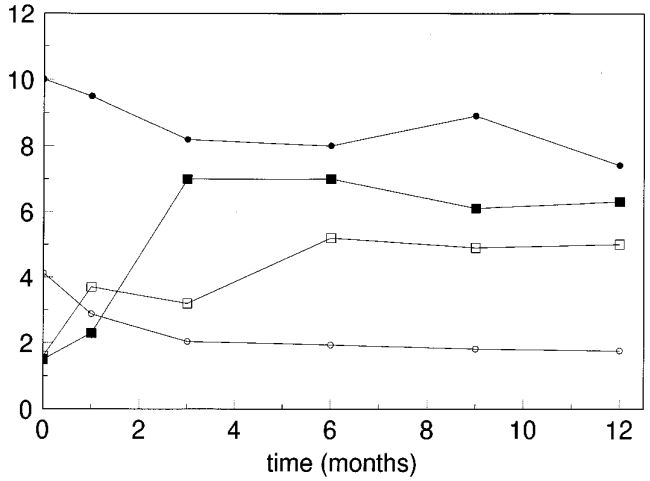

FIG. 1. Time course of lipid oxidation parameters in milk-based infant formula stored at 32°C in air. (●) Remaining essential fatty acids (EFA), percentage × 10^{-1}; (○) remaining tocopherols, ppm × 10^{-1}; (■) thiobarbituric acid-reactive substances (TBARS), μmol/100g; (○) 7-ketocholesterol, ppm.

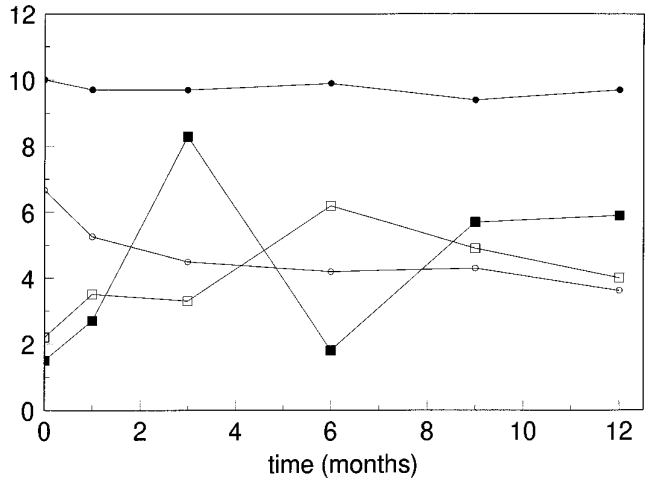

FIG. 3. Time-course of lipid oxidation parameters in hydrolyzed protein-based infant formula stored at 32ºC in air. (●) Remaining EFA, percentage × 10^{-1}; (○) remaining tocopherols, ppm × 10^{-1}; (■) TBARS, μmol/100g; (□) 7-ketocholesterol, ppm. For abbreviations see Figure 1.

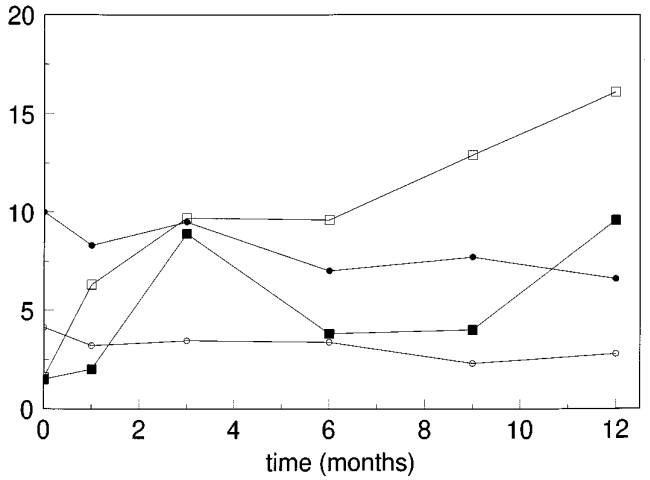

FIG. 2. Time course of lipid oxidation parameters in milk-based infant formula stored at 55ºC in air. (●) Remaining EFA, percentage × 10^{-1}; (○) remaining tocopherols, ppm × 10^{-1}; (■) TBARS, μmol/100g; (□) 7-ketocholesterol, ppm. For abbreviations see Figure 1.

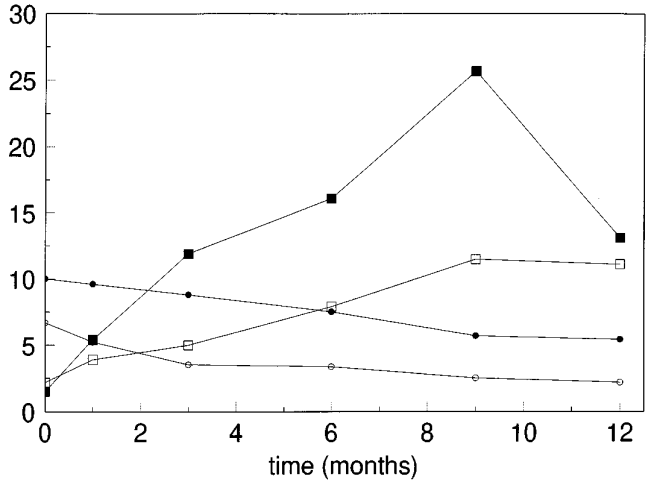

FIG 4. Time-course of lipid oxidation parameters in hydrolyzed protein-based infant formula stored at 55ºC in air. (●) Remaining EFA, percentage × 10^{-1}; (○) remaining tocopherols, ppm × 10^{-1}; (■) TBARS, μmol/100g; (□) 7-ketocholesterol, ppm. For abbreviations see Figure 1.

air. There was an influence of both storage temperature and storage time ($P < 0.01$) on tocopherol losses. Higher losses occurred during the first and third month. Among tocopherols, α-tocopherol and γ-tocopherol suffered the major losses. The tocopherol loss rates differed among infant formulas ($P < 0.001$ in the majority of the cases), at a constant rate, in ppm/mon, of –1.54 and –1.00 for MIF, –2.47 and –2.62 for SIF, and –1.94 and –3.29 for HIF, at 32 and 55°C, respectively.

Effects of storage conditions on EFA losses. Figures 1 to 6 show the time course of the EFA losses during 12 mon of storage of infant formulas at 32 and 55°C packed in air. There was an influence on EFA losses of storage temperature in SIF and HIF ($P < 0.0001$), but not in MIF. Samples stored at 55°C showed an influence ($P < 0.02$) of storage time. There were no significant differences between EFA loss rates of MIF and HIF at 55°C, and SIF and HIF at 32°C. At 55°C, the most important losses occurred in SIF, with only 7% EFA remaining after 12 mon of storage.

Correlation between 7-ketocholesterol concentration and tocopherol losses. When 7-ketocholesterol concentration was plotted against tocopherol losses, a good correlation was found. Squared regression coefficients were 0.753 and 0.660 for MIF, 0.921 and 0.661 for SIF, and 0.837 and 0.917 for HIF, at 32 and 55°C, respectively .

DISCUSSION

Initial concentrations of 7-ketocholesterol in infant formulas agreed with those reported by Zunin *et al.* (8). Other authors have found higher (from 11 to 18 ppm) (9) and lower (<0.1

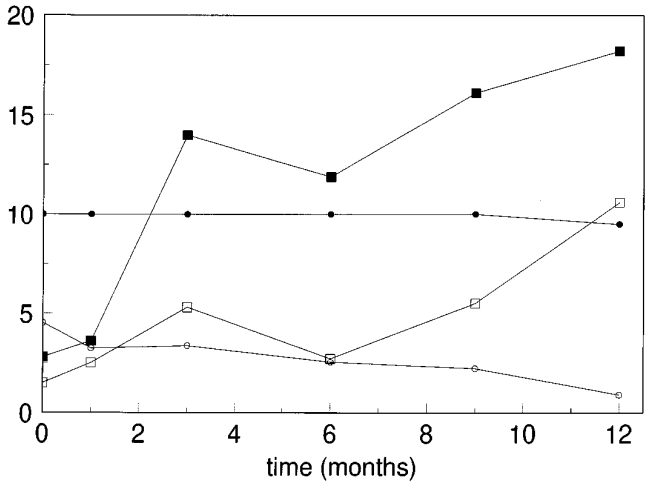

FIG. 5. Time course of lipid oxidation parameters in soy-based infant formula stored at 32ºC in air. (●) Remaining EFA, percentage × 10^{-1}; (○) remaining tocopherols, ppm × 10^{-1}; (■) TBARS, µmol/100g; (□) 7-ketocholesterol, ppm. For abbreviations see Figure 1.

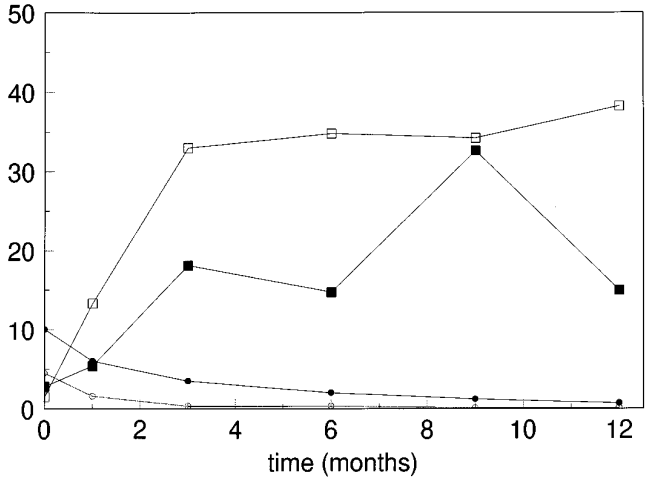

FIG. 6. Time course of lipid oxidation parameters in soy-based infant formula stored at 55ºC in air. (●) Remaining EFA, percentage × 10^{-1}; (○) remaining tocopherols, ppm × 10^{-1}; (■) TBARS, µmol/100g; (□) 7-ketocholesterol, ppm. For abbreviations see Figure 1.

ppm) (10) 7-ketocholesterol concentrations than that reported here. The wide range in concentrations for 7-ketocholesterol in infant formulas may be due to the fact that different products from a number of manufacturers were analyzed, and they used diverse raw materials. Moreover, the period between manufacturing and the analysis date, and between container opening and analysis date may have varied substantially. These facts are supported by Rose-Sallin *et al.* (10), who described that total cholesterol oxides (COPS) concentration in infant formulas multiplied five-fold in 20 d after container opening, and who found a ten-fold difference total COPS concentration in two freshly-opened infant formulas.

Among lipid oxidation parameters studied, 7-ketocholesterol and tocopherol losses were better than the others. Their concentration and losses depended on type of infant formula, storage temperature, and time. 7-Ketocholesterol has been proposed as a cholesterol oxidation index (8), and it seemed to be a good index of lipid oxidation.

Studies involving application of antioxidants to inhibit cholesterol oxidation have been limited. Antioxidants have not been described as inhibiting COPS generation, although they may decrease the rate of cholesterol oxidation (11). Correlation found between 7-ketocholesterol concentration and tocopherol losses reflects that these antioxidants protect cholesterol against oxidation. This protective effect has been described in spray-dried egg powders (11,12), but has not before been detected in infant formulas.

The generation of cholesterol oxidation products in infant formulas seems to be accelerated in comparison with other foods, which has been related to the presence of unsaturated fats and Fe^{2+} (10). In addition, thermal technological processes used in manufactured infant formulas may contributed to this problem. Tocopherols may inhibit cholesterol oxide generation.

Oxidation rate differed among infant formulas. This may be partially due to protein source. Soy protein contains about 5% of remaining fat, as declared by the manufacturer, and is rich in unsaturated fatty acids. It is handled as a fat-free product, which may cause a rapid oxidation of the remaining fat. When this protein source is used during infant formula manufacturing, it may act as a pro-oxidant initiator. The milk protein source is whole milk powder, which is resistant to cholesterol oxidation (9). Hydrolyzed protein is a fat-free product, as declared by the manufacturer. On the other hand, milk protein and hydrolyzed protein, which contains certain peptides and free amino acids, may act as antioxidants (13,14), retarding lipid oxidation.

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